LIPOPOLYSACCHARIDES OF IRON-OXIDIZING *LEPTOSPIRILLUM FERROOXIDANS* AND *THIOBACILLUS FERROOXIDANS*

AKIRA YOKOTA, YORIKO YAMADA, AND KO IMAI

*Institute for Fermentation, Osaka, 17–85, Juso-honmachi 2-chome,
Yodogawa-ku, Osaka 532, Japan*

(Received October 19, 1987)

Lipopolysaccharides were isolated from one strain of *Leptospirillum ferrooxidans* and three strains of *Thiobacillus ferrooxidans*. All strains contained 2-keto-3-deoxyoctonate (KDO), heptose(s) and glucosamine besides neutral sugars, but were devoid of phosphorus. Lipopolysaccharides from iron-grown cells and from sulfur-grown cells had a similar chemical composition. Thus, the chemical properties of the lipopolysaccharide was unaffected by the energy source utilized.

Chemical analysis of lipopolysaccharide from *Thiobacillus ferrooxidans* IFO 14262 revealed the presence of L-rhamnose, D-glucose, L-glycero-D-manno-heptose, KDO, D-glucosamine, and a lipophilic sugar identified as L-acofriose (3-O-methyl-L-rhamnose). 3-Hydroxymyristic acid was the main fatty acid. By hydrolysis in weak acid, the lipopolysaccharide has been separated into the polysaccharide part ("degraded polysaccharide") and lipid A. Presumably the lipid A contains a glucosamine backbone.

The chemical composition of lipopolysaccharides (LPSs) isolated from iron-oxidizing bacteria was studied by WANG et al. (1), VESTAL et al. (2), and HIRT and VESTAL (3). According to the latter two reports, LPSs isolated from *Thiobacillus ferrooxidans* strain TM grown on ferrous sulfate or on glucose were similar in chemical composition but differed from that of LPS grown on elemental sulfur in the content of hexosamine, KDO, and heptoses and in the composition of fatty acids. However, it was pointed out that cultures of many iron-oxidizing bacteria contained contaminants of acidophilic chemoorganotrophs, such as *Acidiphilium cryptum* (4)

Abbreviations used in this text are as follows: GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; KDO, 2-keto-3-deoxyoctonate; LPS, lipopolysaccharide; PAGE, polyacrylamide gel electrophoresis; PC, paper chromatography; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography.

Address reprint requests to: Dr. A. Yokota, Institute for Fermentation, Osaka, 17–85, Juso-honmachi 2-chome, Yodogawa-ku, Osaka 532, Japan.

and *Acidiphilium* sp.(5), or of a heterotrophic chemolithotroph, *Thiobacillus acidophilus* (1,5–10). GUAY and SILVER isolated *T. acidophilus* as a contaminant from the culture of *T. ferrooxidans* strain TM (6). Therefore, little is known about the chemical composition of LPS isolated from pure cultures of iron-oxidizing bacteria. Very recently, RODRIGUEZ et al. (11) reported on the chemical composition of the LPS of *T. ferrooxidans* strain R₂ grown on ferrous sulfate.

A systematic study of a large collection of mesophilic iron-oxidizing bacteria made by HARRISON (12) demonstrated that these bacteria comprise seven different DNA homology groups. To determine the intra- and inter-specific diversity in the chemical composition of the LPS of iron-oxidizing bacteria, we obtained four strains from HARRISON's laboratory and examined the sugar composition. We also report the characterization of the LPS from *T. ferrooxidans* IFO 14262.

MATERIALS AND METHODS

Bacterial strains and cultural conditions. Strains of iron-oxidizing bacteria, *Leptospirillum ferrooxidans* IFO 14244 (= strain BU-1, formerly named *Thiobacillus ferrooxidans* but later identified as *L. ferrooxidans* from its cell morphology and lack of sulfur utilization as a source of energy) (10, 12), *Thiobacillus ferrooxidans* IFO 14245 (= strain Lp) (12), IFO 14246 (= strain BA-4) (12) and IFO 14262 (= strain PH) (12) were obtained from Dr. HARRISON (Missouri University, U.S.A.). They were cultured in a medium containing: (NH₄)₂SO₄, 2 g; K₂HPO₄, 0.5 g; KCl, 0.1 g; MgSO₄ · 7H₂O, 0.5 g; ZnSO₄ · 7H₂O, 0.01 g; Ca (NO₃)₂, 0.01 g; NiSO₄ · 6H₂O, 0.01 g; FeSO₄ · 7H₂O, 40 g or powdered sulfur, 5 g; and distilled water, 1000 ml (12). The pH was adjusted to 3.5 with 2N H₂SO₄. The culture broth (330 ml) in a 1-l Erlenmeyer flask was incubated at 28°C for 5–14 days on a rotary shaker. The cells were harvested by centrifugation, and washed with distilled water.

Isolation and purification of LPS. LPS was extracted by the hot-phenol method of WESTPHAL and JANN (13), and purified by centrifugation at 140,000 × g for 3 h in a Beckman U-2 ultracentrifuge.

To identify L-acofriose in the LPS of *T. ferrooxidans*, LPS was isolated from the cells obtained from 50 l of culture broth. The LPS obtained (195 mg) was then hydrolyzed in 2M HCl at 100°C for 2 h. After the precipitate was removed by centrifugation, HCl was removed by evaporation in vacuo. The residue was chromatographed on a column of cellulose powder (2.2 × 75 cm) with solvent B (see *Analytical methods*). Fractions containing L-acofriose (No. 125–165) were combined and concentrated in vacuo.

Analytical methods. Neutral sugars were liberated by 2M HCl at 100°C for 2 h. HCl was removed by repeated evaporation in vacuo. Descending paper chromatography (PC) (Toyo filter paper No. 50) and thin-layer chromatography (TLC) (cellulose-TLC, precoated and silica gel 60-TLC, precoated) were carried out using the following solvent systems: (A) *n*-butanol–pyridine–water (40:11:6, v/v/v), (B) *n*-butanol–pyridine–water–acetate (60:40:30:3, v/v/v/v), (C) *n*-butyl

acetate–pyridine–ethanol–water (8:2:2:1, v/v/v/v). Sugar spots were detected by spraying the chromatograms with anilinium hydrogen phthalate and alkaline silver nitrate for PC and cellulose-TLC, and with 50% hydrogen sulfate for silica gel TLC. High-performance liquid chromatography (HPLC) was performed on a Shimadzu Model LC-5A pumping system, using a Shim-pack ISA-07/S2504 (4.0 × 250 mm) column according to the method described by MIKAMI and ISHIDA (14). Fluorescence intensities of the effluent were measured with a Shimadzu Model RF-530 spectrofluorometer and a Chromatopac.

To quantitatively determine neutral sugars, the LPS was hydrolyzed in 0.1 M HCl at 100°C for 48 h. The sugars were converted into alditol acetates (15) and were analyzed by gas-liquid chromatography (GLC) on a glass column (0.26 × 100 cm) with Advance DS (Shimadzu) at a column temperature of 160–225°C (3°C/min) and a helium gas flow of 50 ml/min.

Aminohexoses were liberated by 4 M HCl at 100°C for 6 h. HCl was removed by repeated evaporation in vacuo. The sugars were analyzed by cellulose-TLC and quantified in an amino acid analyzer, Hitachi model 838.

To detect KDO, LPS was hydrolyzed in 0.1 M H₂SO₄ at 100°C for 10 min, neutralized with a saturated solution of Ba(OH)₂, and concentrated by evaporation in vacuo. KDO was determined by the method of WEISSBACH and HURWITZ (16). Fatty acids were released from LPS by hydrolysis in 4 M HCl at 100°C for 6 h. Fatty acids, extracted with petroleum ether (30 to 60°C), were weighed and esterified with 5% methanolic HCl at 100°C for 2 h. A chloroform solution of the fatty acids methyl ester was analyzed by GLC on a glass column (0.26 × 200 cm) of diethylene glycol succinate polyester (5% on Chromosorb W, 60 to 80 mesh) at a column temperature of 165°C, or alternatively with a glass column of OV-1 (3% on Chromosorb W, 60 to 80 mesh) at a column temperature of 180°C.

Phosphorus was determined by the method of CHEN et al. (17).

Demethylation of L-acofriose. The sugar (1 mg) was suspended in 1 ml of BCl₃ in CH₂Cl₂ and placed open in a closed dry dessicator for 18 h (18). Boron was removed by several evaporations with methanol. The residue was dissolved in water and passed through a small column of IR 120 (H⁺) and the sugars were examined by HPLC, TLC and by GLC as the alditol acetates.

Degradation of LPS. LPS was treated with 1% acetic acid at 100°C for 2 h. The water-insoluble moiety (lipid A) was separated by centrifugation at 4,000 × g for 5 min, washed with water and acetone, and lyophilized. Water-soluble moiety ("degraded polysaccharide") was further purified by Sephadex G-50 column (1.8 × 115 cm) chromatography with a pyridine–acetic acid–water (10:4:1,000, v/v/v) buffer as eluant.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A polyacrylamide gel was prepared and run with a buffer system of LAMMLI (19). The separating gel contained 15% acrylamide and 0.1% SDS, and the stacking gel 7.5% acrylamide and 0.1% SDS. LPS bands were visualized by silver staining as described by TSAI and FRASCH (20).

Physical and chemical analyses. Optical rotations were measured with a Japan Spectroscopic DIP-181 polarimeter. $^1\text{H-NMR}$ spectrum was measured with a Varian XL 100-A (100.1 MHz) spectrometer with tetramethylsilane as a reference. The electron impact mass spectra were measured with a Hitachi M-80A mass spectrometer and chemical ionization mass spectra were measured with a JMS-DX 300 mass spectrometer.

Chemicals. KDO and *Salmonella typhimurium* LPS were purchased from Sigma Chemical Co. (U.S.A.). 6-Deoxy-L-talose was prepared from the cell-wall of *Microbacterium arborescens* IFO 3750 (21).

RESULTS

Chemical composition of LPS from iron-oxidizing bacteria

LPSs were obtained from all strains with yields of about 0.5 to 1.5%. Neither ribose nor 2-deoxyribose was detected in the HPLC analyses of all LPSs studied, which is proof that they were almost free from contamination with nucleic acids. Since sufficient amounts of the purified LPSs were not available, with the exception of that from strain IFO 14262, the sugar composition was analyzed only qualitatively. The individual sugar components were examined by HPLC or GLC and chemical analyses. The results are summarized in Table 1.

Rhamnose, heptoses, KDO and glucosamine were found as common sugar components in the LPSs of all strains examined. The LPSs from strains IFO 14245, and IFO 14246 contained both D-glycero-D-manno-heptose and L-glycero-D-manno-heptose. As major neutral sugars besides rhamnose, strain IFO 14244 contained mannose, xylose, and an unknown sugar; strain IFO 14245 contained glucose and mannose; strain IFO 14246 contained glucose and xylose; and strain IFO 14262 contained glucose and L-acofriose. Phosphorus, an usual component of lipid A of gram-negative bacterial LPS, was virtually absent from the LPSs of all these strains. Identification of L-acofriose is described later.

Influence of energy source on the chemical composition of LPS

LPSs extracted from cells of strains IFO 14246 and IFO 14262 grown on iron or elemental sulfur showed no striking differences in chemical composition (Tables 1 and 2). These LPSs from strain IFO 14262 were also examined by SDS-PAGE and silver staining. Both LPSs showed a similar migration pattern (Fig. 1).

*LPS of *T. ferrooxidans* IFO 14262*

To determine the chemical composition of the LPS of *T. ferrooxidans* IFO 14262 and its polysaccharide and lipid A moieties in more detail, further studies were carried out.

Analyses for neutral sugars: The neutral sugar fraction obtained by acid hydrolysis of the LPS was analyzed by PC, TLC, HPLC, and GLC after the sugars were converted into their respective alditol acetates. Rhamnose, glucose, and a

Table 1. Effect of energy source on sugar composition of LPS from cultures of iron-oxidizing bacteria.

Strain	Energy source ^a	Neutral sugar composition ^b								Heptose ^c	KDO ^d	Glucosamine ^d	Phosphorus ^d
		Rhamnose	Glucose	Galactose	Mannose	Arabinose	Xylose	Unknown sugar	L-Acofriose				
<i>Leptospirillum ferrooxidans</i> IFO 14244	Fe	57	t ^e	t	11	t	15	17	—	LD	+	+	—
<i>Thiobacillus ferrooxidans</i> IFO 14245	Fe	57	23	—	20	—	—	—	—	DD + LD	+	+	—
<i>Thiobacillus ferrooxidans</i> IFO 14246	Fe	65	6	—	—	t	29	—	—	DD + LD	+	+	—
<i>Thiobacillus ferrooxidans</i> IFO 14246	S	62	14	—	—	t	24	—	—	DD + LD	+	+	—
<i>Thiobacillus ferrooxidans</i> IFO 14262	Fe	32	60	—	—	—	—	—	8	LD	+	+	—
<i>Thiobacillus ferrooxidans</i> IFO 14262	S	34	55	—	—	—	—	—	11	LD	+	+	—

^a Fe, cultured on ferrous sulfate medium; S, cultured on elemental sulfur medium.

^b Analyzed by HPLC and expressed as a percentage of the total sugar fraction.

^c Examined by GLC and tentatively identified as D-glycero-D-manno-heptose (DD) and L-glycero-D-manno-heptose (LD) from the retention times.

^d Examined by chemical method, respectively.

^e Trace amount.

Table 2. Chemical composition of the LPS from *Thiobacillus ferrooxidans* IFO 14262.

Component	Amount of component (mg/100 mg of LPS)	
	Sulfur-grown	Fe-grown
Total neutral sugars	62.6	61.4
L-Acofriose	12.7	11.3
L-Rhamnose	25.0	23.6
D-Glucose	24.6	25.8
L-Glycero-D-manno-heptose	0.3	0.7
2-Keto-3-deoxyoctonate	0.12	ND ^a
D-Glucosamine	2.1	+ ^b
Total fatty acids	16.0	+
3-OH-14:0	(80%)	ND
16:0	(4%)	ND
16:1	(10%)	ND
18:0	(3%)	ND
18:1	(3%)	ND
Phosphorus	<0.01	<0.01

^a Not determined.

^b Present but not quantified.

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the LPSs from iron-grown (1) and sulfur-grown (2) cells of *Thiobacillus ferrooxidans* IFO 14262 and from *Salmonella typhimurium* (3).

lipophilic sugar were present in large amounts; and a small amount of L-glycero-D-manno-heptose was also present. The optical rotation of the isolated sugars, separated by PC, revealed an L-configuration for rhamnose and a D-configuration for glucose and glucosamine.

Isolation and characterization of L-acofriose: The lipophilic sugar, which was contained in the LPS, was stained with anilinium hydrogen phthalate but not with alkaline silver nitrate on PC. It was prepared from LPS hydrolysate by cellulose powder column chromatography. From 193 mg of crude LPS, 12 mg of L-acofriose was isolated as a syrup in pure form.

Chemical ionization mass spectrometric analysis of the sugar with ammonia as reactant gas (Fig. 2) showed the equasimolecular peaks m/z 196 ($M + NH_4$) and 178 ($M + NH_4 - H_2O$), which indicated the molecular weight of the sugar was 178. The 1H -NMR spectrum of the sugar showed a doublet (total 3H) at δ 1.30 and a singlet (total 3H) at δ 3.47, which indicated that the sugar has one C-methyl group and one O-methyl group.

From these results, the sugar could be assumed to be a mono O-methylated deoxyhexose. It was converted into its alditol acetate and analyzed with electron impact mass spectrometry (Fig. 3). Fragmentation at 20 eV gave the following main fragments: m/z 43, 87, 101, 129, 143, 189 and 203. These fragments are typical for the fragmentation of 3-O-methyl ethers of 6-deoxyhexoses (22, 23).

The sugar was then converted into its parent 6-deoxyhexose by demethylation using the BCl_3 technique of ALLEN et al. (18). The demethylated product was analyzed by TLC, HPLC, and GLC; it exhibited the same characteristics as L-rhamnose on the three chromatograms.

The sugar had R_{rha} 1.82 on PC in solvent C, consistent with identification as 3-O-methylrhamnose (acofriose) (Table 3). It gave color reactions of 6-deoxyhexose

Fig. 2. Mass spectrum (chemical ionization mass spectrometry with ammonia) of L-acofriose.

Fig. 3. Mass spectrum (electron impact mass spectrometry) and fragmentation scheme of alditol acetate of L-acofriose.

on PC with anilinium hydrogen phthalate and on TLC with hydrogen sulfate (Table 3).

The optical rotation of the 3-O-methyl-rhamnose was positive ($[\alpha]_D + 36^\circ$ ($c=0.4$, water)) indicating that the sugar has an L configuration. Literature values of L-acofriose are $[\alpha]_D + 30^\circ \sim +39.1^\circ$ (24, 25) and for D-acofriose $[\alpha]_D - 27^\circ$ (26).

Analyses for amino sugars, KDO and phosphorus: Separation of the acid hydrolysate of the LPS on an amino acid analyzer gave only a glucosamine peak, and no further amino sugars could be detected.

The assay for KDO in a mild hydrolysate of the LPS showed the presence of a small amount of KDO. The LPS was virtually free of phosphorus.

Table 3. R_f values and color reactions on paper and thin-layer chromatograms of L-acofriose.

	R_f values on PC ^a		Color reaction on PC		Color reaction on TLC (50% H ₂ SO ₄)
	R_{rha}	(Reference) ^b	Alkaline AgNO ₃	Aniline hydrogen phthalate	
L-Rhamnose	1.00	(1.00)	positive	positive	yellow
3-O-Methyl-D-fucose	—	(1.30)	negative ^c	positive ^c	yellow ^d
6-Deoxy-L-talose	1.59	(1.55)	positive	positive	yellow
3-O-Methyl-L-rhamnose	—	(1.80)	negative ^c	positive ^c	yellow ^d
L-Acofriose	1.82		negative	positive	yellow

^a Solvent C.^b From Ref. (34).^c From Ref. (30).^d From Ref. (35).Table 4. Chemical composition of the lipid A and degraded polysaccharide of LPS from *Thiobacillus ferrooxidans* IFO 14262.

Component	Amount of component	
	Degraded polysaccharide (mg/100 mg of PS)	Lipid A (mg/100 mg of lipid A)
L-Acofriose	13.2	
L-Rhamnose	29.7	
D-Glucose	32.0	
L-Glycero-D-manno-heptose	1.1	
D-Glucosamine	0	9.8
Total fatty acids	0	54.7
Phosphorus	<0.01	<0.01

Analyses for fatty acids: Analysis of the total content of fatty acids, liberated by acidic hydrolysis from the LPS followed by methylation, revealed that 3-hydroxymyristic acid is the main fatty acid component of the LPS. Small amounts of palmitic acid and oleic acid were also present (Table 2).

Degradation studies: Treatment of the LPS with mild acid (1% acetic acid, 100°C for 2 h) resulted in a separation of the water-insoluble lipid A and the water-soluble polysaccharide moiety, "degraded polysaccharide." The degraded polysaccharide fraction was subjected to gel chromatography.

The main sugar of the lipid A was glucosamine. The "degraded polysaccharide" contained L-acofriose, L-rhamnose, and D-glucose in the same ratio as those of the LPS (Table 4).

DISCUSSION

The isolation and identification of LPSs from four strains of iron-oxidizing bacteria are reported. Besides major neutral sugars, heptose(s), KDO, and glucosamine were found in these LPSs. These are known to be regular sugar constituents of the core region and β -1,6-glucosamine disaccharide backbone of lipid A of gram-negative bacterial LPS. Lack of phosphorus among these LPSs is a remarkable feature.

The sugar composition of the LPS of *T. ferrooxidans* strain R₂ (11) was reported to be different from those of the *T. ferrooxidans* strains described here. However, we have recently found the chemical composition of the LPSs from strains IFO 14262 and R₂ are very similar (27).

VESTAL et al. (2) and HIRT and VESTAL (3) have shown that cultural conditions and choice of energy source influence the yield and composition of the LPS of *T. ferrooxidans* strain TM. We found that the LPSs of *T. ferrooxidans* IFO 14246 and IFO 14262 from cells grown on ferrous sulfate and elemental sulfur did not differ in their content of neutral sugars and fatty acids and in their migration pattern on SDS-PAGE.

The LPS from *T. ferrooxidans* IFO 14262 was investigated in more detail. In addition to L-rhamnose, D-glucose, D-glucosamine, L-glycero-D-manno-heptose, and KDO, L-acofriose was found in this LPS. L-Acofriose has been reported in the LPSs from *Klebsiella* K73:010 (28), *Rhodopseudomonas capsulata* 37b4 (29), *Rhodopseudomonas rubrum* 2.4.6. (30), but has not previously been reported in *Thiobacillus*. Since L-acofriose represents 12.7% of the LPS dry weight in the *T. ferrooxidans* strain, it seems to be present in repeating units of the polysaccharide moiety, as in the case of *Anabaena variabilis* (31). As the proportion of L-acofriose/L-rhamnose/D-glucose in the degraded polysaccharide from the LPS is about 1:2:2, the simplest repeating unit conceivable would contain a minimum of five sugar residues. Presumably KDO mediates the connection between lipid A and the polysaccharide in the LPSs of *T. ferrooxidans*.

The presence of sugar O-methyl ethers as characteristic constituents of the O-specific chains in the LPSs of photosynthetic bacteria (29) and chemolithotrophic bacteria *Nitrobacter hamburgensis* (32) and *T. ferrooxidans* IFO 14262 may suggest a possible genealogical relation between photosynthetic bacteria and chemolithotrophic bacteria. The LPSs of other iron-oxidizing bacteria, such as *L. ferrooxidans* IFO 14244, *T. ferrooxidans* IFO 14245 and IFO 14246, do not contain L-acofriose.

Since glucosamine was found in the lipid A, it may be assumed that the lipid A of *T. ferrooxidans* has a backbone of glucosamine units, similar to that of Enterobacteriaceae. The main fatty acid of the *T. ferrooxidans* IFO 14262 LPS is 3-hydroxymyristic acid, as generally found in lipid A of other LPSs as well.

Recent studies (27) on the composition of lipid A and core regions of the LPSs of *Thiobacillus* strains revealed that those from two strains of *T. ferrooxidans* and one strain of *T. thiooxidans* have glucosamine and 2,3-diamino-2,3-dideoxyglucose

as a lipid A backbone and that those from *T. novellus* and *Thiobacillus* sp. IFO 14570 contain 2,3-diamino-2,3-dideoxy-glucose in their lipid A. It would be of interest for phylogenetic reasons (33) to investigate the core region and the lipid A constituents of the LPSs of iron-oxidizing bacteria in more detail.

We wish to thank Dr. A. P. Harrison Jr., Professor of Missouri University, U.S.A., for providing us bacterial strains and for his valuable suggestions. We also thank Mr. H. Takeda, Chemical Research Laboratories of Takeda Chemical Industries, for his excellent mass spectrometry analysis and valuable suggestions. We are grateful to Dr. H. Mayer, Max-Planck-Institut für Immunbiologie (F.R.G.), for his criticism of the manuscript. Grateful acknowledgement is also made to Dr. T. Iijima, Director of this Institute, for his encouragement and valuable discussions.

REFERENCES

- 1) W. S. WANG, M. S. KORCZYNSKI, and D. G. LUNDGREN, *J. Bacteriol.*, **104**, 556 (1970).
- 2) J. R. VESTAL, D. G. LUNDGREN, and K. C. MILNER, *Can. J. Microbiol.*, **19**, 1335 (1973).
- 3) W. E. HIRT and J. R. VESTAL, *J. Bacteriol.*, **123**, 642 (1975).
- 4) A. P. HARRISON, JR., *Int. J. Syst. Bacteriol.*, **31**, 327 (1981).
- 5) D. B. JOHNSON and W. I. KELSO, *J. Gen. Microbiol.*, **129**, 2969 (1983).
- 6) R. GUAY and M. SILVER, *Can. J. Microbiol.*, **21**, 281 (1975).
- 7) G. J. M. W. ARKESTEYN and J. A. M. de BONT, *Can. J. Microbiol.*, **26**, 1057 (1980).
- 8) A. P. HARRISON, JR., B. W. JARRIS, and J. L. JOHNSON, *J. Bacteriol.*, **143**, 448 (1980).
- 9) A. P. HARRISON, JR., *Int. J. Syst. Bacteriol.*, **33**, 211 (1983).
- 10) A. P. HARRISON, JR., *Annu. Rev. Microbiol.*, **38**, 265 (1984).
- 11) M. RODRIGUEZ, S. CAMPOS, and B. GOMEZ-SILVA, *Biotech. Appl. Biochem.*, **8**, 292 (1986).
- 12) A. P. HARRISON, JR., *Arch. Microbiol.*, **131**, 68 (1982).
- 13) O. WESTPHAL and K. JANN, *In Methods in Carbohydrate Chemistry*, Vol. 5, ed. by R. L. WHISTLER, Academic Press, New York (1965), p. 83.
- 14) H. MIKAMI and Y. ISHIDA, *Bunseki Kagaku*, **32**, E207 (1983).
- 15) J. S. SAWADECKER, J. H. SLONEKER, and A. JEANES, *Anal. Chem.*, **37**, 1602 (1967).
- 16) A. WEISSBACH and J. HURWITZ, *J. Biol. Chem.*, **234**, 705 (1959).
- 17) P. S. CHEN, T. Y. TORIBARA, and H. WARNER, *Anal. Chem.*, **28**, 1756 (1956).
- 18) S. ALLEN, T. G. BONNER, E. J. BOURNE, and N. M. SAVILLE, *Chem. Ind.*, 630 (1958).
- 19) U. K. LAMMLI, *Nature*, **227**, 680 (1970).
- 20) C. M. TSAI and C. E. FRASCH, *Anal. Biochem.*, **119**, 115 (1982).
- 21) K. IMAI, M. TAKEUCHI, and I. BANNO, *Curr. Microbiol.*, **11**, 281 (1984).
- 22) H. BJORNDAAL, C. G. HELLERQUIST, B. LINDBERG, and S. SVENSSON, *Angew. Chem. Int. Ed.*, **9**, 610 (1970).
- 23) B. LINDBERG, *In Methods in Enzymology*, Vol. 28, ed. by V. GINSBURG, Academic Press, New York (1972), p. 178.
- 24) V. POZSGAY and P. NANASI, *Carbohydr. Res.*, **81**, 184 (1980).
- 25) H. MUHR, A. HUNGER, and T. REICHSTEIN, *Helv. Chim. Acta*, **37**, 403 (1954).
- 26) I. M. HARRISON, R. YOUNG, M. B. PERRY, and G. A. ADAMS, *Can. J. Chem.*, **45**, 1987 (1967).
- 27) H. BJORNDAAL, B. LINDBERG, W. NIMMICH, *Acta Chem. Scand.*, **24**, 3414 (1970).
- 28) A. YOKOTA, M. RODRIGUEZ, Y. YAMADA, K. IMAI, D. BOROWIAK, and H. MAYER, *Arch. Microbiol.*, **149**, 106 (1987).
- 29) J. WECKESSER, G. DREWS, and H. MAYER, *Annu. Rev. Microbiol.*, **33**, 215 (1979).
- 30) J. WECKESSER, H. MAYER, and G. DREWS, *Eur. J. Biochem.*, **16**, 158 (1970).
- 31) J. WECKESSER, A. KATZ, G. DREWS, H. MAYER, and I. FROMME, *J. Bacteriol.*, **120**, 672 (1974).
- 32) H. MAYER, E. BOCK, and J. WECKESSER, *FEMS Microbiol. Lett.*, **17**, 93 (1983).

- 33) H. MAYER and J. WECKESSER, *In Handbook of Endotoxin*, Vol. 1, ed. by E. Th. RIETSCHEL, Elsevier, Amsterdam, New York, Oxford (1984), p. 221.
- 34) L. D. KENNEDY, *Carbohydr. Res.*, **87**, 156 (1980).
- 35) S. SAKUMA, S. KAWANISHI, and J. SHOJI, *Shoyakugaku Zasshi*, **22**, 23 (1968) (in Japanese).