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Structure of Lipid A from the Marine γ-Proteobacterium *Marinomonas vaga* ATCC 27119^T Lipopolysaccharide

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Abstract—The chemical structure of a novel lipid A obtained as a major component on hydrolysis of LPS from the marine γ-proteobacterium *Marinomonas vaga* ATCC 27119^T with 1% AcOH was determined. Using chemical analysis and NMR data, it was shown to be β-1,6-glucosaminobiose 1-phosphate acylated with R-3-hydroxydecanoic acid (at position 3), and R-3-dodecanoyloxydecanoic (or R-3-decanoyloxydecanoic) acid and R-3-(R-3-hydroxydecanoyl)oxydecanoic acids (at the 2- or 2′-positions). The absence of a fatty acid at the 3′-position and a phosphoryl group at the 4′-position, and also the presence of R-3-acyloxyalkanoic acid with R-3-hydroxyalkanoic acid as the secondary acid are unique features distinguishing the *M. vaga* lipid A from other ones.

Key words: marine proteobacteria, *Marinomonas vaga*, LPS, lipid A

Bacterial endotoxin is a biological antipodal sword. On one side it is an extremely potent toxin, which is associated with severe pathological effects, in some cases leading to lethal outcome; on the other side, it stimulates factors of nonspecific resistance of a macroorganism [1]. Chemically, endotoxins are lipopolysaccharides (LPS) having lipid domains named lipids A (LA), which are responsible for endotoxic properties [2]. At the same time, some nontoxic derivatives of LA were recently shown to block LPS receptors on cell-targets of a macroorganism, preventing development of toxic reactions, and, thus, displaying properties of endotoxin antagonists [3].

The structural forms of LA demonstrating high antagonism to endotoxins have a low degree of phosphorylation and acylation. These are more frequent in bacteria that are phylogenetically distant from Enterobacteriaceae [4]. We have assumed, that marine proteobacteria living in special conditions (increased content of salts, low temperature, high hydrostatic pressure) can synthesize LA of unusual structure, and have carried out corresponding researches. It turned out that LAs isolated from a big group of marine bacteria had a number of features, which distinguished them from terrestrial LAs. They had a high degree of homogeneity, a unique set of fatty acids, mainly pentaacyl and, frequently, monophosphoryl structure types [5]. This gives grounds to expect that marine LAs can function as endotoxin antagonists.

The present research is devoted to studying the structure of LA from the marine bacterium *Marinomonas vaga* ATCC 27119^T, which belongs to γ-subclass of proteobacteria and displays psychrophilic and moderate halophilic properties [6].

MATERIALS AND METHODS

Growth of bacteria and isolation of LPS. The *M. vaga* ATCC 27119^T cells were cultivated at room temperature on rotary shakers in liquid nutrient medium containing (g/liter of 50% natural sea water, pH 7.5-7.8): bactopeptone (Difco, USA), 5; casein-hydrolyzate (Merck, Germany), 2; yeast extract (Merck), 2; glucose, 1; KH_2PO_4, 0.2; $MgSO_4$, 0.05. Upon attainment of late logarithmic phase (18 h), they were harvested by centrifugation, washed consecutively with distilled water, acetone, ethanol, hexane, and twice with chloroform–methanol (2 : 1 v/v) mixture to yield defatted cells (0.68 g/liter). The LPS (25 mg/g of dry cells) was obtained by the hot phenol/water procedure [7] and purified from nucleic acids by precipitation with 40% TCA [8].

Abbreviations: LPS) lipopolysaccharide; LA) lipid A; TCA) trichloroacetic acid; TLC) thin layer chromatography; GLC-MS) gas-liquid chromatography/mass-spectrometry; TEA) triethylamine.

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Isolation of lipid A. To obtain LA, the LPS (300 mg) was hydrolyzed in aqueous 1% acetic acid for 3 h at 100°C and insoluble LA was recovered by centrifugation of the hydrolyzate. The chloroform solution of the sediment obtained was washed with distilled water (3 times), dried with anhydrous sodium sulfate, and precipitated with fivefold volume of acetone to yield crude LA (0.25 mg/mg of LPS). The LA was further purified by Sephadex LH-20 and silica gel column chromatography.

Analytical methods. To determine glycosyl composition of the LA, amino acid analysis of amino compounds using an LKB 4251 Alpha Plus system (Sweden) and the preparation and GLC-MS of alditol acetates were used. Lipid A was converted to free monosaccharides by hydrolysis in 6 N HCl at 100°C for 24 h. In the case of alditol acetates, the monosaccharide samples were reduced with sodium borohydride and acetylated with acetic anhydride in pyridine, and after that, GLC-MS was performed. The total phosphorus generated after LA charring with $HClO_4$ was determined by the ammonium molybdate method. Fatty acids were obtained by alkaline hydrolysis (6 N NaOH at 100°C for 4 h) of LA. The ester bound fatty acids were released by mild alkali treatment of LA (1 mg) with 12% aqueous ammonium hydroxide (200 µl) at 20°C for 18 h as previously described [9]. The released fatty acids were converted to methyl esters with ether solution of diazomethane and identified by GLC and GLC-MS. Calculated amounts of pentadecanoic acid were added to the lipid A prior to hydrolysis as an internal standard.

Chromatography. Gel-permeation chromatography was performed on a column (560 × 15 mm) of Sephadex LH-20 in chloroform–ethanol (3 : 1 v/v). Silica gel chromatography was done with chloroform–methanol mixtures in various ratios. TLC was carried out on ready-made aluminum-backed Sorbfil (Sorbpolymer, Russia) plates using $CHCl_3$–CH_3OH–H_2O–conc. NH_4OH (100 : 50 : 8 : 4 v/v). Bands were visualized by heating the plates at 130°C for 10 min after spraying with 20% H_2SO_4 in methanol. Ninhydrin reagent treatment of the TLC plates was used for detection of free amino groups in lipid A. GLC-MS analyses were carried out on a Hewlett-Packard model 6890 gas chromatograph equipped with HP 5 MS 5% Phenyl Methyl Siloxane capillary column (30 m × 250 µm × 0.25 µm) and connected to a Hewlett-Packard model 5973 mass spectrometer. Samples analyzed were injected in the split mode with a split ratio of 1 : 15 at the injector temperature 250°C. The oven temperature was programmed to increase from 125 to 210°C at rate of 5°C/min. Helium was used as the carrier gas.

Determination of the *R*,*S*-configuration of 3-hydroxy fatty acid. In order to obtain purified 3-hydroxy alkanoic acid, total fatty acids (22 mg) obtained by alkaline hydrolysis of the defatted *M. vaga* ATCC 27119^T cells were fractioned on silica gel column using the following solvent systems: hexane; hexane–ether (1 : 1 v/v); hexane–ether–acetic acid (1 : 1 : 0.1 v/v). To determine *R*,*S*-configuration, the specific rotation of the fraction containing 3-hydroxy fatty acids (5.3 mg) was measured.

NMR spectroscopy. The ^1H-, ^{13}C-, and ^{31}P-NMR spectra of lipid A were recorded at 317 K in the $CDCl_3$–CD_3OD (4 : 1 v/v) mixture at 500, 125, and 202.5 MHz, respectively, with a Bruker Avance DRX-500 spectrometer equipped with a reverse probe. Except for LA in salt form, samples for NMR analysis were prepared as described in [10]. In brief, lipid A (1 mg) was dissolved in ultra pure deionized water (1 ml) by adding 25 µl of 0.36 M TEA in the cold (pH of the solution was adjusted to ≤9.0). Insoluble remnant was separated by centrifugation in an Eppendorf centrifuge and discard. The aqueous supernatant was acidified with 1 N HCl to pH ~ 1 and sediment of lipid A obtained was separated by centrifuging, dissolved in chloroform (in order to made the solution transparent, some drops of methanol were added), washed with deionized water three times, and evaporated. LA was dissolved at the concentration of 4 mg/ml.

Lethal toxicity. The toxic properties of the LA were tested in outbred D-galactosamine-sensitized mice (16-18 g) by the method of Galanos [11]. D-Galactosamine-hydrochloric acid (16 mg per animal) and different amounts of LA (0.004 to 4 mg) were intraperitoneally administered as mixtures in 0.4 ml of phosphate-buffered saline into groups of four animals. A control of 4 mice was injected with saline only (0.4 ml). Mortality was monitored for 48 h. Toxicity was expressed as LD_{50} and was calculated by the Nowotny method [12].

RESULTS

LA isolated from *M. vaga* ATCC 27119^T LPS consisted of GlcN, phosphorus, decanoic acid (10:0), dodecanoic acid (12:0), and 3-hydroxydecanoic acid (3OH10:0) in the amounts shown in Table 1. Generally, 1.2 mol of phosphate and 5 mol of fatty acids fall on 2 mol

Table 1. Chemical composition of the *M. vaga* ATCC 27119^T lipid A

Constituent	Amount of constituent	
	µmol/mg	mol/2 mol GlcN
GlcN	0.1171	2.00
P	0.0687	1.17
10:0	0.0234	0.40
12:0	0.0266	0.45
3OH10:0	0.2415	4.12

of glucosamine (GlcN). No free amino groups were detected by ninhydrin, indicating that no unsubstituted amino compounds were present in LA.

LA of *M. vaga* ATCC 27119^T contained one type of 3-hydroxy acid (3OH10:0) that was shown to be prevalent (75% on the total content of fatty acids, Table 1). The absolute configuration of 3OH10:0 was determined by optical rotatory measurement and this acid was shown to belong to the L-(−)-series ($[\alpha]_D = -14.1^\circ$ (0.5, $CHCl_3$)). *Trans*-Δ^2-unsaturated acids, characteristic for many enteric LA hydrolyzates [13] and indicative of the presence of acyloxy esters in the LA preparations (Δ^2-*trans*-unsaturated derivatives are preferably formed from ester-linked 3-acyloxyacyl residues [13]) occurred only in trace amounts (Table 1). Treatment of LA with weak alkali released 3OH10:0, but no 12:0 or 10:0. These latter fatty acids were therefore assumed to be present in secondary position at C2- or C2′-located amide-linked 3-hydroxydecanoic acid.

The structure of the LA backbone, the pattern of acyl residue distribution, the position of free hydroxyl groups, and the attachment sites of phosphates were analyzed using ^{1}H-, ^{31}P-, and ^{13}C-NMR spectroscopy. The resonance field of C atoms from monosaccharide residues (~50-100 ppm) in the ^{13}C-NMR spectrum of the *M. vaga* LA is represented by 12 signals (Table 2) pointing to its disaccharide nature. Signals at 95.35 and 101.26 ppm belong to two anomers, suggesting that the *M. vaga* lipid A is glucosaminobiose with α- and β-configurations of the anomeric centers of its reducing and non-reducing residues, respectively. A shift of the C1 signal to lower field (in comparison with signal of C1 in the spectrum of non-phosphorylated glucosamine derivative [14]) was observed, indicating that position 1 in the reducing end was esterified by the phosphate residue. In addition, carbon signals at 95.35 and 51.90 ppm were doublets with $J_{C1,P}$ 6.7 Hz and $J_{C2,P}$ 7.6 Hz, respectively, also pointing to presence of phosphate at the C1 atom. ^{31}P-NMR analysis of the *M. vaga* LA, whose spectrum contains only one kind of phosphate groups at ~2 ppm, agrees well with such assumption.

In line with the well established fact that O-acylation at C3 shifts the resonance of C2 in N-acylated glucosamine [15], C2 signal (51.90 ppm) of the reducing end, found in a higher field in comparison with C2 signal (53.9 ppm) in α-methyl-O-(2-deoxy-2-[(*R*,*S*)-3-hydroxytetradecanoylamino]-β-D-glucopyranosyl)-(1→6)-2-(2-deoxy-2-[(*R*,*S*)-3-hydroxytetradecanoylamino]-D-glucopyranoside 6-diphenylphosphate [16], indicates that the reducing end of the *M. vaga* LA backbone has a substituent at the C3 atom. The hydroxyl group at the C3′ atom is free as evidenced by non-reducing end C2′ chemical shift value (56.6 ppm). It coincides closely with non-reducing end C2′ chemical shift value (56.6 ppm) in the above α-methyl-β-1,6-diglucosamine 6-diphenylphosphate, which has no substitute at the C3′ atom.

It should be noted that an acid residue at C3′ of glucosaminobiose is readily liberated even under mild conditions [13]. On preparing LA for NMR spectroscopy, operations, which theoretically can cause a partial degradation of the sample (treatment with TEA and HCl [10]), were used. Fatty acid residue at the C3′ position and phosphate group at C1 are particularly alkali- and acid-labile, respectively. However, additional studies of the *M. vaga* LA by ^{13}C-NMR showed that its salt and acid forms had the same chemical shift values.

The resonance field of C6 atoms of glucosaminobiose was represented only by one signal (61.86 ppm). According to DEPT-135 experiments, resonation of the second C6 atom (68.35 ppm) was shifted to lower field, demonstrating β-1,6-linkage in the backbone of the *M. vaga* lipid A.

Six carbon signals (171.35, 172.69, 173.01, 173.12 with two, at 173.80 and 173.90 ppm, of low intensity) due to ester carbonyl and amide carbonyl carbons were observed in the ^{13}C-NMR spectrum suggesting that, on the whole, the *M. vaga* lipid A has five acyl residues, and are in good agreement with data of total chemical analysis according to which the *M. vaga* LA has pentaacyl type of structure (Table 1). Four signals of equal intensity for the C2 atoms of 3OH10:0 were detected at 42.70, 42.53, 41.64, 41.14 ppm that demonstrates the presence of four residues of this acid in the LA molecule. Analysis of this spectrum field shows that the *M. vaga* lipid A does not contain amide-linked 3OH10:0 with free hydroxyl group (signals at ~44 ppm characteristic for C2 of amide-linked 3-hydroxy acids are absent) and ester-linked acyloxy alkanoic acid (signals at ~38 ppm are also absent).

On the other hand, only two signals are present in the field of resonance of the C3 (68.79 and 68.75 ppm) and

Table 2. ^{13}C-NMR data for glucosamine residues in the *M. vaga* ATCC 27119^T LA

Monosaccharide residue	Chemical shift (δ) for:					
	C1	C2	C3	C4	C5	C6
β-D-GlcN_p(1→	101.26	56.61	74.84	71.18	76.49	61.86
→6)-α-D-GlcN_p	95.35	51.90	73.62	68.85	73.47	68.35

Table 3. Assignment of the selected glucosamine protons in the ^{1}H-^{1}H COSY NMR spectrum of the *M. vaga* LA

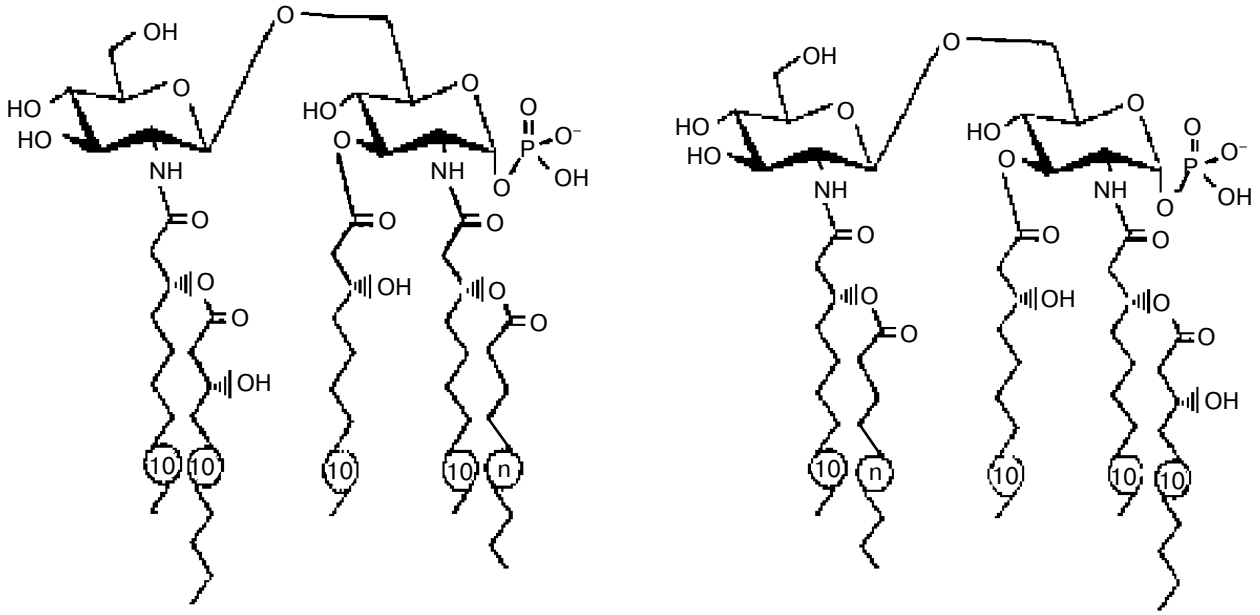
Proton	δ (ppm)	Proton	δ (ppm)
H1	5.49	H1'	4.65
H2	4.19	H2'	3.53
H3	5.15	H3'	4.05
H4	3.53	H4'	3.83

C4 (37.54 and 37.46 ppm) atoms of 3OH10:0 with free hydroxyl group. Resonance of the C3 and C4 atoms of two other 3-hydroxydecanoic acid residues was shifted to lower field (72.24, 71.38, and 34.48 ppm, respectively) because of the substitution of their hydroxyl groups by other fatty acid residues. Two types of 3-acyloxyalcanoic acids, ester- and amide-linked, can be present in LA [2]. As pointed out above, in the ^{13}C-NMR spectrum of the *M. vaga* LA, the signal of the C2 atom at 39 ppm is absent, suggesting that 3-acyloxydecanoic acids are amide-bound.

On the basis of the data obtained it is possible to suggest that the *M. vaga* LA represents glucosamine disaccharide, acylated with one residue of 3-hydroxydecanoic, two residues of 3-acyloxydecanoic acid, and phosphorylated with one residue of phosphoric acid. The further establishment of structure, confirming the assumptions stated above, was achieved using one- and two-dimensional proton nuclear magnetic resonance spectroscopy. The ^{1}H-signal at 5.49 ppm which is shown as a typical doublet of doublets, has been attributed to the anomeric proton of the reducing end having α-anomeric orientation ($J_{H1,H2}$ = 3.4 Hz) and carrying a phosphate group at the C1 atom ($J_{H1,P}$ = 8.6 Hz). The doublet signal at 4.65 ppm was attributed to the anomeric proton H1′ of the β-linked non-reducing glucosamine. The coupling constant $J_{H1',H2'}$ (8.2 Hz) confirms the earlier stated assumption about β-configuration of glycosidic linkage between glucosamine rings in the *M. vaga* LA.

Using ^{1}H-^{1}H COSY NMR spectroscopy and chemical shifts of anomeric protons at 5.49 and 4.65 ppm as a reference point, assignment of signals for the selected protons of both glucosamine residues have been made (Table 3). Chemical shifts of H2 and H2′ appeared at 4.19 and 3.53 ppm, respectively, proving N-acyl substitutions. The methine proton signals of H4, H3′, and H4′ were found between 3 and 4 ppm (Table 3), suggesting no acyl or phosphoryl substitution of the hydroxyl groups at these positions. Based on the above results, two structures of the *M. vaga* ATCC 27119^{T} LA (figure) have been proposed.

Unusual structure of the *M. vaga* ATCC 27119^{T} LA (pentaacyl type, absence of an acid at C3′ position) gives the basis to assume that it can have unusual biological properties, for example, weak toxicity. Therefore we have carried out experiments to determine acute toxicity (in



Proposed structures of the *M. vaga* ATCC 27119^{T} LA

comparison with toxicity of LPS from *Yersinia pseudotuberculosis* O:1b serovariant). In fact, *M. vaga* LA had considerably higher lethal dose (1.46 μg), than *Y. pseudotuberculosis* LPS, whose LD_{50} was 0.063 μg.

DISCUSSION

The data indicate that LA from the marine gram-negative bacterium *M. vaga* ATCC 27119^T has a carbohydrate backbone identical with that of LA from terrestrial bacteria: in both cases it represents β-1,6-linked glucosaminobiose [2]. But the *M. vaga* lipid A has a number of chemically unique characteristics. One of them is presence of extremely short fatty acids (the maximal length of the chain does not exceed 10-12 carbon atoms). 3-Hydroxydecanoic acid, a main acyl residue in the *M. vaga* LA, is rather seldom observed among fatty acids of LA. Until recently, it has been found in LAs only of several bacterial species, such as *Rhodocyclus gelatinosus* [17], *R. tenius* [18], *Rhodobacter capsulatus* [19], *R. sphaeroides* [20], some species of *Pseudomonas* genus (*P. aeruginosa* [21], *P. cichorii* [9], and *P. reactans* [22]), *Sphaerotilus natans* [23], *Comamonas testosteroni* [24], and also *Bordetella pertussis*, *Chromobacterium* sp., and *Rhodopseudomonas* sp. [2]. Only in four of them (*R. gelatinosus*, *R. tenius*, *S. natans*, and *C. testosteroni*), 3-hydroxydecanoic acid occurs both in ester and in amide linkage.

A pattern of phosphate group distribution different from that in enterobacterial LAs was also recognized in the *M. vaga* LA, in which an ester-linked phosphate group attached to the position 4′ of glucosaminobiose completely lacked. Similar structures have been also found in the LAs isolated from *Bacteroides fragilis* [25] and *Porphiromonas gingivalis* [26], *Helicobacter pylori* [27], *Flavobacterium meningosepticum* [28], and, partly, in the LAs from *P. cichorii* [9] and *P. reactans* [22].

The third feature of the *M. vaga* lipid A is that it has low acylation degree and uncommon distribution of acyl substituents over the backbone. Only five fatty acid residues, of which four were 3-hydroxydecanoic acids, were found in its molecule and only one hydroxyl group, at C3 position of the reducing end, was acylated with 3-hydroxydecanoic acid. The other three 3-hydroxydecanoic acid residues, two with amide linkage and one as the secondary acid at C2- or C2′-bound fatty acid, were located at C2 and C2′ positions. Such acyl- and phosphate-deficient non-reducing end was also found in lipid A from *Helicobacter pylori* strain 206-1 but acyloxyalkanoic fatty acid of the latter contained no hydroxy acid [27]. At the same time, A. Molinaro et al. [9, 22] showed that some phytopathogenic bacteria, along with peracylated and perphosphorylated derivatives of LA, synthesize its partly acylated and phosphorylated forms reminiscent of the *M. vaga* LA structure. The further study of these unusual structural forms of LA will answer the question of how widespread they are distributed in nature and whether they can play roles of antagonist of endotoxins. The fact that the *M. vaga* LA has a low toxicity suggests considering such LAs as potential endotoxin antagonists.

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