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EXPERIMENTAL ARTICLES

Capsular and Extracellular Polysaccharides of the Diazotrophic Rhizobacterium *Herbaspirillum seropedicae* Z78

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Abstract—The diazotrophic endophyte *Herbaspirillum seropedicae* Z78 was shown to possess a capsule containing two high-molecular-weight glycolipids, one of which was of a lipopolysaccharide nature. These glycolipids differed considerably in the fatty acid composition of their lipid components. The polysaccharide moiety of these glycans was composed of glucose, galactose, glucosamine, galactosamine, and a noncarbohydrate component, butanetetraol. In the culture liquid of *H. seropedicae* Z78, an extracellular polysaccharide and an extracellular form of lipopolysaccharide were revealed. Fatty acid composition of the extracellular lipopolysaccharide differed from that of the capsular glycoconjugates; the polysaccharide moiety of exoglycans contained only neutral sugars (mannose, glucose, and galactose) and a tetraatomic alcohol, butanetetraol. It is assumed that structural diversity of polysaccharide-containing polymers at the surface of *H. seropedicae* Z78 cells is conditioned by their different roles in plant colonization and formation of efficient symbiosis.

Keywords: Herbaspirillum, endophytic microorganisms, extracellular and capsular polysaccharides, butanetetraol.

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The possible application of diazotrophs as bacterial fertilizers results from the important role of associative nitrogen fixation in the soil nitrogen balance. Bacteria of the genus *Herbaspirillum*, belonging to the β-subclass of *Proteobacteria*, are endophytic associative nitrogen-fixing microorganisms. They were revealed in tissues of such important crops as maize, rice, sorghum, wheat, and sugar-beet [1]. Some *Herbaspirillum* species are able to produce phytohormones stimulating plant growth [1, 2]. Therefore, representatives of the genus *Herbaspirillum* are promising model objects for investigation of plant-microbial associations.

Many microorganisms form capsules around their cell walls, which protect the cells from environmental impact, regulate their water regime, and prevent cell dehydration. Bacteria are also able to excrete a specific slime, in which intercellular interactions occur. Bacterial capsules contain capsular polysaccharides (CPS), whereas microbial slime is composed of extracellular polysaccharides (EPS). All these polysaccharides are K-antigens and play an important role in microbial immunochemical activity [3]. Lipopolysaccharide (LPS, O-antigen) is the obligatory component of the cell wall outer membrane in gram-negative bacteria; it is composed of three structurally different moieties: lipid A (LA), core oligosaccharide, and O-specific polysaccharide (OPS). Capsular glycans of bacteria are composed of LPS [4–6]; some bacteria are able to excrete LPS into the medium [7]. In many soil microorganisms, CPS are involved not only in cell attachment to the root surface but also in the recognition of the host plant. The EPS are necessary for efficient development of legume–rhizobia symbiosis [8]. Plants are known to possess several protective mechanisms against bacterial infections, such as excretion of antibacterial components, phytoalexins, and reactive oxygen species [9, 10]. One of the pathways for defense of phytopathogens and phytosymbionts against the macropartner impact involves the PS of bacterial surfaces, such as CPS, EPS, and LPS. The EPS is assumed to mask the main bacterial antigens at the stage of nodule formation in the legume–rhizobia symbiosis [11]. However, fine mechanisms of plant–microbial interactions are poorly understood; the studies of the composition and structure of biopolymers involved in these processes may provide an insight into this problem. No data on the composition of capsular glycopolymers and extracellular polysaccharides of herbaspirilla are known in the literature. The aim of the present work was to isolate and characterize the CPS and EPS of *H. seropedicae* Z78.

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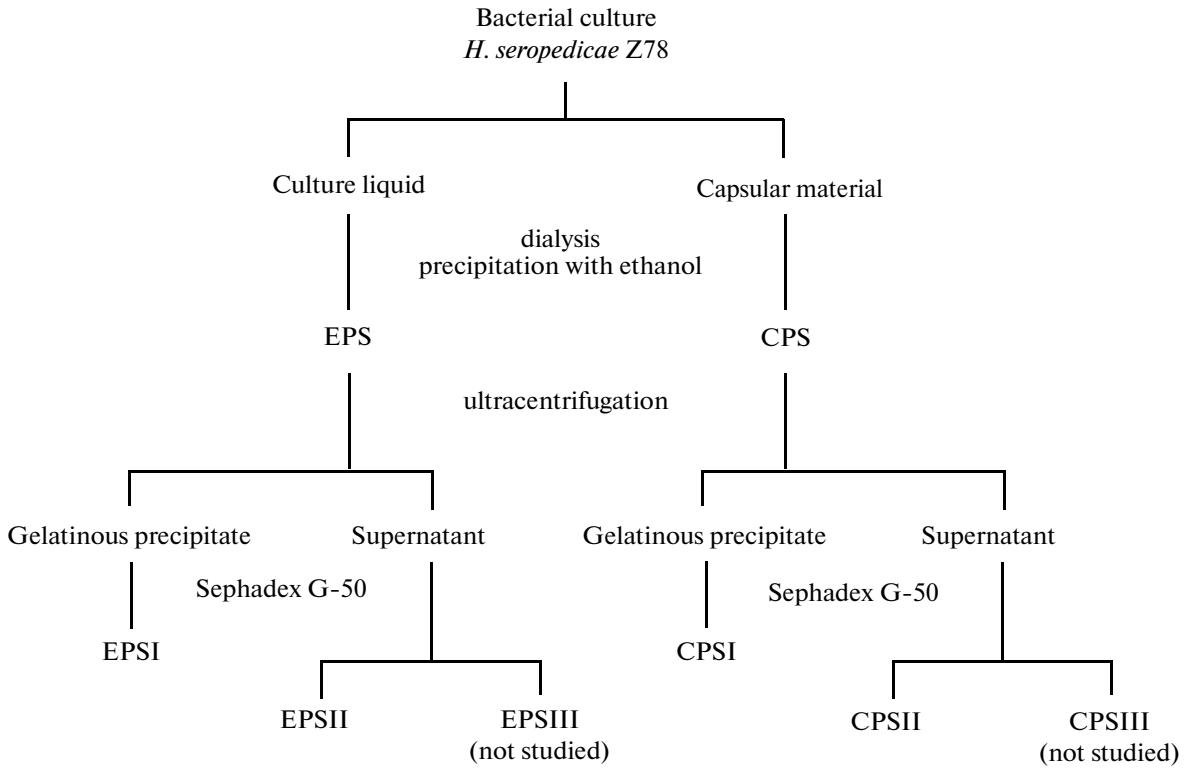

Fig. 1. Scheme of the isolation of capsular and extracellular polysaccharides from *H. seropedicae* Z78.

MATERIALS AND METHODS

The study was carried out with the strain *H. seropedicae* Z78, which was isolated from sorghum roots and presented to the Culture Collection of the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, by J. Döbereiner (Embrapa, Centro Nacional de Pesquisa de Agrobiologia, Seropédica, RJ, Brasil). Bacteria were grown at 30°C to the end of the exponential phase in liquid medium containing the following (g/L): K_2HPO_4, 0.25; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; $Na_2MoO_4 \cdot 2H_2O$, 0.001; $MnSO_4 \cdot H_2O$, 0.002; $FeSO_4 \cdot 7H_2O$, 0.01; $CaCl_2 \cdot 2H_2O$, 0.02; $(NH_4)_2SO_4$, 1.0; malic acid, 5.0; glucose, 5.0; yeast extract, 0.05; biotin, 0.0001; pH 7.2–7.4.

The capsules were visualized by the Gins negative staining method. Drops of fine-dispersed Indian ink and bacterial suspension in 0.15 M NaCl were mixed on a specimen slide, fixed, stained with diluted solution of the Ziehl carbolic fuchsin, washed with water, and air-dried; the specimens were examined under a Leica DM 6000B light microscope (Germany) and photographed using the standard Leica software.

Glycopolymers were isolated according to the scheme (Fig. 1). The biomass was separated by centrifugation (3000*g*); the capsules were washed off the cell surface with 0.15 M NaCl by shaking on a magnetic stirrer for 8 h during 5 days with daily replacement of the washing solution. The culture liquid and capsular material collected during the first two days were concentrated on a Laborota 4000 vacuum rotary evaporator (Germany) and dialyzed against distilled water for 24 h using membranes with a molecular weight cut-off of 12–14 kDa. Ethanol was added to the obtained suspension (3 : 1, vol/vol), and the precipitate containing EPS and CPS was separated by centrifugation, diluted in distilled water, and lyophilized. The 10% water solutions of obtained PS were centrifuged (140000*g*, 6 h, 4°C) on a Beckman Ti 75 ultracentrifuge (Germany). The supernatant and the nonpigmented gelatinous layers of the precipitate were collected; the latter were separated by gel filtration on a column (43 × 2.5 cm, V_0 30 mL) with Sephadex G-50 (Pharmacia, Sweden) using 0.025 M pyridine–acetate buffer (pH 4.1) as an eluent; the eluted substances were detected using an LKB 2142 differential flow refractometer (LKB, Sweden). All carbohydrate-containing fractions which showed no absorption within the 240–260 nm range were combined, concentrated, and lyophilized.

Assays of carbohydrates, 2-keto-3-deoxyoctulosonic acid (KDO), protein, nucleic acids (NA), and phosphorus were carried out by conventional methods [4] using a Specord 40 spectrophotometer (Analytik Jena AG, Germany).

Samples of the glycopolymers were subjected to methanolysis [12]; fatty acids (FA) in the form of methyl esters (FAME) were analyzed on a GC-2010

chromatograph (Shimadzu, Japan) equipped with an EQUTY-1 capillary column (30 m × 0.32 mm) at the temperature gradient from 130 to 250°C at a rate of 4°C/min.

The monosaccharide composition of the polysaccharides (PS) was determined by gas-liquid chromatography–mass spectrometry (GLC–MS) of acetate polyols (AP) and partly methylated AP [12, 13] on a Finnigan Trace DSQ device (ThermoFinnigan, United States) equipped with a TR-5MS column (25 m × 0.15 mm); the temperature was maintained at 160°C for 5 min and then increased to 250°C at a rate of 2°C/min.

Electrophoresis was performed in 15% SDS–PAGE according to the Hitchcock and Brown method [14] at 0.03 A for 1.5 h. Glycans were visualized by staining of the polyacrylamide gel with a silver nitrate-based dye [15].

The results were statistically processed with the confidence interval of 95%.

RESULTS AND DISCUSSION

The strain *H. seropedicae* Z78 was cultivated to the end of the exponential phase (16 h) in liquid synthetic medium with malate and glucose.

Microbial cells grown in carbohydrate-rich media are often surrounded by loose outer layers (capsules), which are usually visualized by the method of negative staining. To study the capsules of *H. seropedicae* Z78, we used the Gins method, which is simple and makes it possible to visualize the capsules of 0.5 μm thick or larger; light oval areas of the capsules containing pink-crimson bacterial cells were well-distinguished against a dark gray background (Fig. 2).

From the culture liquid and the capsular material washed off the cell surface with 0.15 M NaCl, the extracellular and capsular polysaccharides (EPS and CPS, respectively) were isolated by precipitation with a threefold volume of ethanol (1.5 and 0.7% of dry cell mass, respectively).

The preparations of EPS and CPS contained carbohydrates (35.4 and 10.8%, respectively), phosphorus (1.0 and 5.0%, respectively), protein (4.0 and 1.3%, respectively), and KDO (0.3 and 2.0%, respectively). KDO, which is a constituent part of the core oligosaccharide structure, is known as a marker of LPS molecules. However, KDO had been revealed in the CPS of some strains of *E. coli* [5] and *Azospirillum* [4] and in repeating units of CPS in bacteria of the genus *Rhizobium* [16].

The preparations contained saturated acids and unsaturated alkanoic and hydroxyalkanoic acids with chain lengths from C_{10} to C_{18} (Table 1). In the CPS, 3-hydroxydecanoic (3-OH-$C_{10:0}$), 3-hydroxydodecanoic (3-OH-$C_{12:0}$), tetradecanoic ($C_{14:0}$), and hexadecanoic ($C_{16:0}$) acids prevailed; the amounts of dodecanoic ($C_{12:0}$), hexadecenoic ($C_{16:1}$), and octadecenoic ($C_{18:1}$) acids varied from 3 to 6%; the sum of *iso*- and *anteiso*-alkanoic acids was about 1%. The fatty acid composition of the EPS differed from that of the CPS by the absence of 3-OH-$C_{12:0}$, two-fold higher content of both 3-OH-$C_{10:0}$ and $C_{18:1}$ acids and half the content of $C_{14:0,}$ whereas the amounts of $C_{16:0}$ and $C_{16:1}$ acids were similar. In the EPS preparation, *iso*- and *anteiso*-alkanoic FA were revealed: 13-methyltetradecanoic (*i*-$C_{15:0}$), 12-methyltetradecanoic (*a*-$C_{15:0}$), and 14-methylpentadecanoic (*i*-$C_{16:0}$); their total content was 6.7%. In the CPS and EPS preparations, the amount of predominant fatty acids was 86.2 and 85.2% of the total FAME identified, respectively; the sum of hydroxy acids was 39.6 and 45.6%, respectively.

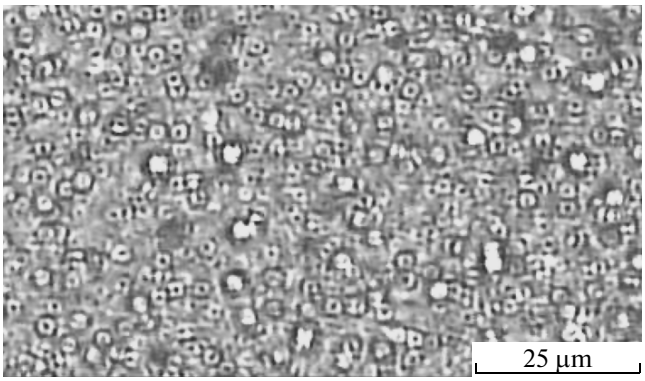

Fig. 2. Cells of *H. seropedicae* Z78 surrounded by capsules. Negative staining by the Gins method. Scale bar, 25 μm.

The even-numbered 3-hydroxy acids (C_{12}, C_{14}) are known as obligatory components of enterobacterial LPS [17]. However, some microorganisms synthesize lipid A (LA) composed of hydroxy acids with a shorter carbon chain length. For instance, 3-OH-$C_{10:0}$ acid was found in the LA of *Comamonas testosteroni* [18] and *Rhodobacter capsulatus* [19]. *Iso*- and *anteiso*-acids were revealed in the membrane LPS of *Flavobacterium meningosepticum* (*i*-$C_{15:0}$) [20] and *Bacteroides fragilis* (*i*-$C_{15:0}$) [21], while the EPS of *Xanthomonas campestris* contains 15-methylhexadecanoic (*i*-$C_{17:0}$) and 14-methylhexadecanoic (*a*-$C_{17:0}$) [22]. It is believed that the presence of such acids in LA promotes the adaptation of microorganisms to environmental impact.

The presence of KDO and hydroxy acids in the total EPS and CPS preparations probably indicated that these PS were extracellular forms of LPS. This assumption, together with revealed differences between the EPS and CPS in FA profiles and the contents of carbohydrates and KDO, indicated the necessity of further investigations.

We isolated the LPS by ultracentrifugation of 10% water solutions of the initial EPS and CPS preparations. The obtained triple-layered sediments included

Table 1. Fatty acid composition of glycolipid preparations isolated from the capsular material and culture liquid of *H. seropedicae* Z78

Components	FAME (% of the sum of identified peak areas)				
	EPS	EPSI	CPS	CPSI	CPSII
2-Hydroxydecanoic, 2-OH-$C_{10:0}$	traces	12.4	traces	–	8.1
3-Hydroxydecanoic, 3-OH-$C_{10:0}$	45.6	17.3	19.7	21.1	–
Dodecanoic, $C_{12:0}$	traces	10.6	2.7	2.6	–
Tetradecanoic, $C_{14:0}$	9.7	6.2	27.7	29.6	–
3-Hydroxydodecanoic, 3-OH-$C_{12:0}$	–	–	19.9	23.2	–
Pentadecanoic, $C_{15:0}$	–	–	–	–	9.0
13-Methyltetradecanoic, *i*-$C_{15:0}$	2.2	5.4	traces	–	12.9
12-Methyltetradecanoic, *a*-$C_{15:0}$	2.7	6.2	traces	–	22.3
14-Methylpentadecanoic, *i*-$C_{16:0}$	traces	4.8	traces	–	18.1
Hexadecanoic, $C_{16:0}$	19.5	15.4	18.9	20.7	12.3
Hexadecenoic, $C_{16:1}$	5.3	5.0	5.9	2.7	–
15-Methylhexadecanoic, *i*-$C_{17:0}$	–	–	traces	–	8.0
14-Methylhexadecanoic, *a*-$C_{17:0}$	–	–	traces	–	9.3
Octadecenoic, $C_{18:1}$	10.4	13.3	3.9	–	–
Octadecanoic, $C_{18:0}$	traces	3.3	–	–	–

Note: "traces" means "not exceeding 2%"; "–" stands for "component is absent".

a cell-containing lower layer and a gelatinous zone with a pigmented lower part and a transparent, slightly colored upper layer. The supernatants containing extracellular and capsular PS and the transparent upper layer of the precipitate, which was assumed to contain extracellular and capsular lipopolysaccharides, were used for analysis. The preparations were separated by gel-filtration on a column with Sephadex G-50. The precipitates of EPS and CPS dissolved in 0.025 M pyridine–acetate buffer showed the elution profile with one symmetric peak at the void volume of the column (Fig. 3). The obtained preparations, which were designated as EPSI and CPSI, amounted to 8.3 and 4.2% of the initial sample weight, respectively. The elution profile of the EPS supernatant differed from that of the precipitate and contained two peaks corresponding to the high-molecular weight fraction eluted at the void volume of the column (EPSII) and the intensely colored fraction of lower molecular weight (EPSIII, not studied) (Fig. 3a). The CPS supernatant was highly heterogeneous in molecular weight; it was conditionally divided into the colorless (CPSII) and a pigmented (CPSIII, not studied) fraction (Fig. 3b). The yields of EPSII and CPSII were 0.9 and 1.3% of the total preparation weight, respectively.

The preparations were also analyzed by electrophoresis in 15% SDS–PAGE (figure not shown). The CPSI preparation formed a series of narrow bands throughout the electrophoretic track that is indicative of its high heterogeneity. The macromolecules of CPSII formed two narrow bands in the middle part of the track. The EPSI profile contained a wide band in the upper part of the track and a series of narrow bands located below. The EPSII showed two narrow, poorly visible bands in the upper part of the track and one intensely colored wide band in the lower part.

In all isolated preparations, carbohydrates, KDO, phosphorus, and protein were revealed with the use of specific reactions (Table 2). Preparations of EPSI and EPSII contained carbohydrates (66.1 and 47.3%, respectively) and KDO (0.4 and 0.2%, respectively), whereas CPSI and CPSII lower carbohydrate contents (almost by an order of magnitude), while their KDO content was 0.7 and 0.5%, respectively. The amount of phosphorus was the largest in CPSII (4.5%) and 3–5-fold lower in EPSI, EPSII, and CPSI. Protein content was relatively high in EPSI (6.9%) and ranged from 0.2 to 1.5% in other preparations (Table 2).

The FAME were revealed in EPSI, CPSI, and CPSII but not in EPSII. In EPSI, the major fatty acids were represented by 2-OH-$C_{10:0}$, 3-OH-$C_{10:0}$, $C_{12:0}$, $C_{16:0}$, and $C_{18:1}$; their sum reached 70%, including 30% of hydroxy acids; the contents of $C_{14:0}$, *i*-$C_{15:0}$, *a*-$C_{15:0}$, *i*-$C_{16:0}$, $C_{16:1}$, and $C_{18:0}$ varied from 3 to 6%. The fatty acid profiles of CPSI and CPSII were similar to that of the CPS in their content of major and minor acids (except for $C_{16:0}$) (Table 1). These results indicated that the lipid moiety of CPSI prevailed in the CPS pool, which was expected since the yield of CPSI was four times higher than that of CPSII. Hydroxy acids

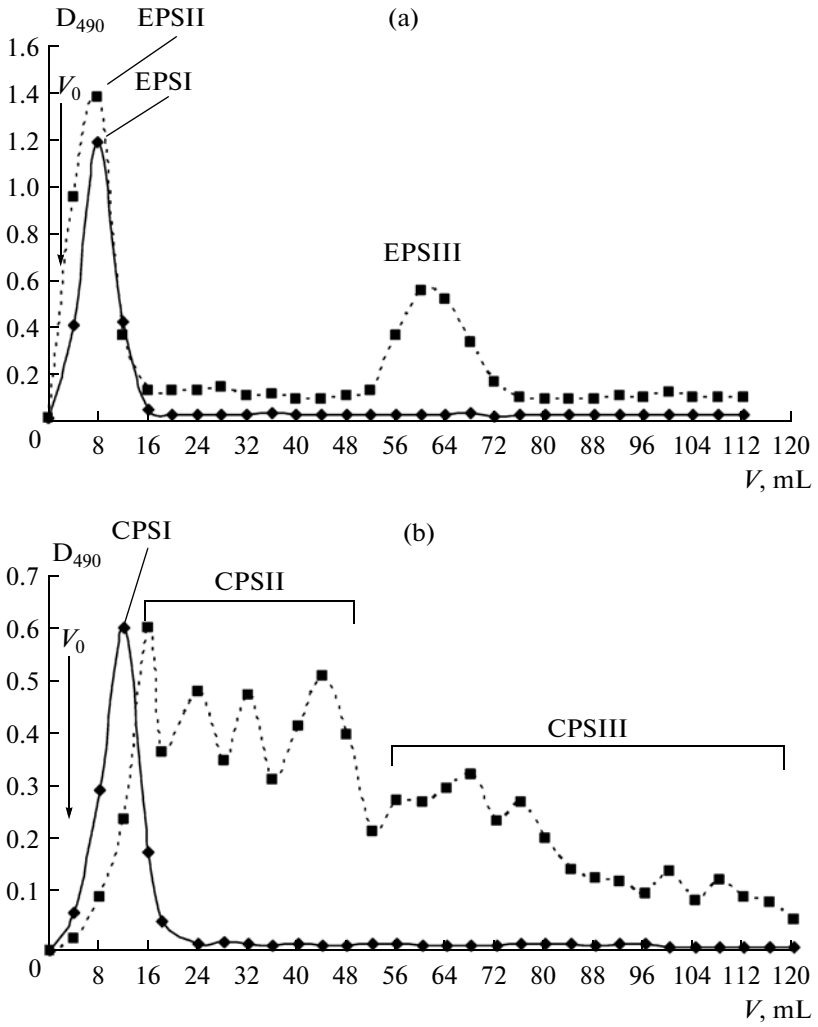

Fig. 3. Gel filtration elution profiles on Sephadex G-50 of glycans isolated from the culture liquid (a) and the capsular material (b) of *H. seropedicae* Z78. Solid and dashed lines indicate gelatinous precipitates and supernatants, respectively. The abscissa shows fraction volumes; the ordinate indicates optical density of the products of carbohydrate reactions with phenol and sulfuric acid.

amounted to 44.3% in CPSI and only 8.1% in CPSII. The low content of hydroxy acids in CPSII probably indicated that this preparation was not an LPS in spite of the presence of KDO, rather a polysaccharide–lipid complex. It should be noted that in the LPS isolated from the outer membranes of capsule-free cells of *H. seropedicae* Z78, 3-OH-$C_{10:0}$, 3-OH-$C_{12:0}$, $C_{14:0}$, and $C_{16:0}$ acids prevailed, similar to EPSI (except for 3-OH-$C_{12:0}$) and CPSI. However, the LPS was shown to contain, apart from these acids, also considerable amounts of 2-OH-$C_{12:0}$ and 2-OH-$C_{14:0}$ acids [23]. The 3-OH-$C_{10:0}$, $C_{12:0}$, 3-OH-$C_{12:0}$, $C_{14:0}$, and $C_{16:0}$ acids were found in the LPS from other strains of herbaspirilla [24].

Monosaccharide composition of the preparations was determined by GLC–MS of acetate polyols. In EPSI and EPSII, mannose (Man), glucose (Glc), and galactose (Gal) prevailed in a ratio of 1 : 3 : 4 and comprised up to 95% of the total identified peaks. Moreover, small amounts of butanetetraol were revealed in EPSI and EPSII; traces (below 0.5%) of glucosamine (GlcN) and galactosamine (GalN) were found in EPSI. In capsular PS, rhamnose (Rha), GlcN, and GalN were found in addition to the above-mentioned neutral sugars and tetraatomic alcohol. The predominant components were represented by Glc, Gal, GlcN, and GalN in a ratio of 3 : 3 : 1 : 1; their sum reached 82%. The amounts of Man, Rha, and butanetetraol did not exceed 3–5% depending on the strain. The presence of noncarbohydrate components in the glycopolymers CPSI and CPSII may explain the low content of carbohydrates in these preparations (Table 2). However, the amount of butanetetraol in the capsular glycans was only twice as high as in the extracellular ones. We assumed that not all butanetetraol residues were acetylated. Indeed, the GLC–MS anal-

Table 2. Biochemical composition and yield of the glycans isolated from the culture liquid and capsular material of *H. seropedicae* Z78

Components		EPSI	EPSII	CPSI	CPSII
Amount, % (wt/wt)	Carbohydrates	66.1 ± 3.6	47.3 ± 1.7	7.3 ± 0.8	4.2 ± 0.4
	Protein	6.9 ± 1.7	0.8 ± 0.1	1.5	0.2
	NA	–	–	traces	–
	KDO	0.4	0.2	0.7 ± 0.1	0.5
	Total phosphorus	1.6 ± 0.1	0.8 ± 0.1	1.1 ± 0.2	4.5 ± 0.2
Yield (% of initial preparation, wt/wt)		8.3	0.9	4.2	1.29

Note: "NA" stands for "nucleic acids"; "–" means "component is absent"; "traces" denotes "below 0.01%".

ysis of partly methylated acetate polyols showed that this polyalcohol comprised up to 80% of the total identified monomers in the capsular PS and about 20% in the extracellular ones; these data are in good agreement with the carbohydrate content of the samples. The monosaccharide composition of the capsular glycans was similar to that of the membrane LPS; however, the latter contained considerable amounts of deoxysugars and heptoses (Hep) in addition to alcohol, neutral hexoses, and amino sugars [23]. It should be noted that residues of polyols (glycerol, ribitol, and mannitol) are essential parts of teichoic acids in gram-positive bacteria. However, alcohol residues were also found in the repeating units of the LPS and CPS of gram-negative microorganisms both as side substituents and within the main chains; they were bound with sugar residues by phosphodiester bonds [25].

Thus, capsule formation by *H. seropedicae* Z78 was established and a glycopolymer of a lipopolysaccharide nature (CPSI) and a polysaccharide–lipid complex (CPSII) were revealed within the capsule. Bacteria excreted an extracellular form of LPS (EPSI) and a polysaccharide (EPSII). The EPS differed from the capsular glycans in the composition of both monosaccharides and the lipid component of EPSI. The polysaccharide moiety of the CPS was composed by 80% of butanetetraol residues, which were most probably bound via phosphodiester bonds (similar to teichoic acids) and characterized by a high negative charge density. Much like the polyanionic EPS of rhizobia and various phytopathogens [26, 27], the CPS of herbaspirilla can chelate Ca^{2+} ions and participate in repression of the plant defensive reactions. LPS of many microorganisms, along with the other microbial elicitors, were shown to induce the defensive mechanisms of plant cells. However, in the case of symbiosis, bacterial PS including LPS often play the role of suppressors inhibiting the output of NO and reactive oxygen species, as well as the input of calcium ions [26]. The CPS and EPS of *H. seropedicae* Z78, being the extracellular forms of LPS, can have a similar effect on plants, optimizing the conditions of endophytic habitats. Heterogeneity of polysaccharide-containing polymers from the *H. seropedicae* Z78 surface is probably conditioned by their different functions in plant colonization and formation of efficient symbiosis, as well as in cell adaptation to the existence within plant tissues.

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