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COMPARATIVE CHARACTERISTICS OF LIPOPOLYSACCHARIDE–PROTEIN COMPLEXES FROM THE NUCLEI OF BACTERIA OF THE FAMILY PSEUDOMONACEAE

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Ethanol-assimilating bacteria of the family Pseudomonaceae are good sources of fodder protein. They give about 5 g of dry biomass per 1 liter of cheap synthetic medium. The study of the cell walls of some bacteria of the family Pseudomonaceae has shown considerable differencs in their chemical compositions, particularly in the composition of the lipopolysaccharides [1].

These facts have led to the necessity of searching for the most effective producing agents of lipopolysaccharide–protein complexes (LPPC's) among the bacteria of this family and for a comparative characterization of the complexes isolated. We have investigated *Pseudomonas fluorescens* 361, *Acinetobacter* sp. 34, 154, and 30, and their mutants. The LPPC's from the various strains of bacteria were isolated by Boivin's method [2]. The general characteristics of the extracts obtained are given below (%):

	P. fluorescens			*Acinetobacter sp.*		
	361	*55*	*154*	*30*	*34*	*18*
Yield of extracts	3,4	1,6	0,6	1,2	3,1	1,0
Yield of lipid A	16,1	2,3	11,7	10,7	9,1	8,1
Monosaccharides	36,9	15,0	18,9	1 ,3	15,5	15,7
Amino sugars	6,6	5,5	2,5	5,9	1,2	0,9
KDO	2,7	Tr.	Tr.	1,1	0,0	0,0
Pentoses	10,7	4,6	0,0	Tr.	0,0	0,0
Protein	16,8	11,5	32,9	30,9	18,9	22,9
Nucleic acids	0,7	0,7	8,1	0,8	1,7	4,5
Phosphorus	3,1	1,4	1,3	0,7	0,5	0,7
Nitrogen	4,6	2,6	4,1	5,1	3,3	4,2
Ash	9,0	4,6	7,3	5,6	17,0	5,9

The yield of extracts amounted to 1-3.5% of the weight of the dry microbial mass. All the extracts contained a relatively small amount of monosaccharides. These facts are in harmony with the results obtained by Fensom and Grey, who showed that the LPS's from bacteria of the family Pseudomonaceae contain a smaller amount of monosaccharides than the LPS's from the family Enterobacteriaceae [3].

The results of a determination of the qualitative monosaccharide composition of the extracts by the PC and GLC methods were as follows:

Bacterium	*X*	*Rha*	*Fuc*	*Xyl*	*Man*	*Gal*	Glc	*Hep*
P. fluorescens 361	+++	+	+++	+	++	+	+++	+++
" 55	+	+	–	+	+	Tr.	++	+
Acinetobacter sp. 154	+	–	+	Tr.	–	+++	++	–
" 34	+	–	–	Tr.	Tr.	+++	+++	–
" 18	–	–	+	+	–	+++	++	–
" 30	+	Tr.	–	+	+	++	+++	+

X) unidentified; Rha) rhamnose; Fuc) fucose; Xyl) xylose; Man) mannose; Gal) galactose; Glc) glucose; Hep) heptose.

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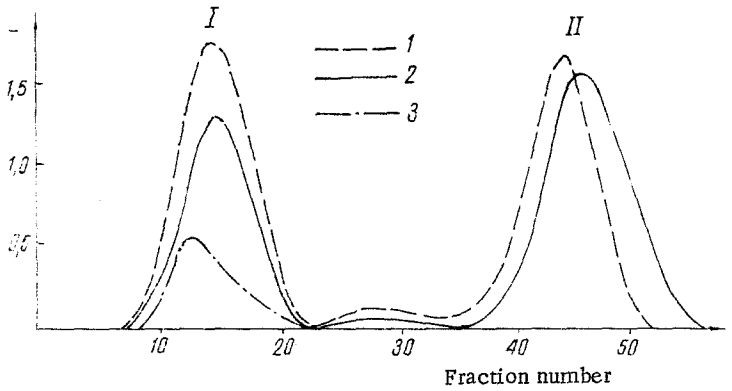

Fig. 1. Fractionation of extracts from *P. fluorescens* 361: I) High-molecular-weight fraction; II) low-molecular-weight fraction; 1) analysis for sugars (at E 480 nm); 2) for proteins (at E 280 nm); 3) at KDO (at E 549 nm).

The amount of 2-keto-3-deoxyoctonoic acid (KDO) varies from species to species. KDO could not be detected in the extract from *Acinetobacter* sp. 154 and 34 by the reaction with thiobarbituric acid (TBA). This fact can be explained either by the absence of KDO or by the assumption that the KDO is partially substituted and does not give the color reaction with TBA [4]. In extracts from mutant strains of *Acinetobacter* sp. 18 and *P. fluorescens* 55 obtained with the aim of raising the yield of proteins, only a very small amount of KDO was isolated. The high heptose content in *P. fluorescens* and its absence in *Acinetobacter* sp. must be noted.

As is well known, in the majority of LPS's studied the lipid component is split off from the polysaccharide component by mild acid hydrolysis. To determine the amount of such lipid component, extracts previously treated with n-butanol to eliminate free acids were hydrolyzed with 1% acetic acid. The yields (see above) amounted to 2-16% of the weight of the extracts.

Thus, the results of analysis showed that extracts from the bacteria *Acinetobacter* sp. 154, 34, and 18 contain no KDO and heptoses, which are characteristic components of the LPS's from Enterobacteriaceae and a number of representatives of the Pseudomonaceae.

Additional information was necessary for a clear assignment of the polysaccharide and lipid components found in extracts from these bacteria, and we therefore studied extracts from *P. fluorescens* 361 and *Acinetobacter* sp. 30.

To purify the LPPC's we used chromatography of the extracts from these bacteria on a column of Sepharose 2B. The samples were deposited on the column and were eluted with Tris-HCl buffer (pH 8). This gave two fractions: a high-molecular-weight fraction issuing with the free volume and a low-molecular-weight fraction (Fig. 1). The fractions (3 ml) were analyzed for sugars, proteins, and KDO.

The total analysis of the LPPC's (fraction I obtained by chromatographing the extracts on Sepharose 2B) gave the following results %:

	P. fluorescens 361	*Acinetobacter 30*
Yield	40,1	45,0
Monosaccharides	23,6	14,2
Amino sugars	1,61	2,3
KDO	6,3	2,1
Heptoses	20,5	0,0
3,6-Dideoxy sugars	0,03	0,01
Phosphorus	1,6	0,6
Protein	18,8	17,4
Nucleic acids	0,06	0,07

According to the analytical results, the whole of the LPPC's is present in the high-molecular-weight fraction. As a result, there was an effective purification from ballast substances and the concentration of KDO rose 1.5-fold and of heptose 2-fold. The first and second fractions differ in their qualitative monosaccharide compositions:

Fraction	X	Rha	Fuc	Xyl	Man	Gal	Glu	Hep
				P. fluorescens				
I	++	+	+	—	++	+	++	+++
II	+	—	++	+	+	+	++	Tr.
				Acinetobacter sp. 30				
I	+	Tr.	—	+	+	Tr.	++	Tr.
II	+	—	—	Tr.	+	++	+	—

Similar results were obtained for *Acinetobacter* sp. 30.

Both for *P. fluorescens* 361 and for *Acinetobacter* sp. 30 both fractions contained an unidentified monosaccharide with a retention time smaller and an R_f value greater than for rhamnose.

The results of amino-acid analyses of fractions I and II from *P. fluorescens* 361 showed a slight predominance of alanine in the first fraction:

Amino acid, %	Fraction I	Fraction II
Aspartic acid	0,65	1,50
Threonine	0,20	0,26
Serine	0,35	0,40
Glutamic acid	0,40	1,00
Proline	0,20	0,30
Glycine	0,45	0,52
Alanine	0,65	0,35
Valine	0,45	0,15
Leucine	0,20	0,23
Tyrosine	0,10	0,08
Phenylalanine	0,10	0,10
Lysine	0,30	0,20
Histidine	0,20	0,12
Arginine	0,16	0,27

Thus, the LPPC from *P. fluorescens* 361 is obtained in higher yield and contains more KDO heptoses, and lipid A than the complex from *Acinetobacter* sp. 30. This LPPC is antigenic for rabbits and mice (indirect agglutination titer 1:10240) and gives one precipitation line in agar with hyperimmune serum.

EXPERIMENTAL

Partition paper chromatography was performed on Filtrak No. 3 and No. 15 papers using the following solvent systems: 1) butan-1-ol—pyridine—water—acetic acid (6:4:3:0.3); 2) butan-1-ol—acetic acid—water (4:1:5). To reveal the monosaccharides we used 1) aniline hydrogen phthalate; 2) an alkaline solution of silver nitrate; and 3) a 0.2% solution of ninhydrin in acetone.

The monosaccharide compositions were determined in the following way. Samples of the LPPC's (5-10 mg) were hydrolyzed with 2 N H_2SO_4 (1 ml) at 100°C for 4 h. The hydrolyzates were neutralized with barium carbonate, deionized with Amberlite IR-120 [H^+], evaporated, and used for PC.

The amino sugars were determined by performing hydrolysis with 4 N hydrochloric acid (1 ml of acid per 10 mg of sample) at 100°C for 4 h. The hydrochloric acid was eliminated by repeated evaporation in vacuum, and the concentrated solution of the hydrolyzate was deionized on Dowex I (HCO_3^-) and used for chromatography and analysis by the Morgan—Elson method [5].

The quantitative analysis of the amino acids (hydrolysis in 6 N HCl, 105°C, 16 h) was performed on a Biocal-3201 automatic amino-acid analyzer from the firm LKB (Sweden).

Gas—liquid chromatography (GLC) was performed on a Pye Unicam-104 chromatograph (U.K.) with a flame-ionization detector and a double system of glass columns (1.5 m × 0.4 cm), and the chromatography of the monosaccharides in the form of the aldononitrile acetates and the corresponding polyol acetates on a column with 3% of QF on Gas-Chrom Q (100-120 mesh) with programming of the temperature from 175 to 225°C (5 deg/min).

To determine the KDO, the LPPC's (1-3 mg) were hydrolyzed with 0.2 N H_2SO_4 (1 ml) at

100°C for 10 min. The KDO was identified by paper electrophoresis in pyridine acetate buffer (100:40:860, pH 5.3) for 3 h in comparison with an authentic sample. On the paper the KDO was detected by the aid of TBA [6]. The KDO was determined quantitatively by the method of Burtseva et al. [7].

For the gel chromatography of solutions (5-6 ml) of samples of LPPC's (50-60 mg) in Tris-HCl buffer (pH 8) we used columns (20 × 65 cm) of Sepharose 2B (Pharmacia, Uppsala, Sweden).

The total amount of monosaccharides was determined by the phenol—sulfuric acid method using glucose as standard [8], the protein content by Lowry's method [9], the nucleic acids by Spirin's method [10], the heptoses by Sinilova's method [11], and the ash by igniting samples at 600°C to constant weight.

The strains *P. fluorescens* 361 and 55 and *Acinetobacter* sp. 30, 34, 154, and 18 were supplied by the Minsk Institute of Microbiology and Epidemiology of the Academy of Sciences of the Belorussian SSR. The strains *P. fluorescens* 55 and *Acinetobacter* sp. 18 are mutants of strains 361 and 34, respectively. To isolate and purify all the cultures we used a synthetic medium of the following composition (g/liter): ethanol, 15.3; urea, 1.91; yeast autolyzate, 2.0; KH_2PO_4, 3.8; H_2PO_4, 3.5; $MgSO_4 \cdot 7H_2O$, 1.3; pH, 7.0.

The microorganisms were grown at 28°C on dry nutrient agar (Dagestan Institute of Nutrient Media) for 48 h. The cells were washed with distilled water, separated by centrifuging, washed with water and acetone, and dried in the air.

Isolation of the LPPC's. The bacterial mass from the various cultures was treated twice with a 10-fold excess of 5% trichloroacetic acid solution by Boivin's method [2]. The extracts were combined and were dialyzed against distilled water. To eliminate free lipids, the extracts were treated with n-butanol [12]. Then they were dialyzed against distilled water and freeze-dried.

Isolation of Lipid A. The LPPC's (20 mg) were hydrolyzed with 1% acetic acid (3 ml) at 100°C for 3 h. This gave lipid A in the form of a precipitate with a yield of 3.2 mg (16%).

Serological Methods. Rabbits were immunized intravenously with the LPPC's three times at intervals of 3 days. The doses of antigen amounted to 50, 100, and 100 mg. Blood was taken on the 4th, 7th, and 10th days after immunization. The titers of the sera were determined by the indirect hemaglutination reaction [13]. For the sensitization of the erythrocytes, the LPPC's (4 mg) were treated with 0.02 N NaOH for 18 h at 37°C. The precipitation reaction was performed by Ouchterlony's method [14].

SUMMARY

Screening performed among representatives of Pseudomonaceae has shown that *P. fluorescens* 361 is the most suitable source of LPPC's.

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