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Absence of a Characteristic Cell Wall Lipopolysaccharide in the Phototrophic Bacterium *Chloroflexus aurantiacus*

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Two strains of the gliding phototrophic bacterium *Chloroflexus aurantiacus* were investigated for the presence of lipopolysaccharide (LPS). With both strains, all fractions of hot phenol-water extracts and the extracted cell residues from whole cells or cell homogenates were found to be free from characteristic LPS constituents, such as 3-hydroxy fatty acids, 2-keto-3-deoxyoctonate, heptoses, or O-chain sugars. Phenol-chloroform-petroleum ether extracts were also free from precipitable LPS. A lipid A fraction could not be obtained, and there was no hint for glucosamine as a possible lipid A backbone amino sugar. Absence of LPS was confirmed by sodium deoxycholate gel electrophoresis.

Phototrophic bacteria are generally believed to have a gram-negative cell wall organization (3). Cells of purple non-sulfur and sulfur bacteria (*Chromatiaceae*), as well as those of green sulfur bacteria (*Chlorobiaceae*), have an outer membrane and contain typical lipopolysaccharides (LPS; 3, 21). Recently, however, peptidoglycan with some characteristics of peptidoglycan from gram-positive bacteria was found in the gliding, thermophilic phototrophic bacterium *Chloroflexus aurantiacus* (9). A complex polysaccharide is likely bound to this peptidoglycan, and a (lipo)protein comparable to that of gram-negative bacteria is lacking (9). The *Chloroflexaceae*, together with the *Chlorobiaceae*, form the order *Chlorobiales*. They share characteristic chlorosome structures. In a phylogenetic sense, however, the *Chloroflexaceae* are deeply separated from the *Chlorobiaceae* (and from other eubacteria as well; 7, 24). In this study, absence of typical cell wall LPS in *C. aurantiacus* was revealed.

MATERIALS AND METHODS

Cultivation of bacteria. *C. aurantiacus* J-10-fl was obtained from R. W. Castenholz, and strain Ok-70-fl (DSM 636) was from J. H. Klemme, Institut für Mikrobiologie, Bonn, Federal Republic of Germany. Strain J-10-fl was grown photoheterotrophically in medium D (2) in a 12-liter Microferm (New Brunswick, N.J.) fermentor at 55°C and a light intensity of 2,000 lx at the surface of the fermentor. Strain Ok-70-fl was cultivated in the medium described by Kaulen and Klemme (10), in 1- or 2.5-liter glass bottles at 50°C and 2,000 lx. Cultures were harvested at the late logarithmic growth phase and washed once with distilled water before storage at −20°C until use.

Preparation of cell homogenates. Cells (15-g wet weight), in 20 mM Tris hydrochloride buffer (pH 8.0) containing 0.3 M sucrose, were broken in a French pressure cell at 16 kPa and 4°C. For collection of a crude cell envelope fraction, the homogenate was centrifuged at 113,000 × *g* (4°C, 60 min) (see fraction CE in Table 2).

Phenol-chloroform-petroleum ether and phenol-water extraction and saline treatment. Unbroken whole cells or cell homogenates obtained by French pressure cell treatment (8) were used for extraction by hot phenol-water (23). The water and phenol phases (after dialysis) were ultracentrifuged (105,000 × *g*, 4°C, 4 h), and the supernatants and pellets (see fractions WPh-P and PhPh-P in Table 2) were lyophilized. The phenol-water interphase (see fraction IPh in Table 2) and the sedimenting extracted insoluble cell residues (see fraction CR in Table 2) were separately lyophilized. Phenol-chloroform-petroleum ether extraction was performed as described by Galanos et al. (6). Cells were treated with 0.9% sodium chloride (saline) at 37°C (2 h) as described elsewhere (22).

Analytical chemical determinations. Amino acids and amino sugars were liberated by 4 M HCl at 105°C for 18 h and determined on an LC 6001 automatic amino acid analyzer equipped with a BT 7040 sample injector (Biotronik, Munich, Federal Republic of Germany) as described elsewhere (4).

Conditions for liberation and gas-liquid chromatographic determination of neutral sugars (as alditol acetates), uronic acids, and fatty acids (as methyl esters) were as described elsewhere (5, 8). Mass spectrometric fragmentation of neutral sugar alditol acetate derivatives ($NaBD_4$ reduced) was performed in a Finnigan 1020B MAT automatic gas chromatography-mass spectrometry system with an SE-54 fused silica capillary column (length, 25 m; inner diameter, 0.25 mm; carrier gas, He; column temperature, 120 to 300°C in a 5°C/min program; injection temperature, 300°C). Mass spectra were taken in the mass range of 43 to 400 *m/e* per s.

Fatty acid methyl esters were separated on an EgSS-X column (15% on Gas-chrom P; 100 to 200 mesh; 165°C; isothermal) and on the SE-54 column (length, 50 m; inner diameter, 0.25 mm; carrier gas, N_2; column temperature, 200 to 285°C in a 4°C/min temperature program). 2-Keto-3-deoxyoctonate (20) and phosphate (13) were determined colorimetrically.

DOC-PAGE. Polyacrylamide gel electrophoresis (PAGE) was performed with sodium deoxycholate (DOC) as the detergent (12; T. Komuro and C. Galanos, unpublished data). The running gel consisted of 13% acrylamide, 0.35% bisacrylamide, 0.5% DOC, and 375 mM Tris hydrochloride buffer at pH 8.8, and the stacking gel was 4% acrylamide, 0.1% bisacrylamide, 0.5% DOC, and 125 mM Tris hydrochloride at pH 6.8. The sample buffer contained 0.25% DOC, 10% glycerol, and 175 mM Tris hydrochloride at pH 6.8, and the electrode buffer contained 0.25% DOC, 192 mM glycine, and 26 mM Tris hydrochloride at pH 8.4. After pre-electro-

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TABLE 1. Yields of fractions upon hot phenol-water extraction of *C. aurantiacus* cells

Fraction	Yield (mg [dry wt]) of fraction from:		
	Strain J-10-fl		Strain OK-70-fl cell homogenate (2 g [dry wt])
	Unbroken cells (55 g [wet wt])	Cell homogenate (15 g [wet wt])	
Water phase supernatant[a]	364	295	188
Water phase pellet[a]	102; 116[b]	17	62
Phenol phase (supernatant)[a]	40	20	33
Phenol phase pellet[a]	13	2	7
Phenol-water interphase	2,781	925	729
Pellet (insoluble)	3,956	395	388

[a] From ultracentrifugation at 105,000 × *g* and 4°C for 4 h.
[b] Amount of pellet from precentrifugation at 2,500 × *g* and 4°C for 30 min before ultracentrifugation.

phoresis at 25 mA, the samples (0.1% in sample buffer) were separated at 18 mA (stacking gel) and 25 mA (running gel) with fresh buffer. Silver staining was done as described by Tsai and Frasch (19).

RESULTS

Absence of LPS-specific compounds. Unbroken whole cells of *C. aurantiacus* J-10-fl and Ok-70-fl were extracted with hot phenol-water. All of the fractions obtained after phase separation (water phase, phenol-water interphase, and phenol phase) and the insoluble cell residue were separately isolated; the water and phenol phase materials were ultracentrifuged (105,000 × *g*, 4°C, 4 h) (for yields of fractions obtained with strain J-10-fl, see Table 1). All of the fractions were analyzed for neutral and amino sugars, 2-keto-3-deoxyoctonate, fatty acids, phosphate, and amino acids (for data obtained with strain J-10-fl, see Table 2).

In none of the fractions were 3-hydroxy fatty acids found as characteristic lipid A constituents. Mass spectrometry of the hydrolytically liberated fatty acid methyl esters showed no *m/e* 103 (cleavage between C atoms 3 and 4 without rearrangement) in any of the fatty acid peaks obtained. The same was true for 2-hydroxy fatty acids. Mass spectrometric fragmentation yielded no peak *m/e* 90 (cleavage between C atoms 2 and 3 with rearrangement of one hydrogen atom). Neither molecular ion peaks (M^+) nor characteristic secondary fragments (e.g., M^+ − amn 50) derived from loss of H_2O (amn 18) and CH_3OH (amn 32) of 2- and 3-hydroxy fatty acids were obtained.

The thiobarbituric acid assay for 2-keto-3-deoxy groups in hydrolysates (conditions ranging from 0.25 M H_2SO_4 at 100°C for 10 min or 1 h to 4 M HCl at 100°C for 10 min or 1 h) (1, 20) of all fractions was negative, indicating absence of 2-keto-3-deoxyoctonate. All fractions were free of L- or D-glycero-D-mannoheptose or other heptoses. These lipopolysaccharide-specific compounds were also lacking in the crude cell envelope fraction obtained by differential centrifugation of cell homogenates of strain J-10-fl. No precipitation was obtained on dropwise addition of water to phenol-chloroform-petroleum ether extracts of whole cells (6). Similarly, saline extracts of whole cells were free of LPS (data not shown).

TABLE 2. Chemical analysis of fractions from *C. aurantiacus* J-10-fl[a]

Compound	Concn (nmol/mg [dry wt]) in fraction:				
	WPh-P	PhPh-P	IPh	CR	CE
2-*O*-methyl-6-deoxyhexose	177	26	28	148	121
2,6-di-*O*-methylhexose	61	7	13	58	63
2-*O*-methylhexoses I and II	Trace	Trace	Trace	20	21
Rhamnose	1,137	19	82	116	382
Ribose	1	—[b]	29	65	13
Arabinose	374	6	33	37	131
Xylose	212	14	30	54	96
Mannose	373	15	40	49	135
Galactose	241	182	112	94	139
Glucose	356	127	90	162	125
Galacturonic acid[c]	+[d]	+	+	+	+
Glucosamine	111	53	4	176	19
Muramic acid[e]	62	31	1	108	10
L-Ornithine	78	52	1	76	Trace
16:0	2	84	32	22	21
18:0	4	168	103	83	11
16:1	—	—	—	—	2
18:1	—	154	50	31	30
19:0[f]	—	31	15	13	3
Phosphate	16	11	10	22	3

[a] For a description of the fractions, see Materials and Methods. Abbreviations: WPh-P, water phase pellet; PhPh-P, phenol phase pellet; IPh, phenol-water interphase pellet; CR, insoluble cell residues left by phenol-water extraction (all obtained by hot phenol-water extraction). The crude cell envelope fraction (CE) was obtained by differential centrifugation of cell homogenates.
[b] —, Absent.
[c] Some glucuronic acid was found additionally.
[d] +, Present but not quantified.
[e] Presumably phosphorylated to a small extent, as indicated by amino acid analysis.
[f] Tentatively identified, showing *m/e* 312 (M^+) on mass spectrometric fragmentation of the methylester derivative.

Absence of LPS-specific constituents was also observed with strain Ok-70-fl, with which most of the extraction methods and fractionations were also performed.

DOC-PAGE. DOC-PAGE confirmed the absence of LPS in representative fractions which are known to contain LPS in other gram-negative bacteria (14). LPS was also not detectable in phenol-water-extracted cell residues. LPS from *Salmonella* sp. strains were used as controls. With the latter, patterns indicating smooth, SR, or rough forms were obtained (Fig. 1). With the ultracentrifugation (105,000 × *g*) pellet of the water phase of phenol-water extracts of *C. aurantiacus*, no staining was observed, even at a 10-times-higher concentration and on prolonged staining. The corresponding pellet from the phenol phase showed a weakly stainable, fast-migrating band (Fig. 1, lane 3). This band is likely ascribable to galactolipids present in this sample as indicated by its chemical composition (Table 2). The phenol-water interphase (Fig. 1, lane 4) and a crude cell envelope fraction (Fig. 1, lane 5) showed no stainable material.

Peptidoglycan-polysaccharide complex constituents and galactolipids. Most of the fractions of the hot phenol-water extracts of *C. aurantiacus* J-10-fl contained significant amounts of neutral sugars, L-ornithine (among other amino acids), muramic acid and glucosamine, and muramic acid phosphate (Table 2). The O-methylated neutral sugar compounds were identified by mass spectrometric fragmentation of their respective alditol acetate derivatives ($NaBD_4$ reduced). Primary fragments *m/e* 87, 118, and 275 confirmed

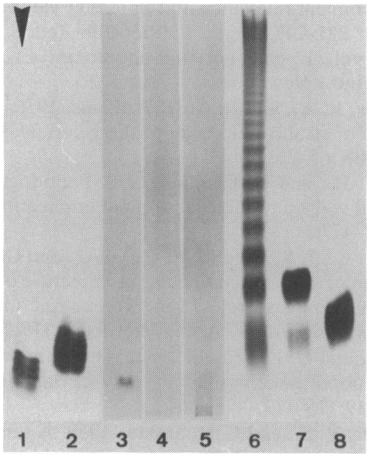

FIG. 1. DOC-PAGE (13%) and silver staining of fractions from *C. aurantiacus* J-10-fl and Ok-70-fl. The fractions applied (Table 2; 20 μg per lane) were as follows (lanes): 3, B; 4, C; 5, E. The LPS from *Salmonella* species used as controls (for chemical structures, see reference 20; 1 to 3 μg per lane) were as follows (lanes): 1, Re mutant *S. minnesota* R595; 2, Rc mutant *S. minnesota* R5; 6, smooth-form *S. typhimurium* var. Copenhagen; 7, SR mutant *S. typimurium* SH777; 8, Ra mutant *S. typhimurium* his386.

the 2-*O*-methyl-6-deoxyhexose configuration, and primary fragments *m/e* 45, 118, and 305 confirmed the 2,6-di-*O*-methylhexose configuration. Two 2-*O*-methylhexoses were identified by primary fragments *m/e* 118 and 333. Neutral and amino sugars, as well as L-ornithine, were found in essentially similar molar ratios as in the peptidoglycan-polysaccharide complex of the same strain (9). This included especially the glucosamine-to-muramic acid ratio, confirming the lack of glucosamine-containing lipid A. A significant relative enrichment of galactose was obtained in the phenol phase pellet and phenol-water interphase materials (Table 2, fractions PhPh-P and IPh, respectively), indicating galactolipids. The fatty acid content of most of the fractions comprised 16:0, 18:0, 18:1, and 19:0 (tentatively identified) as the major fatty acids (Table 2).

The components of the peptidoglycan-polysaccharide complex (9) were also found in the phenol-water-extracted cell residues and in crude cell envelope fraction (fractions CR and CE, respectively [Table 2]). With strain OK-70-fl, qualitative and quantitative results essentially comparable to those obtained with strain J-10-fl (Table 2) were achieved.

DISCUSSION

In accordance with indicative data of Knudson et al. (11), no LPS comparable to those of most gram-negative bacteria (14) was found in the two *C. aurantiacus* strains studied. All fractions of hot phenol-water extracts examined were shown to lack compounds typical for lipid A, core, or O-chains of LPS. In none of them was the glucosamine/muramic acid ratio higher than in the respective peptidoglycan-polysaccharide complex of *C. aurantiacus* J-10-fl (9). Most convincing is the absence of 3-hydroxy fatty acids, which are common constituents of all LPS, in contrast to the variability observed for other constituents of this complex molecule. Thus, absence of 2-keto-3-deoxyoctonate or heptose (or both), lack of O-chains (14), or even a modification of the lipid A backbone amino sugar (15) is observed in some cases, which thus have less value for judging the presence or absence of LPS. Lack of 3-OH fatty acids in the phenol-water extracted cell residues ensures that nonextractable LPS did not remain bound to the cell residues upon phenol-water treatment. Gel electrophoresis confirmed the absence of LPS suggested by the analytical chemical data.

Lack of LPS is in agreement with the structures of the rigid layer and peptidoglycan of *C. aurantiacus* (9), which both have properties of comparable structures in gram-positive bacteria (9, 17). Absence of LPS is a further confirmation for the deep phylogenetic divergence of *C. aurantiacus* from the other phototrophic bacteria, including the also chlorosome-containing *Chlorobiaceae*. The latter have LPS with heptose and 2-keto-3-deoxyoctonate in the core and a D-glucosamine and 3-hydroxy fatty acid-containing lipid A moiety (21). Their peptidoglycan is likely of the A1γ type (9), corresponding to the peptidoglycan of other gram-negative bacteria (17).

Absence of LPS is not necessarily a consequence of the thermophily of *C. aurantiacus*, although a so-called LPS fraction in *Thermus* strains also did not contain heptose, 2-keto-3-deoxyoctonate, or 3-hydroxy fatty acids (16). Significant amounts of LPS with D-glycero-D-mannoheptose, 2-keto-3-deoxyoctonate, 3-OH-14:0, and other characteristic LPS constituents were recently found in another thermophilic strain of the phototrophic bacterium *Chromatium tepidum* (J. Meißner et al., unpublished data). Absence of LPS does not imply absence of an outer membrane in *C. aurantiacus*, although such a structure is not clearly distinguishable on ultrathin sections (18). Possible lack of a typical outer membrane is indicated, but detailed further studies, including isolation of a pure cell wall fraction, will be necessary for determination of the molecular organization of the cell wall of *C. aurantiacus*.

All of the fractions listed in Table 2 contain the compounds from the peptidoglycan-polysaccharide complex of *C. aurantiacus* J-10-fl. The concomitant occurrence of the constituents of both peptidoglycan and polysaccharide in the same fractions is a further confirmation for possible binding of the polysaccharide to peptidoglycan as suggested by Jürgens et al. (9).

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