The cell wall of the filamentous anoxygenic phototrophic bacterium *Oscillochloris trichoides*

Olga I. Keppen,¹ Ruslan N. Ivanovsky,¹* Galina M. Streshinskaya,¹ Natalia V. Lebedeva,¹ Alexander S. Shashkov,² Andrey S. Dmitrenok² and Denis S. Grouzdev³

Abstract

The filamentous anoxygenic phototrophic bacterium *Oscillochloris trichoides* DG-6 has been studied, and it has been shown that there are no lipopolysaccharides on the cell surface. Fatty acids hydroxylated at the C3 position, amino sugars and phosphate-containing compounds characteristic of lipid A have also not been found. The genes encoding for proteins responsible for the synthesis of lipopolysaccharides and the genes for the transport system, usually localized in the outer membrane of Gram-negative bacteria, have not been detected in the genome. The rigid layer of the cell wall contains a peptidoglycan consisting of alanine, glutamine, ornithine and glycine, in the respective ratio 1.8 : 1.5 : 1.0 : 0.6. Thus, the investigated bacterium, *Osc. trichoides*, is a monoderm. The cell wall also contains a branched α-1,4-glucan with a repeating unit consisting of glucose residues linked by α-1→4 bonds (α-1→6 at the branching sites). Such polymers have not previously been reported in phototrophic bacteria.

INTRODUCTION

The structure of the cell envelope is important for the taxonomy and phylogeny of bacteria. The cells of Gram-negative bacteria are surrounded by two membranes – the internal cytoplasmic one and the external one, consisting of a lipopolysaccharide (LPS). Gram-positive bacteria have only the cytoplasmic membrane. Gupta introduced the terms ‘diderm’ for Gram-negative and ‘monoderm’ for Gram-positive bacteria [1]. In the terminology of Cavalier-Smith, these correspond to the negibacteria and unibacteria, respectively [2]. Currently, the problem of identifying the early stages of the origin and evolution of these bacteria is actively being researched [3, 4]. A significant stage in this process is the evolution of the cell envelope. According to Gupta, the filamentous anoxygenic phototrophic bacterium *Oscillochloris trichoides* DG-6 and the *Oscillochloridaceae* family also contain the first cellular organism (the last universal common ancestor – LUCA) that uses the Calvin cycle for autotrophic CO₂ fixation. As a member of the family *Oscillochloridaceae*, *Oscillochloris trichoides* is a photolithoautotrophic mesophilic anaerobic bacterium that uses the Calvin cycle for autotrophic CO₂ fixation [9–12]. Both phototrophic [7, 13, 14] and non-phototrophic [15, 16] representatives of the class *Chloroflexia* stain as Gram-negative bacteria and have a two-layered cell envelope, according to electron microscopy.

At present, the structure and composition of the cellular envelopes of FAP bacteria have been investigated only for phototrophic *Cfl. aurantiacus* [17, 18] and non-phototrophic *Herpetosiphon aurantius* [19] and *Kallotenue papyrolyticum* [15]. According to biochemical studies and analysis of the available nucleotide sequences of the genomes [3, 4], the cell wall of these bacteria lacks the LPS layer and the genes required for LPS biosynthesis.

The aim of this research was to study the presence/absence of LPS on the cell surface of *Osc. trichoides* DG-6 and the...
chemical composition of the cell wall, including the peptido-
glycan (PG) amino acids and the localization and struc-
ture of polysaccharides (PS). These aspects of the cell wall of
Osc. trichoides DG-6 have not been studied to date.

METHODS

Bacterium and growth conditions

Osc. trichoides DG-6 (CM MSU 327, VKM B-10173) was
used in this study. The strain DG-6 was isolated from a
microbial mat in a warm hydrogen sulfide spring in the
Caucasus region of Europe [11, 20]. Osc. trichoides DG-6
was maintained on modified DGN medium [20]. The cul-
ture was grown under anoxic conditions (28°C, 2000 lx) in
completely filled 0.5 l bottles with magnetic stirring. Cul-
tures from the late exponential-growth phase were har-
vested, washed with phosphate buffer (0.01 M, pH 8.0) and
lyophilized.

Morphology and ultrastructure

The size, shape and ultrastructure of the cells were exam-
ined by phase-contrast microscopy and transmission elec-
tron microscopy, as described previously [21]. Staining
procedures were performed as described by Doetsch [22].

Extraction of LPS

The lyophilized cells were extracted with 90% aqueous phe-
nol by stirring and heating (65°C, 30 min) and dialysed
against tap water as described previously [23]. After centri-
fugation (5000 g, 1 h), the supernatant was dialysed against
distilled water and lyophilized (preparation F1). The latter
was used for the determination of the fatty acid (FA)-
containing compounds. The pellet was lyophilized (preparation F2) and used to iso-
lute the PS.

Isolation and identification of fatty acids

Ten milligrams of F1 were treated with 1.5% acetyl chloride
in chilled methanol (100°C, 4 h) in sealed ampoules. The
FA methyl ethers were extracted with hexane. The composi-
tion of FA methyl ethers was analysed using a chromatogra-
phy–mass spectrometry system (Agilent 6890 N/5973) on a
HP-5 MS column (30 m×0.25 mm). Identification of the
components was carried out using a standard mixture of
methyl ester FAs.

The identification of fatty acids from the cell biomass was
performed as described previously [24]. Thirty milligrams
of lyophilized cells were treated with 200 µl of a 5.4 N solu-
tion of anhydrous HCl in methanol at 70°C for 2 h. The
methyl ethers were extracted twice with 100 µl of hexane.
The extract was dried and silylated in 20 µl of N,O-bis(tri-
methylsilyl) trifluoroacetamide for 15 min at 65°C. A 1 µl
portion of the reaction mixture was analysed with a model
HP-5985B gas chromatography–mass spectrometry system
(Hewlett-Packard, Palo Alto, CA), equipped with a capillary
column (25 m×0.25 mm) consisting of fused quartz con-
taining an Ultra-1 non-polar methylsilicone phase.

Determination of phosphate-containing compounds
and monosaccharides

Seven milligrams of F1 were hydrolysed with 2 N trifluoro-
acetic acid (120°C, 2 h). The acid was removed by vacuum
evaporation. Phosphate-containing organic compounds and
the qualitative composition of amino sugars were deter-
mined by paper (Filtrac FN-13, Germany) electrophoresis
and chromatography. Solvent systems and processing meth-
ods have been described previously [25].

Isolation of peptidoglycan

Five grams of lyophilized cells of Osc. trichoides were
heated with 10% TCA (90°C, 20 min) and centrifuged
(5000 g, 15 min). The pellet was repeatedly washed with
water and 0.5 M Tris-HCl buffer (pH 7.8) treated with
trypsin, SDS and 1 M NaOH to remove the non-covalently
bound compounds [26]. The amino acid composition of
the PGs was determined after hydrolysis (6 M HCl, 100°C,
20 h) on an L-8800 analyser (Hitachi, Japan). In the hydro-
lysate, the presence of diaminopimelic acid, its isomers and
ornithine was also detected by chromatography on paper in
the following system: MeOH: H2O: HCl conc.: pyridine
(8:1.75:0.25; 1 v/v) [25].

Fig. 1. Osc. trichoides DG-6. (a) Phase-contrast photomicrograph and (b) electron micrograph of ultra-thin sections.
Isolation of the polysaccharide

One gram of F2 was heated (90°C, 10 min) in 5% TCA and centrifuged (5000 g, 15 min). The supernatant was dialysed against distilled water. The dialysate was lyophilized (preparation F3). A portion of this last preparation was hydrolysed with 2 M HCl (100°C, 3 h). The qualitative composition of the monosaccharides in the hydrolysate was determined. The PS structure of F3 was determined by NMR-spectroscopy.

Preparation of the cell wall and determination of the molecular mass of the glucan

To determine the localization of PS, the cells of Osc. trichoides were autoclaved (110°C, 20 min, pH 4.0) and sonicated using a UP100H disintegrator (30 kHz; Hielscher, Germany) with cooling and subsequent differential centrifugation at 10 000 g for 15 min at 4°C. The resulting pellet was resuspended in 2% SDS, heated for 5 min at 100°C and centrifuged as above. The lower, dense portion of the pellet (unbroken cells) was discarded and the upper, loose layer (native cell wall (CW)), was washed several times with distilled water and freeze-dried (preparation F4) (23). The extraction of the PS and the qualitative composition of the resulting monosaccharides was performed as described above (F3 preparation). The PS structure was determined by NMR spectroscopy. To determine the molecular mass of the glucan, gel chromatography of the PS was conducted on a column (35×3.5 cm) packed with Sephadex G50 (Superfine; Amersham Biosciences) in 0.05 M pyridine-acetate buffer, pH 4.5 (10 ml of pyridine and 4 ml of acetic acid adjusted to 1 l), which resolved a compound with a molecular mass of 10 kDa.

NMR spectroscopy

NMR spectra were recorded on a Bruker AV600 spectrometer (Bruker, Germany) in solutions of 99.96% D2O at 343K. Chemical shifts are reported related to TSP as an internal standard (dH 0.0, dC 3.4). Two-dimensional spectra were recorded using the standard procedures of Bruker. Mixing time in the TOCSY experiments was 100 μs. Spin-lock time in the ROESY experiments was 150 ms. The 1H, 13C-HMBC experiment was optimized for the spin–spin interaction constants JH,C 8 Hz.

Identification of genes responsible for cell wall biosynthesis

A comparative genomic analysis was carried out using the online database services GenBank [27], SEED/RAST [28], IMG [29] and PFAM [30]. The sequence homologues of the Osc. trichoides proteins were retrieved from searching the sequence databases using BLAST [31] with an E value cut-off of 10^{-20}. After removing redundant and nearly redundant homologues, the sequences were aligned using CLUSTAL [32]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA 6.0 [33] and Bioedit 7.0 [34].

RESULTS AND DISCUSSION

Absence of LPS-specific compounds

The cells of Osc. trichoides are bounded by a double-layered envelope (Fig. 1) that stains Gram-negative (Fig. S1, available in the online version of this article). The two-layer outer membrane of diderm bacteria contains LPS, which consists of lipid A, core PS and O antigen in its outer leaflet and phospholipids in the inner leaflet. Lipid A, a

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<th>Fatty acid</th>
<th>%</th>
</tr>
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<tbody>
<tr>
<td>12:0</td>
<td>TR*</td>
</tr>
<tr>
<td>14:0</td>
<td>TR</td>
</tr>
<tr>
<td>14:0 (3OH)</td>
<td>TR</td>
</tr>
<tr>
<td>15:0</td>
<td>TR</td>
</tr>
<tr>
<td>15 alk</td>
<td>TR</td>
</tr>
<tr>
<td>16:1</td>
<td>10.7</td>
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<td>34.9</td>
</tr>
<tr>
<td>16:0 (3OH)</td>
<td>TR</td>
</tr>
<tr>
<td>17:0</td>
<td>0.3</td>
</tr>
<tr>
<td>17 alk</td>
<td>TR</td>
</tr>
<tr>
<td>18:1d9</td>
<td>47.9</td>
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<tr>
<td>18 alk</td>
<td>1.3</td>
</tr>
<tr>
<td>Sum</td>
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</tr>
</tbody>
</table>

Table 1. Fatty acid composition of Osc. trichoides DG-6 cells

*TR (trace) less than 0.5 %. The amounts are given in percentages of the total.

<table>
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<tr>
<th>Amino acid</th>
<th>%*</th>
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<tr>
<td>Ala</td>
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<tr>
<td>Asp</td>
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<tr>
<td>Arg</td>
<td>2.5</td>
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<tr>
<td>Cys</td>
<td>0.3</td>
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<tr>
<td>Glu</td>
<td>19.3</td>
</tr>
<tr>
<td>Gly</td>
<td>7.8</td>
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<tr>
<td>His</td>
<td>0.4</td>
</tr>
<tr>
<td>Ile</td>
<td>2.7</td>
</tr>
<tr>
<td>Leu</td>
<td>5.9</td>
</tr>
<tr>
<td>Lys</td>
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</tr>
<tr>
<td>Orn</td>
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<td>Phe</td>
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</tr>
<tr>
<td>Pro</td>
<td>1.3</td>
</tr>
<tr>
<td>Ser</td>
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</tr>
<tr>
<td>Thr</td>
<td>4.0</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.7</td>
</tr>
<tr>
<td>Val</td>
<td>13.1</td>
</tr>
<tr>
<td>Sum</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Table 2. Amino acid composition of Osc. trichoides DG-6 peptidoglycan

*The amounts are given in percentages of the total.
disaccharide consisting of two residues of glucosamine, contains two phosphate groups and a hydroxylated FA at the third carbon atom [35, 36]. The presence of C3-hydroxylated FA is usually considered a sign of the presence of LPS in bacteria. In the cells of both Osc. trichoides (Table 1) and Cfl. aurantiacus [37], the C3-hydroxylated FA was not found. The products of hydrolysis (trifluoroacetic acid) of the lyophilized preparation F1 did not contain amino sugars, glycerol or phosphorus-containing compounds. Moreover, in the genome of Osc. trichoides, genes tag, tar and dlt, essential for the biosynthesis of teichoic acids, were absent. On the basis of these data, we conclude that lipoteichoic and wall teichoic acids are absent in the cell wall of Osc. trichoides.

The enzymes encoded by genes lpxC, lpxH, lpxB, lpxK, lpxL, lptA, lptDE, lpxM, kdtA, kdsA and kdsB are involved in LPS biosynthesis in diderm microorganisms. Moreover, the outer membrane, specific for diderm bacteria, is expected to contain transport complexes that transfer cytoplasmically synthesized proteins outside of the cell. At least eight types of specialized secretion apparatus are known in different diderm bacteria [38]. The genome analysis of Osc. trichoides revealed the presence of no genes involved in the biosynthesis of LPS that were specific for diderm bacteria transport systems. In addition, Gram-negative bacteria are characterized by the presence of β-barrel membrane proteins in the outer membranes. In the genome of Osc. trichoides, the genes for the β-barrel assembly machinery are absent.

Thus, the results obtained indicate the absence of LPS on the surface of Osc. trichoides cells. In previously studied species of Cfl. aurantiacus and H. aurantiacus, LPS was also absent [18, 19]. For other members of the class Chloroflexia, the conclusion regarding the absence of LPS was made based on the lack of genes responsible for the synthesis of LPS and the outer membrane transport systems in the available genomes [3, 4].

**Peptidoglycan analysis**

Most diderm bacteria contain dianminopimelic acid in their PG [39]. The main PG amino acids of Osc. trichoides are alanine, glutamic acid, ornithine and glycine, in the ratio of 1.8:1.5:1.0:0.6, and some minor amino acids (Table 2). Analysis of the PG hydrolysate by paper chromatography shows that dianminopimelic acid and its isomers are absent. Instead, ornithine is present. Thus, the diamino acid of the Osc. trichoides PG is ornithine.
Previously, ornithine was found in the PG of some chlorophyll-based phototrophic and non-phototrophic representatives of the class Chloroflexia [17, 19, 40]. Ornithine is also found in the PG of Thermus thermophilus (phylum Deinococcus-Thermus) [41] and representatives of the genera Spirochaeta, Borrelia and Treponema (order Spirochaetales) [42]. It is interesting that all of these bacteria, as well as representatives of the class Chloroflexia, do not have LPS.

The Osc. trichoides genome [43] contains a complete set of genes required for PG biosynthesis, and we found 11 genes specific for this process: murA (OSCT_1030), murB (OSCT_2784), murC (OSCT_2783), murD (OSCT_2778), murE (OSCT_2779), murF (OSCT_2780), murG (OSCT_2781), murH (OSCT_2782), murI (OSCT_2785), murJ (OSCT_2786), and murK (OSCT_2787).

Fig. 3. $^1$H,$^{13}$C-HSQC spectrum of the glucan from the cell wall of Osc. trichoides DG-6. Upper case letters denote the carbon atoms in the sugar residues as designated in Table 3.

Fig. 4. Repeating units of the glucan from the cell wall of Osc. trichoides DG-6.
Table 3. $^{13}$C and $^1$H NMR data of preparation F4 from the cell wall of Osc. trichoides DG-6

<table>
<thead>
<tr>
<th>Residue</th>
<th>Chemical shifts ($\delta_{13}^{1}$C, $\delta_{H}^{1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C-1</td>
</tr>
<tr>
<td></td>
<td>H-1,1</td>
</tr>
<tr>
<td>$\rightarrow$-a-d-GlcP-(1 $\rightarrow$ (A))</td>
<td>101.1</td>
</tr>
<tr>
<td>$\rightarrow$-a-d-GlcP-(1 $\rightarrow$ (B) 6)</td>
<td>101.3</td>
</tr>
<tr>
<td>$\rightarrow$-a-d-GlcP-(1 $\rightarrow$ (C))</td>
<td>99.9</td>
</tr>
<tr>
<td>a-d-GlcP-(1 $\rightarrow$ (D))</td>
<td>4.95</td>
</tr>
<tr>
<td></td>
<td>5.33</td>
</tr>
</tbody>
</table>

Table 4. Proteins involved in the biosynthesis of branched $\alpha$-glucan in Osc. trichoides DG-6

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Gene symbol</th>
<th>Product name</th>
<th>Locus tag</th>
<th>Length</th>
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</thead>
<tbody>
<tr>
<td>GlgC-GlgA</td>
<td>glgA</td>
<td>Glycogen synthase</td>
<td>OSCT_1960</td>
<td>407</td>
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<tr>
<td></td>
<td>glgC</td>
<td>Glucose-1-phosphate adenylyltransferase</td>
<td>OSCT_1446</td>
<td>412</td>
</tr>
<tr>
<td></td>
<td>glpP</td>
<td>Glycogen phosphorylase</td>
<td>OSCT_3180</td>
<td>710</td>
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<tr>
<td>GlgC-GlgA TreS-Pep2-GlgE</td>
<td>glgX</td>
<td>Glycogen debranching enzyme</td>
<td>OSCT_1168</td>
<td>696</td>
</tr>
<tr>
<td></td>
<td>glgE</td>
<td>Alpha-1,4-glucan:maltose-1-phosphate maltosyltransferase</td>
<td>OSCT_2027</td>
<td>680</td>
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<tr>
<td></td>
<td>glgB</td>
<td>1,4-alpha-glucan branching enzyme GlgB</td>
<td>OSCT_2029</td>
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<tr>
<td>TreS-Pep2-GlgE</td>
<td>treS-pep2 fusion</td>
<td>Trehalose synthase TreS/maltokinase Pep2 fusion protein</td>
<td>OSCT_2028</td>
<td>1123</td>
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<tr>
<td></td>
<td>treZ</td>
<td>Malto-oligosyltrehalose trehalohydrolase</td>
<td>OSCT_2031</td>
<td>564</td>
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<tr>
<td></td>
<td>treY</td>
<td>Maltooligosyl trehalose synthase</td>
<td>OSCT_2032</td>
<td>951</td>
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<td></td>
<td>treX</td>
<td>Glycogen debranching enzyme TreX</td>
<td>OSCT_1368</td>
<td>715</td>
</tr>
</tbody>
</table>

Analysis of the cell wall polysaccharide

It was shown by electrophoresis and paper chromatography that glucose was the predominant product of the acid hydrolysis (4 M HCl, 100 °C, 4 h) of preparations F2 and F3. To determine the localization of the branched $\alpha$-1,4-d-glucan, the cell wall preparation was obtained (F4). Upon extraction of F4 with 10 % TCA and subsequent dialysis, a polymer preparation was obtained, which was studied by NMR spectroscopy methods (Figs 2–4). These spectroscopic studies showed the identity of the polymers obtained from the F3 and F4 preparations.

The $^{13}$C NMR spectrum of the polymer showed signals (Table 3) only in the sugar region, with three signals in the anomeric region at $\delta C$ 101.3, 101.1 and 99.9. The $^1$H NMR spectrum showed two broad signals at $\delta H$ 5.33 and 4.96 (protons attached to the anomeric carbons), and broad multiplets at 4.0–3.3 ppm. $^1$H NMR and $^{13}$C spectra of the polymer were assigned using $^1$H, $^1$H-COSY; TOCSY; ROESY; $^1$H, $^{13}$C-HSQC and HMBC experiments. Analysis of the homonuclear 2D spectra showed signals of $\alpha$-glucopyranose residues, $\alpha$-GlcP. The $^1$H, $^{13}$C-HSQC spectrum revealed the substitution of the residue on the $\alpha$-GlcP hydroxyl at the C-4 ($\delta C$ 78.9) and partially at the C-6 ($\delta C$ 69.3, weak signal), according to the downfield shifts of the respective carbon atoms as compared with those of $\alpha$-GlcP [44]. The evidence for an $\alpha$-(1$\rightarrow$4) bond between the residues was obtained from the low field shift of the C-4 residue of $\alpha$-d-GlcP (C-6 $\delta C$ 78.9) and the presence of the correlation peaks H-1/C-4 in the HMBC spectra and H1/H4 in ROESY. Analysis of the NMR spectra signals (Figs 2 and 3, Table 3) showed that the main chain of the polymer is formed by repeating units of $\alpha$-d-GlcP-(1$\rightarrow$4)–. The TOCSY spectrum showed correlation signals between the protons at the substituted C6 and the protons at the substituted C4, indicating the presence of the chain branch at position 6 of some (1$\rightarrow$4)-linked glucose residues. The corresponding signal of the glycosylating residue C1/H1 was observed at $\delta C$ 99.9 and $\delta H$ 4.95. The signals of the terminal residue $\alpha$-d-GlcP-[1$\rightarrow$] with the characteristic shifts of the unsubstituted C4/H4 (710, 3.42) also were revealed in the spectra. The ratio of the intensities of the substituted and unsubstituted H4 (approximately 8:1) indicated the presence of frequent branching (every 3–5 units) and a short length (3–5 units) of lateral branches. The molecule of the glucose homopolymer is therefore formed by glucose residues with $\alpha$-1$\rightarrow$4 bonds ($\alpha$-1$\rightarrow$6 at the branching sites, 3–5 residues;
Fig. 4). Thus, the cell wall glycopolymer of Osc. trichoides belongs to the class of α-1,4-D-glucans with a molecular mass of about 10 kDa.

A number of bacteria produce α-1,4/α-1,6-glucans as intracellular reserves [45], but the location of α-1,4-D-glucans on the outer cell envelope surface was described for Mycobacterium species, where they are components of the capsule [46]. The biosynthesis of extracellular α-glucans in M. tuberculosis can occur through two pathways: GlgC-GlgA and Tres-Pep2-GlgE. The mechanism of its transport to the outer cell envelope, however, remains unknown [47]. The genes for both pathways are present in the Osc. trichoides genome (Table 4). α-1,4-D-glucans are presumably processed from α-maltose-1-phosphate by the maltosyltransferase GlgE (OSCT_2027) followed by polymer branching by the GlgB protein (OSCT_2026). It should be mentioned that the presence of genes for branched α-glucan biosynthesis is typical for the representatives of Chloroflexinae suborder only (Fig. 5). In the genomes of chlorosome-less FAP belonging to the Roseiflexinaceae suborder and Herpetosiphonales order, the branched α-glucan biosynthesis genes are absent. Presumably, the acquisition of genes responsible for the branched α-glucan biosynthesis occurred in the evolutionary process in parallel with the genes encoding for bacteriochlorophyll c and chlorosome biosynthesis [48].

Thus, our biochemical and genomic analysis has shown the absence of LPS in Osc. trichoides DG-6 cells. The PG contains ornithine as a diamino acid. This, along with the absence of outer membrane features, makes it possible to classify the studied bacterium, as well as other representatives of the class Chloroflexia, as monoderms. In addition, the cell wall of Osc. trichoides contains a branched α-1,4-D-glucan, the first to be escribed for representatives of the class Chloroflexia.

**Fig. 5.** Phylogenetic tree based on the amino acid sequences of the GlgB and GlgE proteins. The scale bar corresponds to 0.2, the estimated amino acid substitution per sequence position. Bootstrap values from 1000 replicates were included.

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**Conflicts of interest**
The authors declare that there are no conflicts of interest.

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