Chemical Analysis of Peptidoglycans from Species of Chromatiaceae and Ectothiorhodospiraceae

Joachim Meißner, Uwe J. Jürgens, and Jürgen Weckesser

Institut für Biologie II, Mikrobiologie, der Albert-Ludwigs-Universität, Schänzlestraße 1, D-7800 Freiburg i. Br., Bundesrepublik Deutschland

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Rigid layer (sodium dodecylsulfate(SDS)-insoluble cell wall) and peptidoglycan fractions were obtained from the Chromatiaceae (*Thiocystis violacea* and *Chromatium tepidum*) and from *Ectothiorhodospira vacuolata*. Chemical composition of rigid layers from all three species indicated the presence of peptidoglycan-bound protein.

Qualitative and quantitative composition of isolated peptidoglycan indicate an A1γ-type. *meso*-Diaminopimelic acid was the only diamino acid found. Direct cross-linkage of peptide side-chains was confirmed by separation of respective dipeptides (m-A_2pm-D-Ala) from partial acid hydrolysates of peptidoglycan. GlcN and MurN of the sugar strands are completely N-acetylated. All peptidoglycan fractions contained small amounts of Gly.

Introduction

Phototrophic bacteria are phylogenetic diverse [1]. Most, but not all of them are gram-negative. Lipopolysaccharide has been found in purple non-sulfur bacteria, in Chromatiaceae and Ectothiorhodospiraceae as well as in the Chlorobiaceae [2–5]. *Chloroflexus aurantiacus* of the Chloroflexaceae family is lacking this heteropolymer [3].

In contrast to the likely A1γ-type structure of the peptidoglycan of *Chlorobium vibrioforme,* that of *Chloroflexus aurantiacus* has characteristics of peptidoglycan typical for gram-positive bacteria [6]. The data confirm the deep phylogenetic separation within the green bacteria suggested by the 16S-rRNA sequencing studies [1].

Such a deep separation is not observed within the purple bacteria, although 16S-rRNA sequencing studies suggests considerable heterogeneity [1]. Whereas most of the purple non-sulfur bacteria belong to the alpha-subdivision, the purple sulfur bacteria belong to the gamma-subdivision of the proposed phylogenetical tree. Data of lipopolysaccharide composition, especially of the conservative lipid A region, have essentially confirmed 16S-rRNA studies [3, 7].

Reprint requests to J. Weckesser.

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The present paper, together with the few data available on peptidoglycan of purple non-sulfur bacteria [8, 9, 10], reveals A1γ-type structure [11, 12] to be likely common to most if not all purple bacteria. The study includes a mesophilic *(Thiocystis violacea)* and a moderately thermophilic *(Chromatium tepidum)* species of Chromatiaceae as well as the moderately halophilic *Ectothiorhodospira vacuolata* of the Ectothiorhodospiraceae family.

Materials and Methods

Cultivation of strains

Thiocystis violacea 2711 was obtained from N. Pfennig, Universität Konstanz, F.R.G., *Chromatium tepidum* MC from M. T. Madigan, University of Southern Illinois, U.S.A., and *Ectothiorhodospira vacuolata* BN 9512 from U. Fischer, Oldenburg, F.R.G. All strains were grown photoheterotrophically. *Thiocystis violacea,* using the same medium as described in [13], was cultivated in 5 l vessels at room temperature under stirring. Cells were fed with sterile sulfide or acetate solution (final concentration 0.03 and 0.01%, respectively) after adjusting the pH to 7.0. Cells were harvested before they were free of sulfur particles. *Chromatium tepidum* MC was grown in medium given in [14] in a 12 l Microferm fermenter (New Brunswick, New Jersey, U.S.A.) at 50 °C and a light intensity of 2000 lx. For growth of *Ectothiorhodospira vacuolata,* the medium described in [15] was used. Cells were grown at 37 °C and col-

lected after 5 days at the end of the exponential growth phase.

Preparation of rigid layer and peptidoglycan

Lyophilized cells of *Chromatium tepidum* were treated with hot phenol water [16]. The phenol-water interphase obtained was combined with the extracted cell residues and the mixture then extracted three times with 4% (w/v) sodium dodecylsulfate (SDS) in water at 100 °C [17]. With *Thiocystis violacea* and *Ectothiorhodospira vacuolata,* cell homogenates were prepared as described previously [6]. Crude cell envelope fractions were obtained by ultracentrifugation (176,000 × *g*, 4 °C, 1 h) and then extracted with SDS (experimental conditions as above). The rigid layer fraction was obtained as the final sediment of the following repeated centrifugations at 237,000 × *g* (20 °C, 1 h) until the sediment was free of SDS [18].

Treatment of the rigid layers with pronase E (from *Streptomyces griseus,* 6 units per mg of solid, Boehringer, Mannheim) was performed by incubation of 250 mg rigid layer (wet weight, in 20 ml Tris-HCl buffer, pH 7.4) with 2 ml enzyme solution (5 mg enzyme/ml of the same buffer) at 37 °C over night under stirring. The enzyme was removed by 4% (w/v) SDS (100 °C, 15 min) and the peptidoglycan obtained as described above for the rigid layer.

Chemical analyses

Amino acids and amino sugars were liberated by hydrolysis in 4 M HCl, 110 °C for 18 h and were quantitatively determined on the automatic amino acid analyzer [19]. D,L-Enantiomers of amino acids were separated and quantitatively determined as isopropylester/N-tri-fluoro-acetyl derivatives (esterification with isopropanol/HCl), using a 25 m Chirasil-Val fused-silica-capillary column, coated with XE-60-L-valine-(S)-α-phenylethylamide [6]. Combined low voltage thin-layer electrophoresis and thin-layer chromatography (1st dimension: pyridine–ethyl acetate–water, 1:2:250, by vol., pH 4.4, 2.5 h, 400 V, 7–10 mA; 2nd dimension: pyridine–ethyl acetate–acetic acid–water, 35:35:7:21, by vol.) of partial acid hydrolysates (4 M HCl, 105 °C, 30 min) was carried out as described in [20]. Detection was by fluorescamine (0.05%, w/v, in acetone) under ultra-violet irradiation. Neutral sugars and fatty acids were determined as alditol acetate and methylester derivatives, respectively [20], organic phosphorus according to [21].

Results

Thiocystis violacea and Chromatium tepidum

Rigid layer fractions from *Thiocystis violacea* 2711 and *Chromatium tepidum* MC were obtained in yields of 1.8% and 1.5%, respectively, of cell dry weight. They both contained a major protein moiety in addition to the peptidoglycan constituents, fatty acids and presumably contaminating polysaccharides, mainly glucan (Table I). The rigid layer fraction *Chromatium tepidum* MC contained significant amounts of phosphate. Pronase treatment, performed with the rigid layers of both strains, removed in each case the protein moiety. The yields of the peptidoglycan fractions obtained were 0.9% *(Thiocystis violacea)* and 0.8% *(Chromatium tepidum)* of cell dry weight. They contained the peptidoglycan constituents expected for the A1γ-type of peptidoglycan classification, including D-Glu, D- and L-Ala, and *m*-A_2pm as the only diamino acid as well as the amino sugars of the glycan strands. With none of the peptidoglycans, a GlcN-MurN disaccharide peak, in-

Table I. Chemical composition of the rigid layer (RL, SDS-insoluble cell wall fraction) and peptidoglycan (PG) fractions from *Thiocystis violacea* 2711, *Chromatium tepidum* MC, and *Ectothiorhodospira vacuolata* BN 9512. Data are given in nmol per mg of fraction dry weight.

Compound	*T. violacea*		*C. tepidum*		*E. vacuolata*	
	RL	PG	RL	PG	RL	PG
Glu	528	746[a]	483	498[a]	580	402[b]
Ala	772	1050[c]	606	761[d]	739	551[e]
m-A_2pm	432	754	200	504	382	388
MurNAc	299	555	148	375	267	257
GlcNAc	347	628	179	436	329	327
Gly	145	32	232	62	73	53
Other amino acids	772	traces	1487	traces	1092	traces
Glc[f]	103	329	1028	1291	741	836
12:0	–[g]	–	–	–	7	7
14:0	trace	5	3	3	trace	4
16:0	16	18	17	12	16	14
18:0	7	17	4	6	7	11
18:1	1	–	7	–	–	–
Phosphate	–	–	360	503	92	140

[a] About 94% as D-Glu, 6% as L-Glu.
[b] 85% as D-Glu, 15% as L-Glu.
[c] 43% as D-Ala, 57% as L-Ala.
[d] 37% as D-Ala, 63% as L-Ala.
[e] 36% as D-Ala, 64% as L-Ala.
[f] Additional sugars (*T. violacea, C. tepidum:* Rib, Xyl, Man, Rha, Gal; *E. vacuolata:* Rib, Xyl, Man, Gal) were present in trace amounts.
[g] Absent.

dicating incomplete N-acetyl substitution of GlcN [9] was observed on the amino acid analyzer of total hydrolysates (4 M HCl, 105 °C, 18 h). Combined low voltage thin-layer electrophoresis and thin-layer chromatography (for experimental conditions see Materials and Methods section) of partial acid hydrolysates (4 M HCl, 100 °C, 30 min) of peptidoglycan revealed a spot (among others), which migrated like spot No. 8a, b in Fig. 3 shown in [20]. It was obtained with the peptidoglycans of the two species investigated and was shown to consist of *m*-A_2pm and Ala only. It could be separated into two peaks on the amino acid analyzer. They corresponded to peaks No. 8a and b, respectively, in Fig. 4 in [20]. Neither the glucan nor the fatty acid content or the phosphate were removed by the pronase treatment.

Ectothiorhodospira vacuolata

The yield of the rigid layer obtained from *Ectothiorhodospira vacuolata* BN 9512 was 1.5% of cell dry weight. It contained a major protein moiety in addition to the peptidoglycan as well as some fatty acids, phosphate and glucan (Table I). On pronase-treatment of the rigid layer fraction, the peptidoglycan was obtained in a 1.2% yield of cell dry weight. The amino acid and amino sugar composition was similar to those of the peptidoglycans of Chromatiaceae species (see above). Comparable to the peptidoglycans of the Chromatiaceae species studied, on partial acid hydrolysis followed by combined low voltage thin-layer electrophoresis and thin-layer chromatography, one spot (among others) consisting of Ala and *meso*-A_2pm was observed. Again, it could be separated into two peaks as obtained with the Chromatiaceae on analysis on the amino acid analyzer.

Discussion

The rigid layer fraction of all three strains studied herein contained a significant protein moiety in addition to peptidoglycan. This might indicate the presence of peptidoglycan-bound protein, comparable to that found with other gram-negative bacteria [17]. Although fatty acids were found in each case, the tempting assumption of having lipoproteins in the sulfur purple bacteria still lacks experimentell proof. This question is difficult to answer with the data presented, since neither the fatty acids nor the significant amounts of phosphate (indicating contaminating phospholipids) were removed from the rigid layers by the pronase-treatment applied. The risk of having contaminating phospholipids is especially high, since in this study the rigid layers were obtained from hot phenol-water extracts *(Chromatium tepidum)* and not from cell envelope fractions.

As expected for gram-negative bacteria, the qualitative composition and the molar amounts of constituents indicate A1γ-type peptidoglycan for all strains studied. *m*-A_2pm was the only diamino acid detectable. The lower D-Ala (relative to L-Ala) content is likely explained by D-Ala-specific carboxypeptidases or by an already initial partial lack of D-Ala in position 4 of the peptide side-chain [11, 12]. The small amounts of L-Glu found may be due to racemization during acid hydrolysis.

The combined low voltage thin-layer electrophoresis and thin-layer chromatography performed with partial hydrolysates confirmed the A1γ-type structure of all three peptidoglycans studied. The finding of two peaks on the amino acid analyzer, both consisting of *m*-A_2pm and Ala, indicates the fragments *m*-A_2pm-Ala, revealing direct cross-linkage (the degree of cross-linkage has not been determined). There was no indication for an incomplete N-acetyl substitution of amino sugars in the sugar strands of the peptidoglycans studied. Thus, as far as studied, partial lack of N-acetyl substitution of GlcN of peptidoglycan, rendering the cells less sensitive to the action of lysozyme, is restricted to the budding species of purple non-sulfur bacteria within the phototrophic bacteria [9, 10].

It derserves connotation that *Chromatium tepidum,* as the only known – moderate – thermophylic species of Chromatiaceae (growth at 50 °C) has essentially the same peptidoglycan (very likely A1γ-structure) as the mesophilic Chromatiaceae species with respect to both quantity and chemical structure. Accordingly, *Chromatium tepidum* contains lipopolysaccharide with the same characteristics of those of Chromatiaceae [7]. In contrast, some gram-negative strains of *Thermus* spp. lack lipopolysaccharide and, as shown with *Chloroflexus aurantiacus* [6], *m*-A_2pm is replaced by L-Orn in their peptidoglycan [22, 23]. However, a thickened peptidoglycan layer for stabilizing the cell wall as has been discussed and evidenced experimentally for some hydrocarbon-utilizing bacteria [23], has not been observed with either *Chromatium tepidum* MD or *Chloroflexus aurantiacus* [14, 24]. Similarly, the peptidoglycan of the moderately halophilic *Ectothio-*

rhodospira vacuolata (growth in 1% to 6% salt) shows no significant deviation from those of non-halophilic species. Cells of *Rhodospirillum salexigens* (growth in 5% to 20% sodium chloride) [19] contains 100 times less peptidoglycan than that of *Escherichia coli*. Thus, moderate thermophilism or halophilism may not influence significantly the structure and function of peptidoglycan.

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