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Ornithine as a constituent of the peptidoglycan of *Chloroflexus aurantiacus*, diaminopimelic acid in that of *Chlorobium vibrioforme f. thiosulfatophilum*

U. J. Jürgens[1], J. Meißner[1], U. Fischer[2], W. A. König[3], and J. Weckesser[1]

[1] Institut für Biologie II, Mikrobiologie, der Albert-Ludwigs-Universität, Schänzlestr. 1, D-7800 Freiburg i. Br., Federal Republic of Germany
[2] Fachbereich Biologie, AG Geomikrobiologie der Universität Oldenburg, Postfach 2503, D-2900 Oldenburg, Federal Republic of Germany
[3] Institut für Organische Chemie, Martin-Luther-King-Platz 6, D-2000 Hamburg 13, Federal Republic of Germany

Abstract. L-Ornithine is the only diamino acid of the peptidoglycan of the gliding phototrophic *Chloroflexus aurantiacus*. The other constituents are L- and D-alanine, D-glutamic acid, N-acetyl-glucosamine and N-acetyl-muramic acid (in part as muramic acid-6-phosphate), all in approximate equimolar ratios to L-ornithine, aside from small amounts of glycine and histidine. Furthermore unlike typical Gram-negative bacteria, protein is not bound to this peptidoglycan. Instead, the rigid layer (sodium dodecyl sulfate insoluble cell wall fraction) contained large amounts of a complex polysaccharide consisting of sugar O-methyl ethers, hexoses and pentoses. Its binding site is presumably muramic acid-6-phosphate of the peptidoglycan.

In contrast, in *Chlorobium vibrioforme f. thiosulfatophilum*, *meso*-diaminopimelic acid was found as the only diamino acid of this peptidoglycan. As with other Gram-negative bacteria, L- and D-alanine, D-glutamic acid, N-acetyl-glucosamine and N-acetyl-muramic acid (no muramic acid-6-phosphate) were observed in approximate equimolar ratios to *meso*-diaminopimelic acid, except a lower D-alanine content. The rigid layer of *Chlorobium vibrioforme f. thiosulfatophilum* contained protein, and there were no indications for a complex polysaccharide comparable to that of *Chloroflexus aurantiacus*.

Key words: *Chlorobium* – *Chloroflexus* – Cell wall – Diaminopimelic acid – Muramic acid-6-phosphate – Ornithine – Peptidoglycan-polysaccharide complex

The order Chlorobiales comprises the two families of green phototrophic bacteria, the *Chlorobiaceae* and *Chloroflexaceae*. The two families share unique properties. In both, the light-harvesting pigments are located in the characteristic chlorosomes (Cohen-Bazire et al. 1964). Both contain monogalactosyl diglyceride but lack phosphatidyl ethanolamine in their lipids. In spite of the striking similarities, the two families are very different: aside from differences in structure of the photosynthetic reaction center (Feick and Fuller 1984) and possibly in the molecular organization of chlorosomes (Staehelin et al. 1978, 1980), the rod-shaped cells of *Chlorobiaceae*, except the gliding *Chloroherpeton thalussium* (Gibson et al. 1984) are non-motile, mesophilic, strictly anaerobic and photoautotrophic. In contrast, *Chloroflexus aurantiacus* strains form gliding filaments which grow thermophilically, facultatively chemo- or phototrophically in complex media on organic carbon sources (Pierson and Castenholz 1974).

Structures of cell wall macromolecules are useful characteristics of molecular taxonomy. Examples are peptidoglycan (Schleifer and Kandler 1972, 1983) or lipid A (Mayer and Weckesser 1984). Most of the variations of peptidoglycan structure concern the peptide moiety and are found in the Gram-positive bacteria. One of the variations is the replacement of *meso*-A_2pm by L-Lys, by L-Orn in spirochaetes (Schleifer and Joseph 1973), of by *meso*-lanthionine in some fusobacteria (Vasstrand et al. 1979). Peptidoglycans from Gram-negative bacteria show less variability, they have *meso*-A_2pm in the 3-position and are directly cross-linked. (Lipo-) protein but no polysaccharide was found to be linked to the peptidoglycan of Gram-negative bacteria (Braun and Rehn 1969).

The present paper reveals that the lack of phylogenetical relationship between *Chlorobiaceae* and *Chloroflexaceae*, as indicated by a low S_{AB}-value (Gibson et al. 1985), is confirmed by rigid layer and peptidoglycan analysis.

Materials and methods

Cultivation of strains

Chloroflexus aurantiacus J-10-fl was obtained from R.W. Castenholz and grown photoheterotrophically in medium D (Castenholz 1969). Cultures were prepared in a 12 l Microferm fermentor (New Brunswick, NJ, USA) at 55°C and a light intensity of 2,000 lux at the surface of the fermentor. *Chlorobium vibrioforme f. thiosulfatophilum* NCIB 8327 (Deutsche Sammlung von Mikroorganismen, DSM, Göttingen, FRG; DSM-No. 263) was grown photolithoautotrophically at 30°C at 1,000 lux in 20 l carboys in the medium published by Steinmetz and Fischer (1982). Enhancement of cell yield was obtained by feeding the culture twice with a mixture of 62.5 mmol $Na_2S \times 9\,H_2O$ and 94.3 mmol of Na_2CO_3 in 1 l of distilled water and partially neutralized with 40 ml of a 2 M H_2SO_4 solution, each time. The pH was controlled after feeding and was adjusted to 6.9–7.1 with sterilized 2 M H_2SO_4.

Cells of both strains were harvested at the stationary growth phase, washed once with distilled water before storage at −20°C until use.

Abbreviations. Ala, alanine; A_2pm, diaminopimelic acid; GC/MS, combined gas-liquid chromatography/mass spectrometry; GlcNAc, N-acetyl-glucosamine; Glu, glutamic acid; Gly, glycine; HF, hydrofluoric acid; Lys, lysine; MurNAc, N-acetyl-muramic acid; Orn, ornithine; SDS, sodium dodecyl sulfate

Offprint requests to: U. J. Jürgens

Isolation of rigid layer and peptidoglycan

Bacteria (20 g wet weight) were suspended in 20 mM Tris-HCl buffer, pH 8.0, containing 0.3 M sucrose. The cell suspension was passed twice through a French Pressure Cell at 16,000 Pa at 4° C. Following centrifugation at 176,000 × *g*, 1 h, the pellet was washed twice with the same buffer and then extracted with 120 ml 4% (w/v) SDS at 100° C for 15 min (Braun and Rehn 1969). After centrifugation at 237,000 × *g*, 20° C for 1 h, the extraction was repeated three times. Insoluble storage polymers were removed at 12,000 × *g*, 20 min. Rigid layers [4% SDS (100° C, 15 min)-insoluble cell wall fraction], were collected from the supernatant by centrifugation at 235,000 × *g*, 1 h, washed with distilled water until the final supernatant was SDS-free (Hayashi 1975). Protein was removed from the rigid layer by incubation with pronase (from *Streptomyces griseus*, 6 units/mg protein) under stirring at 37° C for 12 h as described elsewhere (Jürgens et al. 1983). Following a 237,000 × *g* (15° C, 1 h) centrifugation, the sediment was extracted twice in 4% boiling SDS for 15 min, each, and finally freed from SDS as above.

For hydrofluoric acid (HF) treatment, peptidoglycan (5 mg dry weight) was suspended in 1 ml ice-cold 48% HF and the suspension kept at 0° C for 48 h. Peptidoglycan was recovered at 14,000 × *g*, 15 min, and washed with ice-cold distilled water until the pH of the final supernatant reached 5.5. The acid was removed from the combined supernatants by dialysis against ice-cold distilled water. HF-insoluble peptidoglycan and HF-soluble polysaccharide were lyophilized.

Analytical-chemical determinations

Amino acids and amino sugars were liberated by 4 M HCl at 105° C for 4, 8, or 18 h and determined on a LC 6001 automatic amino acid analyzer equipped with a BT 7040 sample injector (Biotronik, München, FRG), as described elsewhere (Evers et al. 1986). Absorbance of eluting amino acids and amino sugars was measured at 570 nm and 440 nm (the latter for proline). For determination of amino acid configuration, peptidoglycan acid hydrolysates (see above) were esterified with isopropanol/HCl (gas), 1.5 M and then trifluoroacetylated before separation on a 25 m Chirasil-Val fused-silica-capillary column, coated with XE-60-L-valine-(S)-α-phenylethylamide (König et al. 1981). Combined gas-liquid chromatography/mass spectrometric (GC/MS) identification of isopropylester/N-tri-fluoro-acetyl derivatives was performed on a 5985 A instrument (United Technologies, Packard Instrument GmbH, Frankfurt, FRG), using a SE-54 capillary column (25 m length, 0.2 mm i.d.; Applied Science Europe B. V., Holland) and a temperature program (König et al. 1981). Ornithine configuration was also determined enzymatically using ornithine carbamyltransferase (EC 2.1.3.3; Sigma Chem. Comp., St. Louis, MO, USA; Work 1964).

Muramic acid-6-phosphate was liberated from the rigid layer by partial acid hydrolysis (6 M HCl, 110° C, 2 h) and separated from peptides disaccharides, and monomers formed by cation exchange chromatography on Dowex 50 WX-8 (H^+ form, 0.9 by 20 cm column) using a gradient of 0 to 2 M HCl. MurN-6-P eluted with the water fraction. The latter was evaporated to dryness. The eluate was examined on the amino acid analyzer (see above) without and with an additional hydrolysis (4 M HCl, 105° C, 18 h) for liberation of MurN and phosphate. The eluate was also subjected to a combined low-voltage thin-layer electrophoresis [first dimension; pyridine-acetic acid-water, 1:2:250 (v/v/v), pH 4.4, 20 V/cm] and thin-layer chromatography [second dimension; ethylacetate-pyridine-acetic acid-water, 5:5:1:3 (v/v/v/v)]. Ninhydrin staining was used. The MurN-6-P containing peptidoglycan from *Bacillus cereus* AHU1356 was treated in the same way as the control (Jürgens and Weckesser 1986).

Conditions for liberation and gas-liquid chromatographic determination of neutral sugars (as alditol acetates) and of fatty acids (as methyl esters) were as described elsewhere (Jürgens et al. 1983). Organic phosphate was measured colorimetrically (Lowry et al. 1954).

Results

Peptidoglycan-polysaccharide complex of Chloroflexus aurantiacus

The rigid layer fraction (SDS-insoluble cell wall fraction) from *Chloroflexus aurantiacus* J-10-fl was obtained in a 1.6% yield of whole cell dry weight. It was obtained protein-free, without pronase treatment. Fatty acids were lacking as well. Glu, Ala, Orn, some Gly and His were the only amino acids detectable after hydrolysis on the amino acid analyzer. Separation of their trifluoroacetyl derivatives on a chiral fused-silica-capillary column (XE-60-L-valine-(S)-α-phenylethylamide) allowed the assignment of the major amino acids to D-Glu, L-Ala and D-Ala and L-Orn, respectively, in approximate equimolar ratios (Table 1). Ornithine was confirmed by GC/MS of the trifluoroacetyl derivative. Fragments m/z 324 (M-C_3H_6), 307 (M-OC_3H_7), 306 (M-C_3H_7OH, 279 (M-$COOC_3H_7$), 211 [M-(C_3H_6 + CF_3CONH_2)], 166 [M-($COOC_3H_7$ + CF_3CONH_3)], and 139 (166-CH_2=CH) were obtained, whereby M = 366 is the molecular mass of the Orn-derivative. L-Configuration of Orn was confirmed by enzymatic reaction of the rigid layer hydrolysate with ornithine carbamyltransferase: at least 72% of total Orn were converted to citrulline by the enzyme (the residual percentage may be explained by partial razemization during hydrolysis, see Table 1). The two amino sugars found, GlcN and MurN, are both completely N-acetylated. About 15% of MurN is phosphorylated, as revealed by the detection and respective quantitative determination of MurN-6-P on the amino acid analyzer. The amount of phosphate found is essentially in agreement with that of MurN-6-P. Further proof was obtained by partial acid hydrolysis (6 M HCl, 110° C, 2 h) with a following cation exchange chromatography, whereby MurN-6-P was obtained in the water-eluate. Only a single, symmetrical peak was obtained on the amino acid analyzer on simultaneous injection of the eluate and of a comparable eluate from *Bacillus cereus* AHU 1356. Stronger hydrolysis (4 M HCl, 105° C, 18 h) of both these eluates liberated MurN, and phosphate was found in both of them. MurN-6-P was further confirmed by a combined low-voltage thin-layer electrophoresis (first dimension) and thin-layer chromatography (second dimension): a single spot was obtained with both eluates. No other phosphorylated constituents including GlcN-6-P could be detected in the rigid-layer hydrolysate.

Neutral sugars were found in large amounts in the rigid layer fraction (Table 1). They were identified as rhamnose,

Table 1. Chemical analysis of the rigid layer (SDS-insoluble cell wall fraction), corresponding to the peptidoglycan-polysaccharide complex, of *Chloroflexus aurantiacus* J-10-fl. Note that the rigid-layer is free of protein. Data on the untreated and HF-treated (48% hydrofluoric acid, 0° C, 48 h) rigid layer are given

Compound	Peptidoglycan-polysaccharide complex	
	Untreated	HF-treated
	nmol per mg fraction dry weight	
D-Glu[a]	164	1,004
L- and D-Ala (1:1 ratio)	337	2,010
L-Orn[a]	126	760
Gly	39	211
His	36	37
GlcNAc	200	734
MurNAc	138	518
MurN-6-P	24	trace
2-O-Me-6-deoxy-hexose[b]	274	99
2,6-di-O-Me-hexose[b]	90	19
2-O-Me-hexoses I[b] and II[b]	31	20
Rhamnose	1,173	201
Arabinose	279	17
Xylose	390	67
Mannose	488	482
Galactose	226	80
Glucose	361	505
N-Acetyl	343	ND[c]
Phosphate	32	trace
Fatty acids (14:0, 16:0)	<2	ND

[a] Some Glu was found in L- and some Orn in D-configurations, respectively. This is presumably ascribed to razemisation during hydrolysis
[b] Details of identification not given here
[c] ND, not determined

mannose, glucose, galactose, xylose, arabinose, a 2-O-methyl-6-deoxyhexose, a 2,6-di-O-methyl-hexose, and two different 2-O-methyl hexoses (details of identification by GC/MS not given here). Although the separation of the polysaccharide components was not complete, most of the sugars (except mannose and glucose) were removed from the peptidoglycan fraction by HF-treatment in the cold. Incomplete separation may be explained by co-sedimentation of cleaved polysaccharide with the peptidoglycan. MurN-6-P was dephosphorylated by the HF-treatment (Table 1).

Peptidoglycan of Chlorobium vibrioforme f. thiosulfatophilum

The rigid layer fraction (yield: 1.5% yield of cell dry weight) from *Chlorobium vibrioforme f. thiosulphatophilum* NCIB 8327 contained protein which was not removed by the hot SDS-treatment (Table 2). It was removable, however, by pronase treatment of the peptidoglycan. The protein-free peptidoglycan (yield: 1.2% of cell dry weight) contained Glu, Ala, A_2pm, and a relatively small amount of Gly. Separation of amino acid trifluoroacetyl derivatives on the fused-silica-capillary chiral column showed that Glu has the D- and A_2pm the *meso*-configurations. Ala was found in the L- and D-configurations in an approximate 2:1 molar ratio.

Table 2. Chemical analysis of the rigid layer and the peptidoglycan fraction (derived from the rigid layer by pronase treatment) from *Chlorobium vibrioforme f. thiosulfatophilum*. HF-treatment as with the peptidoglycan-polysaccharide complex from *Chloroflexus aurantiacus* (Table 1). Note that the rigid layer of *Chlorobium vibrioforme f. thiosulfatophilum* contains protein

Compound	Rigid layer fraction	Peptidoglycan fraction	
		Untreated	HF-treated
	nmol per mg fraction dry weight		
D-Glu (total Glu)	(45)	94	1,297
L- + D-Ala	75	107[a]	1,683
meso-A_2pm	31	85	905
Gly	32	8	89
GlcNAc	21	74	655
MurNAc	17	68	482
Other amino acids	145	–[b]	–
Glucose	4,846	4,420	415
Other neutral sugars (arabinose, galactose, mannose)	3	22	142
N-Acetyl	ND[c]	91	ND
Phosphate	ND	2	ND
Fatty acids (16:0, 18:0)	4.8	trace	trace

[a] Molar ratio of L-Ala to D-Ala = 2:1
[b] –, None
[c] ND, not determined

GlcN and MurN were the only amino sugars found. Both are likely completely acetylated, as indicated by the acetyl determination of the peptidoglycan fraction and by the lack of respective disaccharides of GlcN-β(1 – 4)MurN as this is obtained in acid hydrolysates depending on partial lack of N-acetylation of the peptidoglycan amino sugars (Araki et al. 1972). The molar ratios of amino acids and amino sugars in the *Chlorobium vibrioforme f. thiosulfatophilum* peptidoglycan are essentially in agreement with the A1γ peptidoglycan structure of Gram-negative bacteria. The 2:1 molar ratio of L- to D-Ala is explained by partial splitting off of the D-Ala in the 4 position of the peptide moiety by D-Ala carboxypeptidases, a phenomenon well known from other Gram-negative bacteria as well (Schleifer and Kandler 1972).

Ornithine was not detectable in the peptidoglycan of *Chlorobium vibrioforme f. thiosulfatophilum*. Most of the large amounts of glucose found was a contamination. It could be removed by HF-treatment of the peptidoglycan fraction (Table 2, the respective supernatant of peptidoglycan sedimentation contained essentially glucose only). MurN-6-P was not detectable in the rigid layer or in the peptidoglycan fraction as well. Lack of MurN-6-P was confirmed by partial acid hydrolysis (6 M HCl, 110° C, 1 or 2 h) of the rigid layer followed by cation exchange chromatography. No amino sugar or amino acid and no phosphate was detectable in the water-eluate. Thus, removal of glucan by the HF-treatment could not be explained here by cleavage of phosphodiester bridges, as confirmed by a negligible phosphate content of the peptidoglycan of *Chlorobium vibrioforme f. thiosulfatophilum* (Table 2).

Discussion

The finding of L-Orn to replace *meso*-A_2pm in the peptidoglycan of *Chloroflexus aurantiacus* is exceptional among phototrophic bacteria so far studied (Drews et al. 1978) and underlines the isolated genealogical position of *Chloroflexus* (Fox et al. 1980; Gibson et al. 1985). The reverse finding (*meso*-A_2pm but no Orn or other diamino acids) in peptidoglycan of *Chlorobium vibrioforme f. thiosulfatophilum* confirms the divergence between the two green-bacteria *Chloroflexus* and *Chlorobium*. *Chloroflexus* but not *Chlorobium* contains wax esters (Beyer et al. 1983; Knudson et al. 1982). Accordingly, based on ribosomal RNA cataloging data, *Chloroflexus* and *Chlorobium* have no close phylogenetic relationship (Gibson et al. 1985).

The difference between *Chlorobium* and *Chloroflexus* concerns major structral principles in the cell wall. The data available on *Chlorobium* indicate a typical Gram-negative cell wall organization: cell wall fine-structure (Cohen-Bazire 1964; Staehelin et al. 1980), lipopolysaccharide in the outer membrane (J. Meißner, unpublished data), protein in the rigid layer, and a likely A1γ-type structure of peptidoglycan. With *Chloroflexus aurantiacus*, however, it is difficult to define clearly the peptidoglycan layer and an outer membrane on ultrathin sections (Pierson and Castenholz 1974; Staehelin et al. 1978). Furthermore, the complex polysaccharide found in the rigid layer fraction of *Chloroflexus aurantiacus* seems to be bound to MurN-6-P of the peptidoglycan via phosphodiester-bridges. This, together with the observed lack of (lipo-)protein in the rigid layer of *Chloroflexus*, are properties known from Gram-positive bacteria only (Schleifer and Kandler 1983). Lipopolysaccharide has not been found so far in significant amounts in *Chloroflexus aurantiacus* J-10-fl on application of the common hot phenol-water extraction (J. Meißner, unpublished data).

Amino acid and amino sugar composition of the *Chloroflexus* peptidoglycan is comparable to that of the L-Orn-containing peptidoglycan of *Spirochaeta stenostrepta* and possibly *Spirochaeta litoralis* (Schleifer and Joseph 1973). Here, peptidoglycan has been proven to be directly cross-linked. In the mono-layered, only 5 nm thick Orn-containing peptidoglycan of *Treponema pallidum*, at least part of Gly found is believed to be involved in cross-linkage of peptide side-chain (Ota 1980; Umemoto et al. 1981). Interestingly, like in the rigid layer fraction of *Chloroflexus*, polysaccharides with genus-specific very complex compositions were found in cell walls of spirochaetes (Azuma et al. 1975; Yanagihara et al. 1984). As with *Chloroflexus aurantiacus*, the polysaccharide dominates by far over the peptidoglycan portion in these cell walls (Azuma et al. 1975).

Other thermophilic bacteria such as some *Thermus* strains (groups B and C in Merkel et al. 1978; Pask-Hughes and Williams 1978), having a Gram-negative cell wall fine-structure, also contain Orn in their peptidoglycan. Recently, A3β-type structure (interpeptide bridges, Gly_2) was identified in various *Thermus* species (Hensel et al. 1986). However, such a structure is unlikely for *Chloroflexus aurantiacus*, due to the lower Gly content (relative to Orn) of this peptidoglycan. Also, the large amounts of sugars in the rigid layer of *Chloroflexus aurantiacus* seem not to belong to glycolipids, comparable to those found in *Thermus* strains (Pask-Hughes and Shaw 1982) and their cell wall preparations (Hensel et al. 1986). Such glycolipids are unlikely, due to the isolation procedure and to the low fatty acid content of the rigid layer of *Chloroflexus aurantiacus*.

It has been discussed that the fact of having Orn in peptidoglycan, together with other properties, justifies to consider *Thermus* species as Gram-positive eubacteria, inspite their Gram-negative reaction (Hensel et al. 1986). Accordingly, characteristic lipopolysaccharide constituents such as β-hydroxy fatty acids, 2-keto-3-deoxyoctonate or heptose were not detectable in hot phenol-water extracts of *Thermus* cells (Pask-Hughes and Williams 1978). Supported by experiments, it was discussed that an increased amount of peptidoglycan and amino sugars in thermophilic strains might stabilize the cells at high temperatures (Merkel et al. 1968). However, with the thermophilic *Chloroflexus aurantiacus* studied herein, there is no evidence for an enhanced peptidoglycan moiety in the cell, as judged from the rigid layer and peptidoglycan yields obtained.

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