Partial Lack of N-Acetyl Substitution of Glucosamine in the Peptidoglycan of the Budding Phototrophic *Rhodomicrobium vannielii*

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The rigid layer and peptidoglycan fractions from two strains (ATCC 17100 and Rm5) of the budding phototrophic *Rhodomicrobium vannielii* were isolated. Rigid layers of both strains contain protein in addition to peptidoglycan. They were free of polysaccharides and fatty acids. The respective peptidoglycan fractions contain glucosamine, muramic acid, L- and D-alanine, D-glutamic and *meso*-diaminopimelic acid in approximately equimolar ratios except for a significant lower relative D-alanine content. Analysis of partial acid hydrolysates revealed A1γ-type structure of *Rhodomicrobium vannielii* peptidoglycan (shown with strain ATCC 17100). An about 10–30% lack of N-acetylation of glucosamine was indicated. The degree of cross-linkage was found to be about 60%.

No differences in peptidoglycan composition and degree of cross-linkage were found between swarmer- and chain-cells as examined with strain Rm5.

Introduction

Only in a few cases, composition of peptidoglycan from purple bacteria is known. The amino acid pattern of the *Rhodospirillum rubrum* peptidoglycan [1] is characteristic of the A1γ-type structure of the peptidoglycan classification [2]. An influence on the extent of cross-linkage and on the cell morphology by the D-Ala metabolism was suggested [3]. Partial lack of N-acetyl substitution of GlcN was detected in isolated peptidoglycans of the budding-like species *Rhodopseudomonas viridis*, *Rhodopseudomonas palustris*, and *Rhodopseudomonas sulfoviridis*, but not with *Rhodocyclus gelatinosus* or *Rhodocyclus tenuis* [4]. Exceptionally small amounts of peptidoglycan are present in the cell wall of the halophilic phototrophic *Rhodospirillum salexigens* [5].

Abbreviations: Ala, alanine; A_2pm, diaminopimelic acid; GC/MS, combined gas-liquid chromatography/mass spectrometry; GlcNAc, N-acetyl-glucosamine; Glu, glutamic acid; MurNAc, N-acetyl-muramic acid; SDS, sodium dodecyl sulfate.

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Rhodomicrobium vannielii cells are typically budding and have a complex cell cycle. Flagellated swarmer cells, non-motile filamentous budding cells (chain-cells) and exospores are sequentially produced in the cell cycle [6]. High carbon dioxide concentration and low light intensity change the complex cell cycle to the so-called simple cell cycle consisting of swarmer and filamentous two-cell forms (chain-cells) only [7]. Lipopolysaccharides of various *Rhodomicrobium vannielii* have major structural principles in common aside from smaller, strain-specific differences [8–10]. A structural analogon of the respective lipid A was synthesized [11]. The present paper describes the chemical composition of the rigid layer (SDS-insoluble cell wall fraction) and of the isolated peptidoglycan from the *Rhodomicrobium vannielii* type strain (ATCC 17100) and from separated swarmer- and chain-cells of strain Rm5.

Materials and Methods

Growth of cells, isolation of swarmer- and chain-cells

Rhodomicrobium vannielii ATCC 17100 was obtained from the American Type Culture Collection,

Rockville, USA, strain Rm5 was from the culture collection of the Department of Biological Sciences, University of Warwick, UK. Mass-cultures were grown in 1 l flasks in the light in R8ÄH-medium as described elsewhere [8]. Cells were collected at the end of exponential growth phase and washed with distilled water. Swarmer and filamentous two-cell form cells (chain-cells) were separated by a glass-wool column [6]. Swarmer- and chain-cells of strain Rm5 were lyophilized before being stored, those of strain ATCC 17100 were used freshly.

Isolation of rigid layers and peptidoglycan

Bacteria (20 g wet weight) were suspended in 60 ml of 20 mM Tris-HCl buffer, pH 8.0, containing 0.3 M sucrose and 2 mM ethylene diamine tetra-acetate. After the addition of about 2 mg DNase and RNase, cells were broken by a two times passage through a French Pressure Cell at 16,000 psi at 4 °C. After centrifugation at 48,000 × *g* (4 °C, 30 min), the pellet was suspended in 20 ml Tris (hydroxymethyl) aminomethane-hydrochloric acid buffer, pH 8.0. It was added dropwise into 200 ml boiling SDS (4%, w/v, in distilled water), boiled for 15 min and after cooling to room temperature allowed to stir for 12 h [12]. SDS-insoluble storage polymers were separated by low-speed centrifugation (3,000 × *g*, 15 °C, 15 min). The sediment of a following centrifugation at 176,000 × *g* (15 °C, 1 h) was suspended in 20 ml distilled water and the extraction repeated 4–6 times until the final supernatant was colourless. The final sediment contained the rigid layer (SDS-insoluble cell wall fraction). It was freed from residual sodium dodecyl sulfate (determined according to [13]) by repeated resuspension in distilled water and ultra-centrifugation.

Peptidoglycan was obtained from rigid layers (50 mg) by removal of protein by incubation with pronase (5 mg, from *Streptomyces griseus*, 8 units/mg protein; Sigma Chemical Co., St. Louis, Missouri, USA) under stirring at 37 °C for 12 h as described elsewhere [12, 14]. Following a 176,000 × *g* (15 °C, 1 h) centrifugation, the sediment was extracted two times in 4% boiling SDS for 15 min, each, and finally freed from SDS as given above.

Identification and determination of components

Amino acids and amino sugars were liberated by 4 M HCl at 105 °C for 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 [5]. For the determination of configuration of amino acids, peptidoglycan acid hydrolysates (see above) were esterified with isopropanol/HCl (gas), 1.5 M and then trifluoroacetylated before separation on a 25 m fused-silica-capillary column, coated with XE-60-L-Valine-(S)-α-phenylethylamide [15]. Combined gas-liquid chromatography/mass spectrometric (GC/MS) identification of isopropylester/N-trifluoroacetyl derivatives was performed on a Hewlett-Packard 5985 A instrument using a SE-54 capillary column (25 m length, 0.2 mm i.d.; Nordion/Helsinki, Finland) and a temperature program.

Conditions for liberation and gas-liquid chromatographic determination of neutral sugars (as alditol acetates) and of fatty acids (as methyl esters) as well as for organic phosphate determination were as given in [14].

N-Acetylation, dinitrophenylation

Peptidoglycan (about 5 mg) was N-acetylated with 5 ml of acetic anhydride saturated with $NaHCO_3$, at 4 °C for 18 h [16]. The sediment of a following centrifugation at 12,000 × *g*, 4 °C, 30 min was washed 5–7 times with ice-cold distilled water before being lyophilized.

For dinitrophenylation, about 2 mg of peptidoglycan were suspended in 1 ml of a 4% solution of triethylamine in ethanol–water (1:10, v/v) and 1 ml of a 5% ethanolic solution of 1-fluoro-2,4-dinitrobenzene (v/v) were added [17]. The reaction mixture was kept at 60 °C for 30 min. After evaporation *in vacuo*, the residue was dissolved in 1 ml of distilled water and extracted 7 times with diethyl ether before the aqueous phase, containing the dinitrophenylated peptidoglycan, was lyophilized.

Separation of partial acid hydrolysates

Partial acid hydrolysates (4 M HCl, 100 °C, 30 min) of peptidoglycan were separated by a combined low-voltage electrophoresis–thin-layer chromatography on cellulose plates (20 by 20 cm, Merck, Darmstadt): first dimension, electrophoresis in pyridine–acetic acid–water (1:2:250, v/v/v), pH 4.4, 20 V/cm, 4 °C, 3 h; second dimension: ascending chromatography in ethyl acetate–pyridine–acetic acid–water (5:5:1:3, v/v/v/v). Staining was with

ninhydrin of fluorescamine for preparative purposes [14]. Spots containing fluorescamine-coupled compounds were scraped off, eluted from the cellulose with distilled water (5 times with 100 µl water, each) and lyophilized after separation of cellulose by centrifugation. Composition of fragments was determined after a following total hydrolysis (4 M HCl, 100 °C, 18 h).

Enzymatic digestion

Peptidoglycan (50 mg) was suspended in 50 ml of 20 mM ammonium acetate buffer, pH 4.8. The suspension was adjusted to an $OD_{578\,nm}$ of 0.23 and warmed up to 37 °C before the addition of 40 µl N,O-diacetyl muramidase (EC 3.2.1.17, from *Chalaropsis* sp., 53,000 U/mg of protein [18]). Enzymatic degradation of peptidoglycan was followed by determining the decrease of optical density at 578 nm and of the concomitant increase of reducing groups [19] during a 19 h incubation at 37 °C.

Electron microscopy

Rigid layers were fixed in 2% glutaraldehyde and 1% osmium tetroxide and embedded in Epon as described in [20]. Staining of ultra-thin sections was performed with uranyl acetate and then by lead citrate [20]. The specimens were examined in a Philips EM 400 microscope at 80 kV.

Results

Rigid layer of Rhodomicrobium vannielii ATCC 17100

The rigid layer (SDS-insoluble cell wall fraction, *i.e.* peptidoglycan with bound polymers) from *Rhodomicrobium vannielii* ATCC 17100 was obtained from cell homogenates by hot SDS-extraction in an about 5% yield of cell dry weight. The rigid layer contained protein in addition to peptidoglycan constituents (Table I). Neutral sugars, fatty acid, or phosphate were not found. Ultra-thin sections of the rigid layer fraction showed electron-dense typical sacculus-like structures indicating high purity of the fraction (Fig. 1).

Composition of peptidoglycan from strain ATCC 17100

Treatment of the rigid layer with pronase yielded a pure peptidoglycan fraction. The yield of isolated peptidoglycan was 1–2% of cell dry weight. Separation of hydrolytically liberated and derivatisized (isopropylester/N-trifluoroacetyl derivatives) amino acids on a chiral XE-60-L-valine-(S)-α-phenylalanine capillary column allowed the assignment of the total of A_2pm to the *meso*- and that of Glu to the D-configurations. Ala is present both as L-Ala (80%) and D-Ala (20%). The approximate molar ratios found in the 4 M HCl (105 °C, 18 h) hydrolysates were GlcN : MurN : *meso*-A_2pm : D-Glu : D-Ala : L-Ala = 0.8:0.6:1.0:0.9:0.3:1.0. In addition, two peaks (a major one eluting at t_R 0.966 and a smaller one at t_R 1.096, both relative to GlcN) were observed on the amino acid analyzer. Both were also observed in respective hydrolysates of the peptidoglycan from *Bacillus cereus* AHU 1356, which is known to show a partial lack of N-acetyl substitution of glucosamine and, thus, an incomplete hydrolysis of the sugar strands of peptidoglycan [16]. Both peaks were absent in the hydrolysates when the peptidoglycan of

Table I. Amino acids and amino sugars in hydrolysates (4 M HCl, 105 °C, 18 h) of rigid layer fractions (SDS-insoluble cell wall) of *Rhodomicrobium vannielii*, strains ATCC 17100 and Rm5. With strain ATCC 17100, the cells from the complex cell cycle were examined, with strain Rm5 swarmer- and chain-cells were separately investigated.

Compound	nmol per mg fraction dry weight		
	Strain ATCC 17100	Strain Rm5	
	Complex cell cycle cells	Swarmer-cells	Chain-cells
Glu (total)	498	599	721
Ala (total)	653	855	895
A_2pm	375	471	423
GlcN	214	229	224
MurN	206	248	241
Peaks I and II[a]	+[b]	+	+
Amino acids (others than Glu and Ala)	2132	2329	3540
Aspartic acid	412	375	584
Threonine	197	186	297
Serine	145	178	267
Glycine	409	476	681
Valine	178	176	281
Isoleucine	78	135	187
Leucine	194	203	302
Tyrosine	141	164	276
Phenylalanine	130	167	259
Histidine	69	51	59
Lysine	96	131	200
Arginine	83	87	147

[a] See Table II.
[b] +, Present but not quantified.

Fig. 1. Ultra-thin sections of the rigid layer fraction from *Rhodomicrobium vannielii* ATCC 17100 showing peptidoglycan sacculi. Bar represents 1.0 μm.

either *Rhodomicrobium vannielii* ATCC 17100 or *Bacillus cereus* AHU 1356 was N-acetylated prior to hydrolysis or when very drastic hydrolysis conditions (6 M HCl, 105 °C, 48 h) were applied for hydrolysis of the peptidoglycan. Concomitantly, the relative amounts of both GlcN and MurN increased under the latter two conditions. Thus, partial lack of N-acetylation of GlcN of the *Rhodomicrobium vannielii* peptidoglycan was revealed. Accordingly, both peaks were shown to contain free amino groups, since they were absent after dinitrophenylation using 1-fluoro-2,4-dinitrobenzene. The percentage of nonacetylated GlcN to the total of GlcN was estimated to be about 22 to 27%.

Structural studies on the peptidoglycan

A partial acid hydrolysate (4 M HCl, 100 °C, 30 min) was subjected to a combined low-voltage electrophoresis–thin layer chromatography (Fig. 2). The fragments GlcN–MurN (or MurN–GlcN), MurN–GlcN–Ala, GlcN–Ala–Glu–A_2pm, Ala–Glu–A_2pm, MurN–Ala, Ala–Glu, Glu–A_2pm were found. Two different spots (identical to spots No. 8a, b in Fig. 3 in [14]) were both found to consist of A_2pm and Ala, as proven by total hydrolysis (4 M HCl, 105 °C, 18 h) of the separately isolated spots. They were separable on the amino acid analyzer (see also [14]) and represent both dipeptides with A_2pm, the one with terminal D-Ala and the other one with the D-Ala involved in the direct cross-linkage. All the other fragments obtained were also according to expectation for A1 structure of peptidoglycan.

The degree of cross-linkage (molar quotient of diamino acid with blocked amino group divided by total diamino acid amount [21] as determined by dinitrophenylating the free amino groups of A_2pm not being involved in cross-linkage) was found to be about 60% in *Rhodomicrobium vannielii* ATCC 17100 peptidoglycan.

Sensitivity towards lysozyme

The isolated peptidoglycan of *Rhodomicrobium vannielii* ATCC 17100 was cleaved to soluble products with a concomitant release of reducing groups by N,O-diacetyl muramidase from *Chalaropsis* sp. (Fig. 2). The degradation, however, was incomplete, although this enzyme is much more effective in peptidoglycan digestion than lysozyme from hen egg-white.

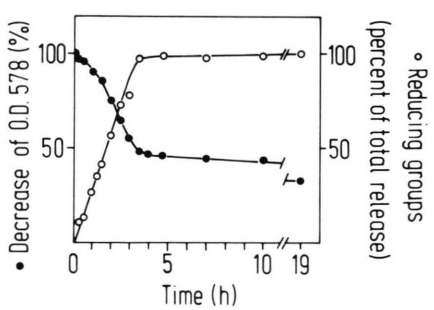

Fig. 2. Enzymatic degradation of peptidoglycan (50 mg) from *Rhodomicrobium vannielii* ATCC 17100 (complex cell cycle cells) by N,O-diacetyl-muramidase (from *Chalaropsis* sp.; EC 3.2.1.17, 53,000 U/mg of protein) in 20 mM ammonium acetate buffer, pH 4.8 at 37 °C. The enzyme-substrate ratio was 1:50 (wt/wt). Closed circles, decrease in optical density at 578 nm (at time zero = 100%). Open circles, increase in reducing groups (percent of total release).

Rigid layer and peptidoglycan from swarmer- and chain-cells from Rhodomicrobium vannielii Rm5

With *Rhodomicrobium vanielii* Rm5, swarmer- and chain-cells were separately investigated for rigid layer and peptidoglycan composition. As with strain ATCC 17100, rigid layer fractions of both swarmer- and chain-cells of strain Rm5 contain protein(s) in addition to peptidoglycan but are free of neutral sugars, fatty acids and phosphate. The molar ratios of peptidoglycan constituents in the isolated peptidoglycan fractions of both cell types were similar to those obtained with strain ATCC 17100 (Table I). Thus, comparable D- and L-configurations of amino acids can be assumed (Table II). Again, incomplete N-acetylation of GlcN was observed (techniques of detection as above). The percentage of nonacetylation of GlcN was estimated to be about 22% (swarmer-cells) and 27% (chain-cells). There was no significant difference in the degree of cross-linkage between the peptidoglycans of swarmer- (57%) and chain-cells (54%). Structural studies were not performed with the peptidoglycans of the two cell types of strain Rm5. With the swarmer-cells, however, an identical pattern as for strain ATCC 17100 was obtained with the comparable partial hydrolysate of peptidoglycan from the swarmer-cells. A1γ-type structure can be assumed for both swarmer- and chain-cells as well based also on the data of qualitative and quantitative composition.

Table II. Amino acids and amino sugars in hydrolysates (4 M HCl, 105 °C, 18 h) of the isolated peptidoglycans of *Rhodomicrobium vannielii*, strains ATCC 17100 and Rm5 (for cell types examined see legend to Table I).

Compound	nmol per mg fraction dry weight		
	Strain ATCC 17100	Strain Rm5	
	Complex cell cycle cells	Swarmer-cells	Chain-cells
D-Glu	822	866	866
L- + D-Ala[a]	1076	1190	1223
meso-A_2pm	912	940	938
GlcN	748	773	767
MurN	546	547	585
Peak I[b]	97	96	94
Peak II[b]	42	36	40
Glycine	62	29	47
Aspartic acid	57	20	30
Lysine	42	39	55

[a] Molar ratio of L- to D-Ala = 4:1.
[b] For elution times on the amino acid analyzer see text; quantitative values given are based on the response factor of GlcN.

Discussion

The data of chemical composition and partial acid hydrolysis fragments reveal A1γ-type structure [22] of the peptidoglycan of the *Rhodomicrobium vannielii* type strain (ATCC 17100). The degree of cross-linkage, however, is in the range of that of Gram-positive bacteria. The same structure is likely for strain Rm5. The finding of A1γ-type peptidoglycan is in agreement with the Gram-negative cell wall organization of both cell types of *Rhodomicrobium vannielii*, swarmer cells and budding cells, which both contain lipopolysaccharide in their outer membrane [9]. Furthermore, as with other Gram-negative bacteria, protein is likely to be bound to the peptidoglycan of *Rhodomicrobium vannielii* as is indicated by the nonpeptidoglycan amino acids in the rigid layer fraction. Although no fatty acids were found in this fraction in this study, lipoprotein as that of *Escherichia coli* [23] should not be excluded at the moment due to a possibly very small lipid moiety. It should be noted that not all phototrophic bacteria have a Gram-negative type cell wall. Lack of lipopolysaccharide, together with a – protein-free – rigid layer fraction was recently observed with the thermophilic, gliding *Chloroflexus aurantiacus* [22]. The respective peptidoglycan contains L-ornithine instead of *meso*-A_2pm, and a complex polysaccharide is presumably covalently bound *via* MurN-6-P to this peptidoglycan.

Cells of *Rhodomicrobium vannielii* are sensitive to lysozyme, although the sensitivity is low. Similarly, the N,O-diacetyl muramidase from *Chalaropsis* sp. is able to degrade the peptidoglycan, but incompletely. This reminds to the findings obtained with the cells and the isolated peptidoglycan of the budding-like *Rhodopseudomonas viridis* [23]. In this case, an about 70% lack of N-acetyl substitution of GlcN was observed explaining the nearly complete lysozyme insensitivity (lysozyme reaction requires specifically N-acetyl substitution of GlcN [24]). The finding of an about 10–30% lack of N-acetyl substitution of GlcN in the peptidoglycan of *Rhodomicrobium vannielii* might explain this intermediate lysozyme insensitivity. Partial lack of N-acetyl substitution of GlcN might have a phylogenetical implication. *Rhodomicrobium vannielii* belongs to the alpha-subdivision of the non-sulfur purple bacteria and its non-phototrophic relatives [25] and, there, is closely related to *Rhodopseudomonas viridis*. Partial lack of N-acetyl substitution of GlcN has been observed only in bud-

ding-like species of purple bacteria, but not in those with binary fission, as studied so far [4].

There were no significant differences between swarmer and budding (chain-cells) of *Rhodomicrobium vannielii* in peptidoglycan and rigid layer composition including cross-linkage, as examined with strain Rm5. Similarly, the lipopolysaccharides of the two cell types are essentially identical in composition [9]. Interference by exospores of the two cell types examined can be excluded, since exospores were completely absent from the cultures of both strains, ATCC 17100 (mixture of swarmer- and budding-cells) and Rm5 (swarmer- and chain-cell cultures). Differences in lipopolysaccharide composition between photo- and chemotrophically grown *Rhodomicrobium vannielii* cultures were recently found to be restricted to the finding of an additional 2-O-methyl pentose and of palmitic acid in the chemotrophically grown cells only and glucan was produced in phototrophically cultures only [26].

In the chemotrophic stalk-forming *Caulobacter crescentus* CB13, the peptidoglycan structures with molar ratios of Ala : A_2pm : Glu = 2:1:1 was assumed to be essentially similar for swarmer- and stalked cells [27]. With the stalked cells, the peptidoglycan seems to be differentiated: the cellular peptidoglycan is relatively rich in MurN, that of the stalk rich in GlcN [28]. In accordance with *Rhodomicrobium vannielii*, the peptidoglycan of *Caulobacter crescentus* was suggested to have A1γ-type structure [29], although a relative high pentapeptide moiety with a partial replacement of the terminal D-Ala by glycine together with a lack of carboxypeptidase were found [30, 31]. The molar ratio of D-Ala : L-Ala = 1:4 in the *Rhodomicrobium vannielii* peptidoglycan also indicates partial lack of terminal D-Ala. Little glycine, aspartic acid and lysine were present in the peptidoglycan fraction of *Rhodomicrobium vannielii*. Their structural role, however, was not examined.

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