Peptidoglycan and Protein, the Major Cell Wall Constituents of the Obligate Halophilic Bacterium *Rhodospirillum salexigens*

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The obligate halophilic member of Rhodospirillaceae, *Rhodospirillum salexigens*, does not contain lipopolysaccharide in its thin and delicate gram-negative type cell wall. Major constituents are protein (approximately 70% of dry weight) and peptidoglycan (murein).

Introduction

The phototrophic bacterium *Rhodospirillum salexigens* has been described recently [1, 2]. Viability and growth are obligatory depending on 5–20% NaCl in the growth medium [1, 2]. Halophilic and halotolerant bacteria seem to follow different strategies to preserve osmotic balance and stability of cell walls (3). Under the phototrophic bacteria *Ectothiorhodospira halophila, E. halochloris* and *Rhodospirillum salexigens* have been found to be salt dependent. We began to investigate the cell wall of *R. salexigens* in order to determine the macromolecules building up the cell envelope. Electronmicroscopical studies showed a regular surface pattern and a very delicate outer membrane but no peptidoglycan-(murein) sacculus of a Gram-negative type [1]. However, inhibitors of murein biosynthesis effected growth and cell shape of *R. salexigens* [2]. Therefore we searched for peptidoglycan. The present study showed peptidoglycan and proteins as major constituents of the envelope fraction.

Material and Methods

Culture of R. salexigens

The organism was cultivated anaerobically in the light in an acetate-glutamate-mineral medium as described recently [1]. The cells were harvested in the exponential growth phase and used directly for analytical work or stored frozen at −70 °C.

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Isolation of peptidoglycan

24 g cells (wet weight) were suspended in 5 ml distilled water. The suspension was added dropwise to boiling solution of sodium dodecyl sulfate (SDS, 8% w/v, 99% pure, Serva, Heidelberg). The mixture was kept boiling for 30 min under stirring and was stirred for several hours during cooling. The mixture was kept over night at room temperature and centrifuged for 2 h at 28,000 × *g*. The sediment was washed 3 times with 700 ml distilled water at room temperature and was finally freeze-dried.

5.8 g of the dried material was extracted with methanol and chloroform as described [4].

The extracted material was resuspended in 1 ml distilled water and added to a boiling solution of SDS (4%; [5]). The suspension was stirred for 2 h while it cooled and kept over night at room temperature. The mixture was centrifuged 20 min at 78,000 × *g*, and washed at least 3 times with dist. water.

The isolated material was washed with 60% trifluoracetic acid, in order to remove unbound protein and washed with water.

A part of the material was freeze dried and subjected to amino acid analysis. Another part was subjected to proteolytic treatment.

Protease treatment

The protein content of the murein-protein complex was determined according to Lowry *et al.* [6]. An enzyme-substrate ratio of 1:50 (w/w) was used. The reaction mixture contained 10 μM Tris-buffer adjusted to pH 8.2 for Trypsin and pH 7.4 for Pronase, respectively. The material was treated 16 h at

37 °C. The digested material was removed from the complex by washing with distilled water.

Amino acid analysis

The material was freeze dried and hydrolyzed in 4 N HCL at 105 °C ± 1 °C for 16 h and subjected to amino acid and amino sugar analysis on an automatic amino acid analyzer (Durrum, model D-500).

Lysozyme treatment of whole cells

The cells were resuspended in the culture medium minus organic compounds. In some experiments ethylene diamintetraacetate (EDTA) was added in a final concentration of 0.5 mg/ml and the cells incubated for 20 min in this solution. Egg white lysozyme (E.C. 3.2.1.17); Sigma Chemical Co. St. Louis, Mo, U.S.A.) was added in a final concentration up to 40 µg/ml). Cells were incubated 45 min at 30 °C. In some experiments cells were frozen in liquid nitrogen and kept for 30 min at −80 °C after addition of lysozyme.

Isolation of a cell wall fraction

The cells were homogenized as described [1] and the extract centrifuged for 3 min at $120 \times g$. The supernatant was spun for 30 min at $25{,}000 \times g$. The sediment was resuspended in Tris-salt-buffer (50 mM, pH 7.6; 6% NaCl, 0.05% $MgCl_2$, 0.05% phenylmethylsulfonylfluoride, 2% Triton X-100) and layered on a sucrose step gradient (30, 50, 60% in the above mentioned Tris-salt-buffer). The gradient was spun for 16 h at $28{,}000 \times g$, 4 °C, in the rotor Ti 60 (Beckman L5–65 ultracentrifuge).

Results and discussion

Table I gives the composition of the SDS-insoluble material from *Rhodospirillum salexigens*. The peptidoglycan specific components diaminopimelic acid, glucosamine and muramic acid are present in about equal molar ratios and were enriched during steps A–D of purification. In spite of an intensive treatment with SDS, methanol, chloroform and proteases a high protein portion is still in the preparation. It has to be determined whether besides alanine other amino acids are constituents of the peptidoglycan and whether a polypeptide is covalently linked to the peptidoglycan.

Table I. Amino acid and amino sugar content of the peptidoglycan protein complex isolated from *Rhodospirillum salexigens* as described under Materials and Methods. The values are given in mol%. The material contained 75.6 mg protein per 100 mg dry weight. A: Samples analyzed after first SDS extraction. The following values are from material which was additionally treated. B: by chloroform-methanol (MeOH), 4% SDS, 60% trifluoroacetic acid (TFA); C: by chloroform-MeOH, 4% SDS, 60% TFA, and pronase: D: by chloroform-MeOH, 4% SDS, 60% TFA and trypsin.

	A	B	C	D
Lys	2.06	2.63	2.29	1.81
His	1.33	1.77	1.10	0.82
Arg	3.68	3.72	2.02	1.48
Asp	7.08	5.99	8.90	7.10
Thr	4.46	3.62	2.59	3.32
Ser	4.88	4.28	4.12	4.58
Glu	6.54	17.27	27.55	21.86
Pro	5.27	2.22	2.08	3.48
Gly	16.57	13.48	11.71	12.63
Ala	14.35	14.94	13.50	16.35
Cys	0.5	N.D.	N.D.	N.D.
Val	4.53	5.43	3.44	3.59
Met	2.14	0.95	+	+
Ile	3.28	2.82	1.12	1.43
Leu	9.22	5.84	2.82	4.02
Tyr	2.95	1.99	0.90	1.04
Phe	4.14	3.01	1.35	1.56
DAP	1.97	3.40	4.31	5.16
Glucosamin	1.53	3.90	3.96	4.84
Muramic acid	1.50	3.36	4.17	4.92

N.D. not determined.
\+ Present in small amounts, but not determined.

Alanine and glycine are the amino acid with the highest molar ratio in the preparation. Its molar ratio does not decrease during protease digestion. Electron microscopically this material did not show sacculus structure (J. R. Golecki; unpublished).

The role of peptidoglycan in the stability of the cell wall

In order to study the role of peptidoglycan for cell stability, cells were treated with lysozyme (see Materials and Methods). Digestion of cells with lysozyme either after pretreatment with EDTA or without EDTA did not change the spirilloid form of the cells. Freezing and thawing of cells resulted in about 10% spheroplasts. Fifty percent of the cells were transformed into spheroplasts after freezing and thawing in the presence of lysozyme. The percentage of spheroplast formed was lower when the cells were

frozen and thawed in the presence of EDTA. However, 95% of cells became spheroplasts after freezing and thawing if they were pretreated with EDTA and frozen in the presence of lysozyme. The additive effect of EDTA, lysozyme and freezing supports the idea that peptidoglycan has a function in cell wall stability of *R. salexigens*, but other macromolecules, presumably, proteins contribute also to cell wall stability.

Isolation and composition of a cell wall fraction

The crude envelope fraction of *R. salexigens* was treated with Triton X-100 in order to dissolve the cytoplasmic membranes and layered on a sucrose density gradient. After centrifugation (see Materials and Methods) a milky-white band was observed in the lower part of the gradient. This band was isolated and dialyzed against distilled water. The yield was 90 µg per mg cell dry weight. Electronmicrographs of this material showed small particles of 15–35 nm in diameter which were irregularly aggregated (see Fig. 7 in [1]). A sacculus structure could not be seen. The fraction did not show activity of NADH oxidation (membrane fraction contains NADH oxidase activity, unpublished result). The major component of the cell wall fraction was protein. Carbohydrates are minor components. Neutral sugars were not detectable (Table II). SDS polyacrylamide gel electrophoresis of this fraction revealed several polypeptides with an apparent m_r higher than 20,000. The pattern was different from that of the intracytoplasmic membrane fraction [1].

The peptidoglycan content of this fraction was lower as compared with the SDS insoluble fraction.

Table II. Composition of the cell wall fraction of *R. salexigens* in mg per 100 mg dry weight.

Protein	66.5
Total carbohydrate*	0.8
Neutral sugars	n.d.
Amino sugars (glucosamine, muramic acid)	1.3
Galacturonic acid	+
Phosphate	0.6
Fatty acids, determined after acid hydrolysis	0.9
or alkaline hydrolysis	1.5
Diaminopimelic acid	0.6

n.d.: not detectable.
* Glucose as standard; amino sugars were only partially covered by the anthron method [7].

It is possible that during the isolation procedure of the cell wall fraction parts of the peptidoglycan were lost by degradation and that the fraction contains some proteins which do not belong to the cell wall.

The low fatty acid and phosphate content indicate that phospholipids are a minor component or absent from the cell wall. Major fatty acids were C_{14}, C_{16} and C_{18}. The data summarized in Tables I and II as well as earlier analytical results [1] show that the cell wall of *R. salexigens* contains peptidoglycan (murein) and protein as major constituents, lipopolysaccharides are absent.

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