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Peptidoglycan of *Rhodopseudomonas viridis*: Partial Lack of *N*-Acetyl Substitution of Glucosamine

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A lack of at least 70% of *N*-acetyl substitution of glucosamine in the glycan strands of the peptidoglycan from the gram-negative bacterium *Rhodopseudomonas viridis* is reported. A disaccharide, very likely GlcNβ(1 → 4)Mur, was observed in hydrolysates of the isolated peptidoglycan. The disaccharide was not observed when peptidoglycan was *N*-acetylated before hydrolysis. The peptidoglycan of *R. viridis* was resistant to lysozyme but became sensitive after *N*-acetylation with acetic anhydride. The disaccharide was found with peptidoglycan from all *R. viridis* strains investigated, as well as with *R. sulfoviridis* P1 and *R. palustris* strains, but not with peptidoglycan from *R. gelatinosa*, *Rhodospirillum tenue*, and *Pseudomonas diminuta* NCTC 8545.

Peptidoglycans of gram-negative bacteria are believed not to show much structural variation. Quite a number of strains belonging to different taxonomical groups have been found to uniformly contain the A1-type of peptidoglycan (20). No modifications within the glycan strands, except some *O*-acetylations (5, 14), are known. In the interpeptide bridges, *meso*-diaminopimelic acid can be replaced by ornithine in *Spirochaetaceae* (10) or by lanthionine in *Fusobacterium nucleatum* (11).

Little is known about the chemical composition of peptidoglycan from the gram-negative *Rhodospirillaceae* (7, 17). Within the *Rhodopseudomonas* genus of this family, three morphologically different groups of species can be recognized (19). One of them, to which *R. viridis*, *R. sulfoviridis*, and *R. palustris* belong, has rod-shaped cells that multiply by budding (19). It will be shown in the present paper that peptidoglycan isolated from *R. viridis*, as well as from *R. sulfoviridis* and *R. palustris* strains, differs from that of other gram-negative bacteria by a partial lack of *N*-acetyl substitution of glucosamine in the glycan strands.

MATERIALS AND METHODS

Growth of bacteria and isolation of peptidoglycan. *R. viridis* ATCC 19567 and other *Rhodospirillaceae* strains used were taken from the strain collection of the Institut für Biologie 2, Mikrobiologie, der Universität Freiburg i.Br. Mass cultures of *R. sulfoviridis* P1 (13) were obtained from H. Biebl, Institut für Mikrobiologie, Göttingen, Federal Republic of Germany. *Pseudomonas diminuta* NCTC 8545 was obtained from the Department of Chemistry of the University of Hull, Hull, Great Britain, and was grown chemotrophically in nutrient broth. Phototrophic bacteria were grown anaerobically in the light (21). The cells were collected in the late log phase.

A French pressure cell was used for disrupting frozen cells (20 g [wet weight]) at 16,000 lb/in^2. Sodium EDTA and SDS were added to the homogenate (final concentrations, 10 mM and 0.4%, respectively.) After centrifugation at 22,000 × *g* for 1.5 h, unbroken cells were removed from the sediment by sedimentation at 12,000 × *g* for 4 min. The two centrifugation steps were repeated twice. The final sediment, suspended in 60 ml of distilled water, was added by drops into 300 ml of aqueous, boiling SDS-mercaptoethanol (4 and 0.1%, respectively). After cooling to room temperature, standing overnight, and centrifugation (22,000 × *g*, 20°C, 2 h), the sediment was extracted again (same conditions). The resulting sediment was centrifuged at 12,000 × *g* for 4 min (20°C), and the supernatant was dialyzed against distilled water before sedimentation at 22,000 × *g* for 2 h, yielding the rigid-layer fraction as the sediment. Peptidoglycan was obtained from the rigid-layer fraction by pronase treatment followed by SDS extraction according to Braun and Rehn (6). The peptidoglycan, obtained as the SDS-insoluble fraction, was additionally purified by centrifugation at 12,000 × *g*, 4 min. Finally, the SDS was removed by dialysis.

Analytical-chemical determinations. Amino sugars and amino acids were released by 4 N HCl at 110°C for 18 h, unless otherwise noted, and were determined on an automatic amino acid analyzer (Durrum, model D-500). The elution program was modified as follows: 0 min, 0.2 M sodium citrate (pH 3.28); 27 min, 0.2 M sodium citrate (pH 4.25); 43 min, 0.38 M sodium citrate (pH 4.25); 57 min, 1.1 M sodium citrate (pH 7.9). Ninhydrin reaction was measured at 590 and 440 nm. Thin-layer chromatography on cellulose plates (0.1 mm, E. Merck AG, Darmstadt) was applied by the buffer system (i) pyridine–*n*-butanol–water = 4:6:3

(vol/vol/vol). Neutral sugars, fatty acids, and phosphorus were determined as described earlier (21).

Determination of *O*-acetyl. Selective cleavage of *O*-acetyl was performed with 0.05 N NaOH at 22°C for 4 h. Acetyl was determined as acetic acid by gas-liquid chromatography (8) in a Varian aerograph, series 14445-01, with a Porapak QS column at temperatures of 170°C (injection port), 200°C (column), and 240°C (detector).

***N*-Acetylation and dinitrophenylation.** For *N*-acetylation, peptidoglycan (50 mg in 7.5 ml of water) was treated with 3 ml of cold saturated $NaHCO_3$ in 10% aqueous acetic anhydride at 4°C for 12 h and then dialyzed for 24 h. For dinitrophenylation, 1 ml of 2.5% (wt/vol) aqueous $NaHCO_3$ and 6 ml of 2.5% (vol/vol) 1-fluoro-2,4-dinitrobenzene in freshly prepared ethanol were added to an aqueous suspension of peptidoglycan (5 mg in 2 ml). The mixture was kept at 37°C in the dark for 16 h and centrifuged at 90,000 × *g* for 60 min, and the sediment was washed twice with 66% ethanol and distilled water.

Enzymatic assays. Incubation with α-amylase, type II from *Bacillus subtilis* (Sigma Chemical Co., St. Louis, Mo.), was performed as described by Mayer et al. (15). *R. viridis* and, as a control, *Escherichia coli* B cells were treated with lysozyme (hen egg white, Serva Feinbiochemica) according to Braun and Rehn (6). For lysozyme treatment of peptidoglycan, 40 μl of a lysozyme (1 mg/ml) solution was added to 4 ml of 10 mM Tris-hydrochloride buffer (pH 7.4) containing 2 mg of peptidoglycan. The reaction (34°C) was recorded by measuring the optical density (578 nm) and by determination of the reducing groups (18).

RESULTS

Isolation of peptidoglycan. Peptidoglycan of *R. viridis* ATCC 19567 was obtained by SDS extraction of cell envelopes followed by pronase treatment of the rigid-layer fraction. Peptidoglycan was obtained in a 0.4% yield of cells (dry weight). Some loss of cell wall material during the differential centrifugation for cell envelope isolation cannot be excluded.

The main contaminant in the peptidoglycan fraction (6 to 60% of dry weight) was a glucan which was sensitive to α-amylase treatment. The fraction was nearly free of protein and lipopolysaccharide (21). The phosphorus content amounted to less than 0.5% of the dry weight.

Chemical analysis of peptidoglycan. Large amounts of *meso*-diaminopimelic and glutamic acids, as well as of alanine, but, unexpectedly, little glucosamine and muramic acid were released on hydrolysis of the *R. viridis* ATCC 19567 peptidoglycan in 4 N HCl at 110°C for 18 h (Fig. 1, Table 1). An unknown constituent (X) eluting just behind glucosamine (glucosamine, 52 min, 55 s; X, 54 min, 49 s) on the amino acid analyzer was present as a main component in this hydrolysate.

No *O*-acetyl was found in the isolated peptidoglycan.

Identification of X as GlcNβ(1 → 4)Mur. When very strong hydrolytic conditions were applied, X decayed, whereas a simultaneous increase in glucosamine and muramic acid was observed. The increase was equal for both glucosamine and muramic acid. At 6 N HCl at 100°C for 34 h, the amount of X was nearly zero, whereas glucosamine and muramic acid reached maximum values. Thus, it was assumed that X might represent an oligosaccharide of the glycan strands of the *R. viridis* peptidoglycan. A GlcNβ(1 → 4)Mur disaccharide was formed on hydrolysis of the peptidoglycan from *Bacillus cereus* AHU 1356 due to an 80% lack of *N*-acetyl substitution of glucosamine in this peptidoglycan (4). We *N*-acetylated the peptidoglycan of *R. viridis* before hydrolysis in 4 N HCl at 110°C for 18 h. The amount of X decreased to almost zero with a concomitant increase in both glucosamine and muramic acid (Fig. 1, Table 1). Thus, a partial lack of *N*-acetyl substitution of glucosamine and, as a consequence, the identity of X with GlcNβ(1 → 4)Mur were indicated. Identical migration values (R_{Ala} = 0.4, solvent [i]) were observed for X and authentic GlcNβ(1 → 4)Mur from *B. cereus* AHU 1356 peptidoglycan on thin-layer chromatograms. Both spots stained characteristically gray with ninhydrin. Isolated X and authentic GlcNβ(1 → 4)Mur eluted at exactly the same position from the amino acid analyzer. In view of the data for lysozyme sensitivity (see below), chemical identity of X with GlcNβ(1 → 4)Mur was highly suggested.

Sensitivity against lysozyme. No spheroplasts were formed on treatment of *R. viridis* ATCC 19567 cells with lysozyme. Isolated peptidoglycan of the strain was also not hydrolyzed by the enzyme (Fig. 2). However, after *N*-acetylation the peptidoglycan became lysozyme sensitive. Reducing groups were released only with the *N*-peracetylated peptidoglycan during the action of the enzyme (Fig. 2).

Dinitrophenylation of peptidoglycan. The peptidoglycan was dinitrophenylated, hydrolyzed in 4 N HCl at 110°C for 18 h, and the amounts of non-dinitrophenylated amino sugars were determined on the amino acid analyzer. The molar ratio of glucosamine to *meso*-diaminopimelic acid present in the *N*-acetylated peptidoglycan (Table 1) revealed a lack of at least 70% of *N*-acetyl substitution of glucosamine in the peptidoglycan. For muramic acid, about 20% of free amino groups were calculated. Note, however, that lower muramic acid values might also be explained by some degradation of muramic acid in strong acid.

Detection of X in peptidoglycan from other strains. Compound X was also found in hydrolysates of peptidoglycan of other *R. viridis*, of *R. sulfoviridis* P1, and of *R. palustris* strains (Table

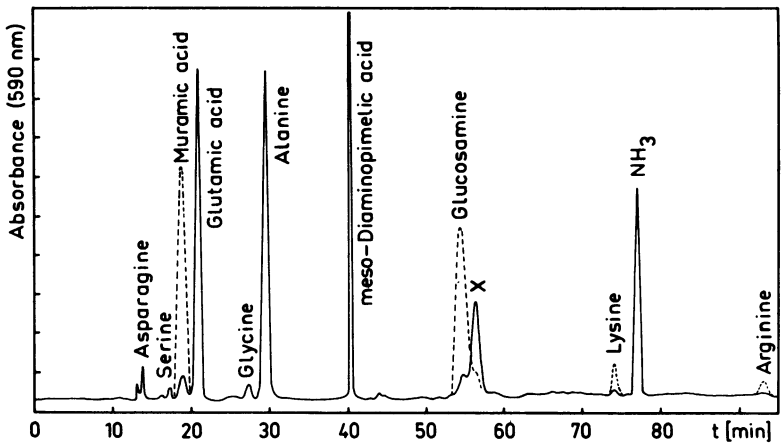

FIG. 1. Amino acid and amino sugar analyses of hydrolysates (4 N HCl, 110°C, 18 h) of peptidoglycan of *R. viridis* ATCC 19567 on the amino acid analyzer. Unbroken line, untreated; dashed line, *N*-acetylated before hydrolysis.

2). It was not found with peptidoglycan from strains of *Rhodospirillum tenue*, *Rhodopseudomonas gelatinosa*, and *P. diminuta* NCTC 8545.

DISCUSSION

The lack of *N*-acetyl substitution in the glycan strands of the *R. viridis* peptidoglycan concerns mainly the glucosamine residues. Analysis of the dinitrophenylated peptidoglycan revealed that free amino groups were present in at least 70% of the glucosamine units. Some lack of *N*-acetyl substitution of muramic acid, however, cannot be excluded.

The findings obtained with *R. viridis* ATCC 19567 are similar to those described for peptidoglycan from *Bacillus* species where 70 to 80% of glucosamine, but none of the muramic acid residues, lacks *N*-acetyl substitution (4, 9). Vice versa, in *Micrococcus lysodeikticus*, muramic acid lacks *N*-acetyl substitution (16), in contrast to glucosamine.

It has been reported that a lack of *N*-acetyl substitution of glucosamine in the glycan strands of peptidoglycan causes resistance to lysozyme (9). *R. viridis* cells, as well as the respective isolated peptidoglycan, are resistant to lysozyme. It is known that the lysozyme reaction specifically requires *N*-acetyl substitution of the glucosamine residues (1, 2). Hydrogen bonds between the acetamido group of glucosamine and amino acid residues 107 and 59 of subsite C of the enzyme were found. Thus, peptidoglycans of the above *Bacillus* strains but not of *M. lysodeikticus* are resistant to lysozyme treatment (16). Diminished *N*-acetyl substitution is presumably not due to a defective biosynthesis of peptidoglycan in *B. cereus*. Instead, an enzyme was found which cleaves off the acetyl group from *N*-acetyl glucosamine residues (3). This enzyme can be distinguished from *N*-acetylglucosamine-6-phosphate deacetylase. Recently, an endoglucosaminidase was found in *B. cereus* AHU 1356 which hydrolyzes the glycosidic linkage of *N*-unsubstituted glucosamine in peptidoglycan (12).

Sensitivity of peptidoglycan against lysozyme can also be diminished by the presence of *O*-acetyl in the sugar strands, the occurrence of free amino groups in the peptide units, the

TABLE 1. Amino sugars and amino acids in hydrolysates (4 N HCl, 110°C, 18 h) of isolated peptidoglycan of *R. viridis* ATCC 19567

Peptidoglycan	Mol%[a] of:					
	Muramic acid	Glucosamine	X	*meso*-Diaminopimelic acid	Glutamic acid	Alanine
Untreated	4.7	5.0	17.8[b]	20.6	32.1	34.5
N-Acetylated before hydrolysis	12.9	16.4	—[c]	14.4	22.3	29.0
Dinitrophenylated before hydrolysis	12.8	4.3	—	12.1	27.9	36.9

[a] Sum in the hydrolysate = 100.
[b] Based on the response factor of glucosamine.
[c] —, None.

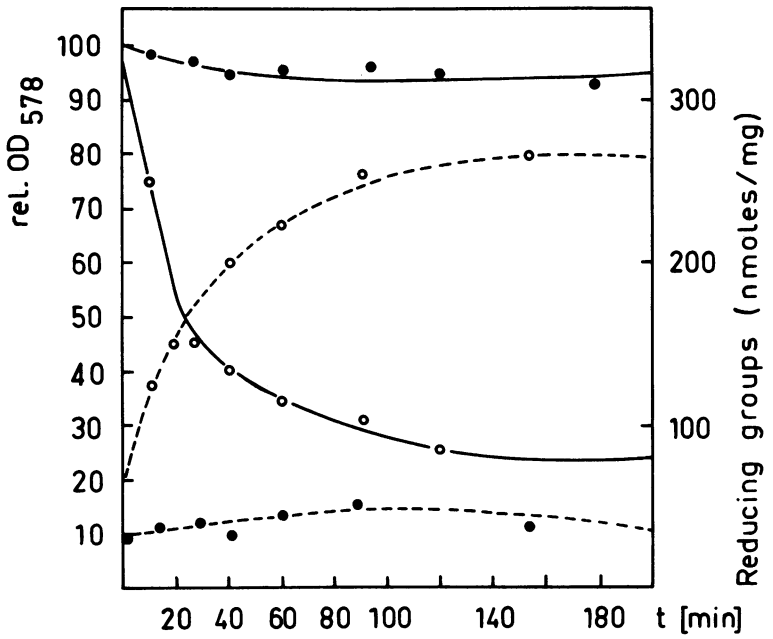

FIG. 2. Lysozyme sensitivity (10 μg/ml) of suspensions of *R. viridis* ATCC 19567 peptidoglycan (0.5 mg of 0.01 M Tris-hydrochloride, pH 7.4) at 34°C. Unbroken line, decrease in optical density at 578 nm (at time zero = 100%). Dashed line, increase in reducing groups (nmol/mg of peptidoglycan [dry weight]). Without (●) and with (○) *N*-acetylation before lysozyme treatment.

degree of peptide cross-linking, or the attachment of other polymers on peptidoglycan (4). There has been no indication so far of any of these possibilities in *R. viridis*. The observed lysozyme sensitivity of the peracetylated *R. viridis* peptidoglycan would also not be explained by them.

To our knowledge, the lack of *N*-acetyl substitution of amino sugar residues in peptidoglycan has not been observed before with gram-negative bacteria. It is remarkable that the disaccharide indicating this lack was found with peptidoglycan of other *R. viridis*, of *R. sulfoviridis* P1, and of *R. palustris* strains but not with *R. gelatinosa* or *R. tenue*. It was also not reported in an analysis of peptidoglycan from *Rhodospirillum rubrum* (17). There are additional major modifications in the cell wall macromolecules of

TABLE 2. Amino sugars and amino acids in hydrolysates (4 N HCl, 110°C, 18 h) of peptidoglycans of various *Rhodospirillaceae* strains and of *P. diminuta* (non-peptidoglycan amino acids are deleted)

Strain	Molar ratio of:					
	meso-Diaminopimelic acid	X[a]	Muramic acid	Glucosamine	Glutamic acid	Alanine
R. viridis Fv103	1	1.1	0.3	0.4	1.1	1.9
R. viridis 9350	1	0.8	0.3	0.4	2.1	1.7
R. sulfoviridis P1[b]	1	1.0	0.3	0.4	2.6	4.3
R. palustris le5[b]	1	0.8	1.2	1.2	1.7	2.5
R. palustris 8/1[b]	1	0.8	0.9	1.2	2.2	3.2
R. palustris 2/2[b]	1	1.0	1.0	1.3	1.8	3.1
R. gelatinosa 29/1	1	—[c]	0.7	0.8	2.8	2.0
R. tenue 2761	1	—	0.7	0.8	1.1	1.8
R. tenue 3761	1	—	0.7	0.9	1.3	2.1
P. diminuta NCTC 8545	1	—	0.8	1.1	1.2	2.3

[a] Based on the response factor of glucosamine.
[b] Peptidoglycan fractions containing essential amounts of contaminating protein. This will explain the relatively high amounts of alanine and glutamic acid in these samples.
[c] —, None.

the three former budding-like species. First, lipid A of the lipopolysaccharides of *R. viridis* and *R. palustris* is remarkably different from lipid A of other gram-negative bacteria (7). Second, there is no serological activity of the cells of the two species in antisera against lipoprotein of the *E. coli* B cell wall (7). Thus, a possible taxonomical relevance of a partial lack of *N*-acetyl substitution in peptidoglycan is indicated. However, a lack of *N*-acetyl substitution of glucosamine was not indicated in peptidoglycan from *P. diminuta*. *P. diminuta* and *Pseudomonas vesicularis* have a type of lipid A similar to that of *R. viridis* and *R. palustris* (22), as well as of *R. sulfoviridis* P1 (manuscript in preparation).

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