SHORT COMMUNICATIONS

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A comparative study of the cell walls of transformable mutants of *Micrococcus lysodeikticus*

The structure of the cell wall of *Micrococcus lysodeikticus* has been extensively investigated[1,2]. In all these studies, the strain used was the one isolated originally by FLEMING[3] in 1922 and kept in culture collections as ATCC 4698 or NCTC 2665. Recently, mutants of *M. lysodeikticus* ATCC 4698 have been described which can undergo genetic transformations (L. GROSSMAN AND I. MAHLER, private communication, and ref. 4), a property not shared by the parent strain. This report describes studies on the composition and structure of the cell walls of three such transformable mutants, in comparison with those of the parent strain.

The transformable strains were obtained through the courtesy of Prof. L. Grossman and Dr. Inga Mahler of Brandeis University. They are designated as 7^- (yellow) and 3^- (white), both ultraviolet-sensitive mutants derived from the parent strain by nitrosoguanidine treatment, and WT ISU, a variant which apparently resulted from subculture of the original Fleming strain[4,5].

The bacteria were grown in a medium containing Bacto peptone (0.5%, Difco), yeast extract (0.1%, Difco), beef extract (0.3%, Difco), NaCl (0.5%) and glucose (1%). Inocula were obtained from subcultures of stocks on agar slants. Cultures were grown in batches (350 ml) in 1.5-l erlenmeyer flasks mounted on a Gyratory shaker (New Brunswick) at 200 rev./min for 24 h at 30°. At the end of this period the culture reached a turbidity of 320–350 Klett units (filter No. 66). The contents of an erlenmeyer flask served as the inoculum for growing the bacteria on a larger scale, in a 20-l carboy containing 14 l of the above medium, to which antifoam was added. Bacteria were harvested at the stationary phase after 24 h of growth with a refrigerated Sharples centrifuge and rinsed with cold water.

Bacterial cell walls were prepared according to SHARON AND JEANLOZ[6]. They were analyzed for nitrogen[7], phosphorous[8], neutral sugar[9] and amino acids. In addition, the walls were digested by lysozyme, the disaccharide *N*-acetylglucosaminyl–*N*-acetylmuramic acid (GlcNAc–MurNAc) and tetrasaccharide GlcNAc–MurNAc–GlcNAc–MurNAc were isolated by gel filtration and ion-exchange chromatography, and their quantities were estimated.

The cell walls (100 mg) were dispersed in 4 ml of 0.1 M ammonium acetate, pH 6.8, lysozyme (hen egg white, 2 × crystallized, Worthington) was added (0.125 ml, 2 mg/ml in water) and the mixture was incubated for 18 h at 37° under a thin layer of toluene. The mixture was then centrifuged for 15 min in a refrigerated Sorvall centrifuge at 9000 rev./min. The supernatant was applied to a 1.5 cm × 120 cm column of Sephadex G-50 (coarse, bead form). The column was eluted with water at a rate of 30 ml/h, and 2.5-ml fractions were collected. Samples (0.5 ml) were taken

Abbreviations: MurNAc, *N*-acetylmuramic acid; GlcNAc, *N*-acetylglucosamine.

from every third fraction for the Morgan–Elson reaction, using a heating time of 35 min and the cell-wall disaccharide GlcNAc-β-(1→4)-MurNAc as standard[10,11]. The major Morgan–Elson positive peak (Tubes 50–86) was pooled, freeze dried, dissolved in water (2 ml) and applied to a 0.8 cm × 32 cm column of Dowex 1-X8, 200–400 mesh, acetate form. Separation was carried out with a linear gradient of acetic acid (mixing chamber, 250 ml water; reservoir, 250 ml of 1 M acetic acid). Fractions (3 ml) were collected at a rate of 30 ml/h. Every second tube was dried in a vacuum desiccator over KOH. The residues were dissolved in water (0.5 ml each) and the samples were used for the Morgan–Elson reaction (0.2 ml) and the reducing sugar test[12] (0.05 ml). The remaining tubes containing half the original amount of the disaccharide (Fractions 50–80) and tetrasaccharide (Fractions 87–130) were pooled separately and dried in vacuum.

To further identify the di- and tetrasaccharides, they were examined by high-voltage paper electrophoresis, pH 6.5 (ref. 13). In addition, they were also examined by thin-layer chromatography on silica gel G (Merck), 0.25 mm, with methanol as developer, and spots were revealed by spraying with sulfuric acid followed by heating at 100° for 10 min. The tetrasaccharide migrated with $R_{GlcNAc-MurNAc}$ 0.55.

In both tests, the disaccharide migrated as a single spot, at a rate identical to that of GlcNAc-β-(1→4)-MurNAc. The rate of migration of the tetrasaccharide fraction isolated from the different mutants was the same as that of the authentic cell-wall tetrasaccharide[11]; however, trailing was usually observed, suggesting contamination by higher oligosaccharides. Quantitative estimation of the disaccharide content gave reproducible results (± 8%). On the other hand, large variations were found in the estimation of the tetrasaccharide fraction when the same cell-wall preparation was used, presumably because no satisfactory separation of the tetrasaccharide from higher oligosaccharides has been achieved. The percentage of the tetrasaccharide found for the different strains was in the range of 3–5.

TABLE I

CELL WALL COMPOSITION IN STRAINS AND MUTANTS OF *M. lysodeikticus*

Cells were harvested after 24 h (stationary phase). The cell walls were prepared as described by SHARON AND JEANLOZ[6]. Nitrogen was determined according to Dumas[7]. Phosphorous was determined by the method of KING[8]. Neutral sugars were determined by the phenol–sulfuric acid method[9] with D-glucose as standard. Amino acids were determined on a Beckman-Spinco amino acid analyzer, after acid hydrolysis (6 M HCl, 22 h, 110°) in ampules sealed under vacuum. Disaccharide GlcNAc–MurNAc was determined as described in text. Reproducibility of the method was established to be ± 8%, with cell walls obtained from commercial organisms. Rate of digestion of cell walls (62–85 μg/ml in 0.1 M ammonium acetate, pH 6.8) by hen's egg white lysozyme (7.5 μg/ml) at 22°, estimated according to SHUGAR[15], $k = 10^2 \times (\ln A_t - \ln A_{30})/t$, where A_{30} is the absorbance at 450 nm after 30 sec, and A_t absorbance at 450 nm at time t.

Strain or mutant	*N* (%)	*P* (%)	*Neutral sugars* (%)	*Amino acid* (μmoles/mg)				*Disaccharide* (%)	*k* (sec^{-1})
				Ala	*Glu*	*Gly*	*Lys*		
7⁻ (yellow)	8.3	0.19	13.9	1.07	0.52	0.51	0.54	4.0	1.38
3⁻ (white)	9.2	0.22	8.6	1.59	0.75	0.77	0.77	4.0	1.19
WT ISU	10.2	0.14	12.2	1.31	0.69	0.65	0.60	6.8	1.45
ATCC 4698	8.6	0.16	9.6	1.21	0.64	0.63	0.63	5.1	1.14
ATCC 4698*	8.7	0.13	15.7	1.00	0.53	0.51	0.50	4.7	1.25

* Freeze-dried cells from Miles Laboratories.

The results of the analyses are summarized in Table I. For comparison, analyses of cell walls of *M. lysodeikticus*, ATCC 4698, prepared from organisms grown in the laboratory or commercially available (Miles Laboratories, Elkhart, Ind.), are also presented.

Qualitatively, no differences between the various cell walls analyzed could be observed. They were all low in phosphorous, suggesting the virtual absence of teichoic acids, and contained neutral sugars present probably as a glucose-aminomanuronic acid polymer[14]. The reason for the high content of neutral sugars in the cell walls of the commercially produced organism is not clear. The amino acids characteristic for cell walls of *M. lysodeikticus* were all present in normal proportions (alanine, 1.88–2.12; glycine, 0.94–1.02; lysine, 0.86–1.03; relative to glutamic acid = 1.00). Other amino acids were found in quantities smaller than 3% of glutamic acid.

The preparations examined contained *N*-acetylmuramic acid residues, devoid of peptide moieties, in the form of the disaccharide GlcNAc–MurNAc, or the corresponding tetrasaccharide. In the cell walls of mutants 7^- (yellow) and 3^- (white), the quantity of disaccharide found was very close to that of the parent strain. The disaccharide content of the walls of WT ISU was higher. If the quantity of free disaccharide is taken as a measure for the relative proportion of glycan chains which are not cross-linked, it may be concluded that the different strains do not differ markedly in the degree of cross-linking in their walls. This is also in agreement with the finding that the rate of digestion[15] of the different cell wall preparations (62–85 μg/ml in 0.1 M ammonium acetate, pH 6.8) by lysozyme (7.5 μg/ml) was very similar.

It can be concluded from these data that the transformable strains possess a normal cell wall which does not differ markedly from that of the parent strain.

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