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Electron Microscopy of Isolated Cell Walls of *Bacillus subtilis* var. *niger*

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Abstract. Isolated cell walls of *Bacillus subtilis* have a striated appearance in the electron microscope. The structure persists when teichoic acids are removed. It is inferred that the structure bears on the arrangement of the peptidoglycan chains.

Key words: Cell wall – Peptidoglycan – Electron microscopy – *Bacillus subtilis.*

Bacillus subtilis cell walls contain as main macromolecular components peptidoglycan and teichoic acids. Both macromolecules can be present in about equal amounts, and the teichoic acids are covalently attached to the muramic acid parts of the peptidoglycans (Reviews: Rogers, 1970; Reaveley and Burge, 1972; Fiedler and Glaser, 1973). Information about the spatial arrangement of these macromolecules in the cell walls of Gram-positive bacteria is limited. Models have been proposed which are based more on reasoning than on experiments. For the peptidoglycan we refer to Kelemen and Rogers (1971) and for the teichoic acids to Archibald et al. (1973). From thin sections it has been inferred that the two components occur as separate layers (Nermut and Murray, 1967) or intermingled (Millward and Reaveley, 1974). In the present paper we are specifically concerned with the organisation of peptidoglycan in the cell wall of *B. subtilis.*

B. subtilis var. *niger* was grown under magnesium limitation in a chemostat (Evans et al., 1970) or as batch culture in Spizizen medium with glucose (Spizizen, 1958). Cell walls were isolated in the following manner. The bacteria were sonified in an ice-salt bath for 2×9 min at 80 W with a model B 12 sonifier equipped with a micro tip (Branson Sonic Power Comp., Danbury, Conn.). Cell walls were prepared according to Fan (1970). The disrupted bacteria were incubated for 15 min in 1% SDS at 100° C. The resulting cell wall suspension was centrifuged for 30 min at $30000 \times g$. The walls were then incubated overnight at approx. 20° C with traces of DNase and RNase. The suspension was centrifuged once more for 30 min at $30000 \times g$, and the pellet was resuspended in 0.1 M Tris-KCl buffer (pH 8). To extract the teichoic acids the cell walls were suspended in 10% trichloroacetic acid in 0.1 M Tris-KCl buffer and incubated for 3 h at 60° C. To check the removal of the teichoic acids from the peptidoglycan the phosphate determination of Chen et al. (1956) was applied. Samples of 0.1 ml were mixed with 0.2 ml 10% $Mg(NO_3)_2 \cdot 6\ H_2O$ in ethanol, and heated over a sandbath until the brown fumes had disappeared. The white ashes were dissolved in 2 ml 1 N HCl and incubated for 15 min at 100° C, whereafter 2 ml of Chen's reagent C was added.

We examined the cell walls by means of positive staining with 1% aqueous uranyl acetate, darkfield illumination and shadow casting. Micrographs were taken with a Philips EM 300 electron microscope. When isolated cell walls or their fragments are stained with uranyl acetate they show striations and ruptures perpendicular to the length axis of the cell (Fig. 1). To deduce whether the substructure is due to the presence of peptidoglycan or teichoic acid the latter was extracted with hot trichloroacetic acid. From Table 1 it can be concluded that this treatment removes almost all teichoic acids. It can be observed that the striated

Table 1. Determination of phosphate

Fraction	µg phosphate/mg cell wall dry weight
Walls before trichloroacetic acid treatment	113.3 ± 6.8
Walls after trichloroacetic acid extraction	8.3 ± 1.8
Supernatant of extracted cell walls	111.6 ± 6.7

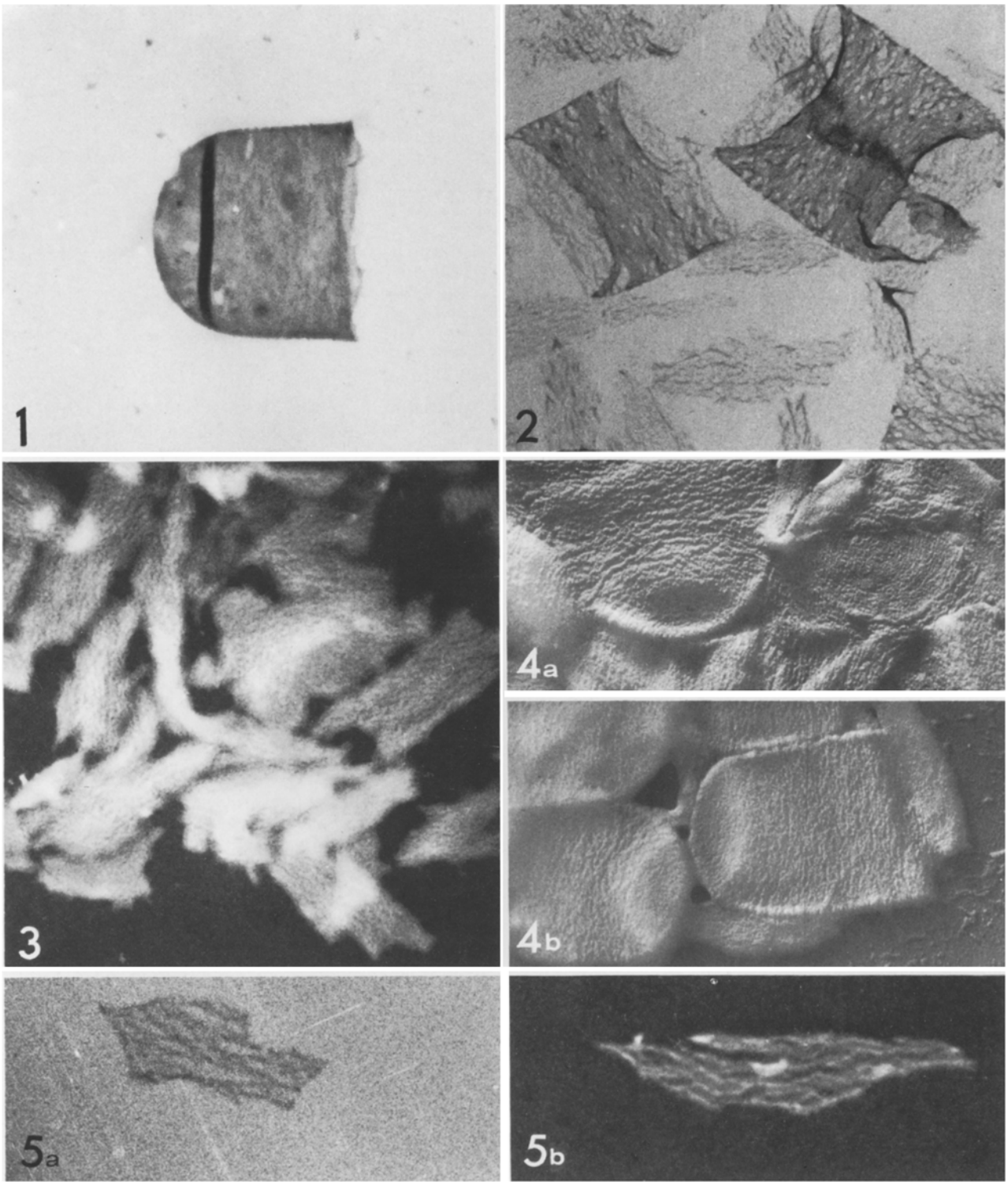

Fig. 1. Cell wall fragment before treatment with trichloroacetic acid. Stained with uranyl acetate. 37000×

Fig. 2. Cell wall fragments after trichloroacetic acid extraction. Stained with uranyl acetate. 37000×

Fig. 3. Darkfield micrograph of unstained wall fragments. 38000× with platinum. 47000×

Fig. 4a and b. Unstained cell wall fragments after shadow casting

Fig. 5a and b. Cell wall fragments after trichloroacetic acid extraction. (a) Stained with uranyl acetate. (b) Darkfield picture of unstained fragment. 44000×

pattern has been retained, though the whole structure looks much thinner (Fig. 2).

A similar pattern has been observed by Preusser (1969) in isolated sacculi of *Spirillum serpens* after staining with uranyl acetate. He suggested that the glycan chains were drawn closer towards each other by the UO_2^{2+}-ions. We have checked this interpretation by visualizing unstained cell walls by darkfield electron microscopy (Fig. 3). It appears that striations are present, and consequently in our case they are not merely due to the specific action of the UO_2^{2+}-ions. Shadow-casting also shows up the striations very well (Fig. 4a and b) and in particular the circular arrangement at the poles of the cells (Fig. 4a). The striations are likewise present in unstained teichoic acid extracted walls (cf. Fig. 5a and b).

We think that the observed structure is related to the organisation of the peptidoglycan chains in the cell wall. We consider the structure as such as an informative artefact which has arisen from the rupture of the weakest bonds during the isolation and preparation procedure. With respect to the arrangement of the peptidoglycan chains one may envisage three possibilities. The chains are arranged as follows: 1. radially, 2. longitudinally, or 3. tangentially. The first possibility seems unlikely: peptidoglycan chains may have a length of 80–700 Å, whereas the wall thickness is in the order of 200 Å (Ward, 1973). The second possibility implies the rupture of the peptidoglycan chain along distances of about 200–500 Å (the thickness of the bands). The third possibility implies ruptures in the peptide bridges connecting the peptidoglycan chains. The second alternative suggests that the peptidoglycan chains are broken at weak points lying in register and perpendicular to the length axis of the cell. To our knowledge there exist to-date no information to confirm that this idea is plausible. We therefore prefer possibility three. We do not imply that the arrangement is strictly regular. But we think that the tangential orientation is the predominant one. However, further evidence is required to substantiate this notion.

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