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The Composition of the Spore Wall and the Wall of Vegetative Cells of *Bacillus subtilis*

BY M. R. J. SALTON AND BETTY MARSHALL*

Department of Bacteriology, University of Manchester

SUMMARY: The spore wall of *Bacillus subtilis* is composed largely of structural protein, in contrast to the peptide+amino sugar+sugar complex of the wall of vegetative cells. Alanine, glutamic acid, α -diaminopimelic acid, glucosamine, muramic acid, glucose, ribitol compounds and O-ester groups are the principal constituents of the vegetative cell walls. All of the compounds characteristic of the peptide+amino sugar+sugar complex have been detected in the spore wall preparations but they account only for a minor part of the spore wall. The walls of the vegetative bacteria have a high phosphorus content and contain appreciable amounts of a ribitol phosphate polymer. Spore walls and vegetative cell walls differ also in the nature of their N-terminal amino acids; glycine is the principal N-terminal amino acid of the spore wall protein whereas alanine is the main amino acid found in the wall of vegetative cells.

The composition of isolated spore walls or spore 'coats' of several members of the genus *Bacillus* was first investigated by Strange & Dark (1956*a*). Apart from a qualitative study by Yoshida, Izumi, Tani, Tanaka, Takaishi, Hashimoto & Fukui (1957) there have been no reports defining the differences in composition of spore walls and the walls of vegetative cells of spore-forming bacteria. It was apparent from the studies of Strange & Dark (1956*a*) that the spore wall with its high nitrogen content and variety of amino acids would differ from the peptide+amino sugar+sugar complexes encountered in the walls of many Gram-positive bacteria (Cummins & Harris, 1956; Salton, 1953, 1958). The results presented in the present paper summarize some of the chemical differences between the spore wall and cell wall of a strain of *Bacillus subtilis*.

METHODS

Isolation of spore walls and walls of vegetative bacteria. *Bacillus subtilis* was grown with aeration in nutrient broth at 30°. For the preparation of walls of vegetative bacteria, the organisms were harvested after 16 hr. growth, washed with distilled water and disintegrated by the procedures previously described (Salton & Horne, 1951). After sporulation of *B. subtilis* in the nutrient broth the spores were harvested by centrifugation and washed three times with distilled water. Spores were then crushed by shaking for *c.* 2 hr. with ballotini beads (*c.* 0.13 mm. diameter) on a reciprocating shaker at 0°. The spore walls were washed with distilled water on the centrifuge. Ultraviolet absorption spectra of the spore-wall preparations did not reveal any absorption maxima corresponding to nucleic acids or dipicolinic acid (Powell, 1953) although

* Present address: Division of Food Preservation and Transport, Commonwealth Scientific and Industrial Research Organization, Homebush, N.S.W., Australia.

several very small 'humps' were apparent at wavelengths of *c.* 240 and 280 m μ . Digestion of the spore walls with trypsin eliminated the substances responsible for the ultraviolet absorption. For all experiments reported below, spore walls and cell walls were digested with trypsin (1 mg. trypsin/ml.; 0.1 M-phosphate buffer, pH 8; 2 hr. at 37°) before analysis.

Chemical analyses

Nitrogen. The nitrogen content was determined on triplicate samples by the procedure described by Johnson (1941) after digestion as in the Kjeldahl method.

Phosphorus was estimated colorimetrically by the Fiske & SubbaRow (1925) procedure.

Amino sugars were determined by the colorimetric method of Rondle & Morgan (1955) after hydrolysis of the wall preparations in sealed ampoules with 2N-HCl for 2 hr. at 100°. For the individual estimation of glucosamine and muramic acid, the hydrolysates were applied to Whatman paper 3 MM and separation of the basic glucosamine from the neutral muramic acid was achieved by electrophoresis (12 volts/cm., 2 hr., 0.05 M-ammonium acetate buffer, pH 6.8). Muramic acid was eluted from the 'neutral' zone and glucosamine from the 'basic' zone of the electrophoresis paper. Both amino sugars were then estimated by the method of Rondle & Morgan (1955), with glucosamine hydrochloride as the standard. Expressing the muramic acid as glucosamine equivalents underestimates the quantity of this amino sugar in the preparations. Strange & Dark (1956*b*) showed, with crystalline muramic acid, that its colour yield under the conditions of the amino sugar colour reaction is less than that of glucosamine. In the absence of a standard of muramic acid, the glucosamine equivalent at wavelength 580 m μ was multiplied by a factor of 1.78 (Strange & Dark, 1956*b*) to give the muramic acid contents.

Lipid contents were determined by extraction with ether or by ether extraction after hydrolysis of the walls with 6N-HCl as previously described (Salton, 1958).

O-ester groups were estimated quantitatively by the method of Hestrin (1949); the results are expressed for comparative purposes as O-acetyl contents although the nature of the O-ester groups has not been established.

Diaminopimelic acid (DAP) contents were determined on hydrolysed wall preparations by the colorimetric procedure of Work (1957).

Identification of wall constituents by paper chromatography. Amino acids and amino sugars were identified after two-dimensional paper chromatographic separation (Whatman paper No. 1, pyridine+water, 4:1, v/v; *n*-butanol+glacial acetic acid+water, 6:1:2 v/v) of the 6N-HCl hydrolysates (16 hr. at 107°). For estimating the relative molecular proportions of the amino acids, the chromatograms were dipped in a solution of ninhydrin (5%, w/v in acetone+phosphate buffer [9.5:0.5 by volume]) and the colours were eluted with a mixture of acetone and water (3:1, v/v) and the extinctions determined at wavelength 570 m μ .

Wall constituents possessing free amino groups were identified by reacting the preparations with 1-fluoro-2:4-dinitrobenzene (FDNB) and determining the nature of the dinitrophenyl (DNP) derivatives by paper chromatography and paper electrophoresis of the products liberated by 6N-HCl hydrolysis as described by Ingram & Salton (1957).

Detection of ribitol compounds. The presence of ribitol phosphate compounds in our cell-wall (but not in the spore-wall) preparations was reported by Baddiley, Buchanan & Carss (1958). With the detection of muramic acid and DAP in the spore-wall preparations it was necessary to examine the spore-wall preparations again, using the Buchanan, Dekker & Long (1950) spray for the detection of polyols. Spore wall (30 mg.) was hydrolysed with 2N-HCl for 3 hr. at 100°, after removal of the HCl by evaporation *in vacuo* over conc. H₂SO₄ and NaOH pellets, the products were taken up in distilled water and applied to Whatman paper No. 1 as a band (5 cm. wide). The papers were irrigated with *n*-butanol+glacial acetic acid+water (3:1:1, v/v) and the dried papers treated as described by Buchanan *et al.* (1950).

Sugar constituents were identified with the aniline phthalate spray reagent on paper chromatograms of hydrolysates of wall preparations (0.5N-H₂SO₄, 16 hr. at 100°; followed by neutralization with Ba(OH)₂ and de-ionizing with a mixed resin bed of Amberlite IR 120 (H)+IR 4B(OH)). Paper chromatographic separation was achieved on Whatman paper No. 1, using ethyl acetate+pyridine+water (Jermyn & Isherwood, 1949) as the solvent system.

Lysozyme sensitivity was determined as described by Salton (1956).

RESULTS

The results of the quantitative analysis of the spore walls and the walls of vegetative *Bacillus subtilis* are presented in Table 1. The nature of the major cell-wall and spore-wall constituents identified in acid hydrolysates

Table 1. *Analysis of spore walls and walls of vegetative Bacillus subtilis*

	Spore walls	Cell walls
	Dry weight (%)	
Total N	12.9	4.6
Total P	1.4	4.2
Total amino sugars (as glucosamine)	2.2	10.7
Glucosamine	1.5	7.9
Muramic acid	0.6 (1.1)*	2.8 (4.1)*
Diaminopimelic acid	c. 1	5.6
Lipid		
<i>a.</i> ether extractable	1.4	0.2
<i>b.</i> total†	3.0	0.7
O-ester groups (as O-acetyl)	0.027	0.255

* Values in brackets represent muramic acid contents corrected by the factor of 1.78 (Strange & Dark, 1956*b*).

† 'Total lipid' estimated by ether extraction after hydrolysis according to Salton (1953).

together with the identification of compounds possessing free amino groups are summarized in Table 2.

Digestion of the walls of vegetative *B. subtilis* with lysozyme liberated 98% of the weight of the walls as soluble components, leaving behind an insoluble wall residue accounting for 7% of the original wall material.

DISCUSSION

The results of the analysis of the spore walls of *Bacillus subtilis* reported above are in close agreement with those obtained by Strange & Dark (1956a). Although the spore-wall preparations contain the components characteristic of the peptide + amino sugar complex of the wall of vegetative organisms, these constituents represent only a minor part of the spore wall. It seems unlikely that these components arise from contamination with vegetative walls, since glucose, a constituent of the latter, was not detectable in the spore-wall preparations. Strange & Dark (1956a) also observed the presence of the 'spore peptides' in the spore-coat fractions of other *Bacillus* species.

Table 2. *A comparison of some of the products identified in acid hydrolysates of walls of spores and of vegetative cells of Bacillus subtilis*

	Spore walls	Cell walls
Amino acids (Relative molecular proportions in brackets)	lysine (6); glycine (5); serine (4); alanine (4); glutamic acid (3); valine/methionine (2); leucine/isoleucine (2); tyrosine (1.5); aspartic acid (1); DAP (1); threonine (1); arginine, cystine, histidine, proline and phenyl-alanine present in smaller amounts.	alanine (4); glutamic acid (2); DAP (1); traces of aspartic acid, serine, valine and leucine.
Amino sugars	glucosamine, muramic acid	glucosamine, muramic acid
Sugars	{ none detectable in 30 mg. hydrolysed wall	glucose
Products of reaction with FDNB	{ DNP-glycine; trace DNP-alanine; mono DNP-DAP (1)*	DNP-alanine; mono DNP-DAP (5)*
Ribitol compounds†	{ Small amounts detectable in 30 mg. hydrolysed wall	Readily detectable in 5 mg. hydrolysed wall

* Relative proportions of mono DNP-DAP in the preparations.

† Detectable mainly as the anhydro-ribitol compound.

The marked difference between the phosphorus contents of spore walls and of vegetative cell walls can be accounted for by the ribitol phosphate polymer as a major wall component of the vegetative cells (Baddiley *et al.* 1958). However, the spore wall contained 1.4% P and the results of the paper chromatographic detection of ribitol compounds gave the impression that there was more phosphorus than could be accounted for by the small amounts of the ribitol phosphate polymer found. It is evident therefore that the spore wall contained phosphorus compounds other than the ribitol phosphate polymer (the teichoic acids of Armstrong, Baddiley, Buchanan, Carss & Greenberg,

1958) and lipids. Yoshida *et al.* (1957) observed a small difference between the P-content of the walls of spores and of vegetative cells of *Bacillus cereus*. The presence of O-acetyl groups in bacterial cell walls was first reported by Abrams (1958). Armstrong *et al.* (1958) found alanine as an O-ester group in the teichoic acids. We have not determined whether the O-ester groups of the spore and cell-wall preparations are exclusively O-alanyl groups or whether there are also O-acetyl groups.

The amount of lipid that we found in the walls of *Bacillus subtilis* is comparatively low; it agrees with the data of Strange & Dark (1956*a*). However, a lipid content of 3 % could readily account for the hydrophobic properties of bacterial spores if the lipid were oriented on the outer surface of the spore wall.

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