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Preliminary communication

Specific determination of ribitol teichoic acid in whole bacteria and isolated walls of *Bacillus subtilis* W23*

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Studies on the mode of assembly of the cell wall of *Bacillus subtilis* have shown[1,2] that new wall material is incorporated first at the inner surface of the wall and becomes exposed at the cell surface as a result of subsequent growth. An indication of the time taken for this translocation can be obtained by observing the interval that elapses between the addition of excess of phosphate to a phosphate-limited culture and the development of the ability of the bacteria to bind bacteriophages that are specific for wall material that contains teichoic acid[3]. Detailed study of this phenomenon requires the accurate determination of teichoic acid incorporated as a result of the addition of excess of phosphate. The amount of phosphate added must be limited to only a small excess, so as to minimise perturbation in growth rate. For this reason, a sensitive assay for teichoic acid was required. Although walls of the phosphate-limited bacteria lack teichoic acid[4], they contain teichuronic acid and small proportions of associated organic phosphates that preclude the use of phosphate analysis for the accurate determination of small amounts of teichoic acid incorporated. An analytical procedure has been described[5] in which ribitol, obtained by acid hydrolysis of the isolated repeating structures of the teichoic acid, is determined enzymically. This method suffers from the disadvantage that 1,4-anhydroribitol is formed during the acid hydrolysis[6] and is not detected enzymically. We have therefore used a g.l.c. procedure to determine the ribitol and glucosylribitol [2-*O*-β-D-glucopyranosyl-L-ribitol] residues that are formed quantitatively on degradation[7] of the teichoic acid with 60% (w/v) hydrofluoric acid. The procedure is simple, specific, and sensitive, and can be used to determine the content of ribitol teichoic acid for isolated walls or whole bacteria.

Bacillus subtilis W23 was grown under phosphate limitation in 3-litre chemostat culture at dilution rate 0.186 h^{-1}, as described previously[3]. After equilibration of the culture, a solution of $NaH_2PO_4 \cdot 2H_2O$ (200 mg) in water (10 ml) was injected into the growth chamber, and samples of culture were collected into cooled receivers for periods of

*Dedicated to the memory of Sir Edmund Hirst, C.B.E., F.R.S.

TABLE I

PHOSPHORUS CONTENT OF WALLS AND GLUCOSYLRIBITOL CONTENT OF WALLS AND OF WHOLE BACTERIA HARVESTED[a] AT INTERVALS AFTER THE ADDITION OF INORGANIC PHOSPHATE TO A PHOSPHATE-LIMITED CULTURE OF *B. subtilis* W23

Time after introduction of phosphate pulse (h)	*Wall phosphorus (% dry weight of wall)*	*Wall glucosylribitol (% dry weight of wall)*	*Cell glucosylribitol (% dry weight of cell)*
0	0.19	0.03	0.02
0–0.5	0.23	0.05	0.06
0.5–1.0	0.30	0.93	0.21
1.0–1.5	0.40	1.33	0.33
1.5–2.0	0.37	1.14	0.26
2.0–2.5	0.37	1.08	0.25
2.5–3.0	0.33	0.83	0.23
3.0–3.5	0.32	0.85	0.23
3.5–4.0	0.40	0.69	0.18
4.0–4.5	0.31	0.61	0.19
4.5–5.0	0.28	0.56	0.14
5.0–5.5	0.29	0.45	0.10
5.5–6.0	0.29	0.39	0.09
6.5–7.0	0.21	0.28	0.07
7.5–8.0	0.28	0.15	0.12
8.5–9.0	0.23	0.11	0.03
10.0–10.5	0.38	0.06	0.03

[a]Samples were treated with HF and analysed by g.l.c., as described in the text.

30 min at the times shown in Table I. Bacteria were recovered by centrifugation, and wall samples were prepared and analysed for phosphorus as before[2]. Accurately weighed samples of dry walls (~5 mg) or of whole bacteria (~10 mg) were suspended in 60% (w/v) hydrofluoric acid (0.25 ml) and stirred for 24 h in stoppered polypropylene tubes at 0°. The contents of the tubes were then washed rapidly into stirred aqueous suspensions of Dowex 2 X8 (20–50 mesh, HCO_3^-) resin (15 ml) in a total volume of 30 ml. An appropriate quantity of trehalose (5–100 μg) was added to each suspension as internal standard. After evolution of CO_2 was complete, the resin was removed by filtration, and the combined filtrate and water washings were concentrated by rotary evaporation and then freeze-dried in glass tubes (6 x 50 mm). Trimethylsilyl ether derivatives were prepared by adding silylation reagent [20 μl; prepared by mixing chlorotrimethylsilane (0.1 ml) and hexamethyldisilazane (0.3 ml)] to the freeze-dried sample in dry pyridine (20 μl). The tubes were sealed and incubated at 37° for 2 h, and then the contents were analysed on a Packard model 417 chromatograph. Samples (3–5 μl) were injected onto columns (3.2 mm x 1 m) packed with 10% of SE30 on Chromosorb WHP (80–100 mesh), and N_2 was used as carrier gas (15 ml.min^{-1}). The chromatograph was programmed to give an initial period of 5 min at 130°, rising at 10°.min^{-1} to 240°. The Me_3Si derivatives of ribitol, glucosylribitol,

and trehalose had retention times of 12.5, 27, and 35 min, respectively. The amounts of ribitol and glucosylribitol in samples were calculated by comparison with standards containing known amounts of these compounds.

Preliminary experiments were performed on a wall sample that was rich in teichoic acid (P, 2.74%). Analysis of eleven separate samples of these walls, using the procedure described above, gave reproducible results and showed that they contained 19.5 ± 0.5% of glucosylribitol and 2.02 ± 0.25% of ribitol; in the wall, each of these structures carries a phosphate group, and therefore ~85% of the phosphorus in these walls is present as ribitol teichoic acid. At least part of the other phosphorus may be present in the short chains of glycerol phosphate residues that are thought to be located between the ribitol teichoic acid and the peptidoglycan[8]. Teichoic acid in this wall sample was also analysed by extraction into alkali followed by alkaline hydrolysis and quantitative enzymic dephosphorylation[9]. Ribitol (2.51 ±0.31%) and glucosylribitol (20.1 ±0.4%) were formed in quantities closely similar to those formed on degradation with hydrofluoric acid. However, the alkali procedure is less specific than the hydrofluoric acid degradation, and was not satisfactory for analysis of whole bacteria or of walls that contained only small amounts of teichoic acid. Analysis of the glucosylribitol content of such walls by the hydrofluoric acid procedure gave highly reproducible results; thus, four separate analyses of walls prepared from bacteria that were collected between 1.5 and 2 h after the addition of the phosphate pulse showed that they contained 1.12 ±0.03% of glucosylribitol. Ribitol was less well-resolved in these samples, although the ratio ribitol:glucosylribitol appeared to be approximately constant in all wall samples that were examined. Therefore, the content of glucosylribitol can be taken as a fairly accurate measure of the total content of ribitol teichoic acid. The analytical results given in Table I show that the maximum content of ribitol teichoic acid was present in samples harvested between 1.0 and 1.5 h after addition of the phosphate pulse. Walls of these bacteria contained ~1.8% (w/w) of ribitol teichoic acid; this is less than 5% of the amount of teichoic acid present in walls of bacteria that are grown in phosphate-rich media. Phage-binding measurements (unpublished) showed that the bacterial sample containing the maximum content of wall teichoic acid had only a small amount of exposed receptor material, and that the sample showing maximum exposed receptor is that collected between 4 and 4.5 h after addition of the phosphate pulse. The interval between the incorporation of teichoic acid and its maximum exposure at the cell surface is thus at least 3 h, which corresponds to at least three quarters of a generation time under the growth conditions used.

In these bacteria, the wall constitutes ~25% of the dry weight of the cell. Since ribitol teichoic acid is found only in the wall, whole bacteria would be expected to contain only 25% as much (w/w) ribitol teichoic acid as do isolated walls. Analysis of the whole bacteria shows that this is so (Table I). As the analytical procedure described here can be applied to whole bacteria, as well as to isolated walls, it should be of value for studies on the regulation of teichoic acid synthesis in intact bacteria. This analytical procedure can also be used (unpublished observations) to determine teichoic acid in walls of bacteria that contain glucosylated glycerol teichoic acid.

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