**Influence of Culture pH on the Content and Composition of Teichoic Acids in the Walls of *Bacillus subtilis***

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**SUMMARY**

Teichoic acids were invariably present in the walls of *Bacillus subtilis* var. *niger* and *B. subtilis* w23 grown in chemostats at $D = 0.25 \, h^{-1}$, $35^\circ C$, pH 7-0 in media containing non-limiting concentrations of phosphate, but they possessed little ester-bound alanine. However, varying the culture pH between 8-0 and 5-0 effected changes not only in the amount but also in the ester-bound alanine content of wall teichoic acids of both organisms. The walls of phosphate-limited *B. subtilis* var. *niger* were devoid of teichoic acid when grown at pH 7-0, but not at lower pH, when this polymer again contained ester-bound alanine. The magnesium binding properties of these wall preparations were also determined. The results showed that walls containing ester-bound alanine in the teichoic acid bound the same amount of magnesium as walls in which the teichoic acid was unsubstituted by alanine.

**INTRODUCTION**

The walls of bacteria change substantially in response to relatively small changes in the growth environment (see Ellwood & Tempest, 1972). This is most evident with organisms grown in chemostat cultures which, besides varying phenotypically in response to parameters such as growth rate and growth limitation (Ellwood & Tempest, 1969; Ellwood, 1970), seemingly differ substantially from organisms grown in batch cultures. For example, chemostat-grown *Bacillus subtilis* had wall teichoic acids that contained little ester-bound alanine (0-1 mol alanine/mol P), whereas those of batch-grown organisms often contained much more (0-2 mol alanine/mol P). This may be because chemostat organisms have almost invariably been grown in simple salts media, whereas batch-grown organisms have often been cultured in nutrient broth, but it is more likely that alanylation is affected by the culture pH, and this we sought to check experimentally.

During growth of batch cultures, a progressive change in pH generally occurs due to either the disproportionate uptake of cations and anions or a net synthesis of acidic end-products of metabolism or both. In these cultures, particularly those grown in plugged flasks, where aeration may be minimal, the drift in pH may be substantial and, indeed, often accounts for the cessation of growth and the onset of the stationary phase. Although this change in the pH of the culture can be minimized by adding a suitable buffer to the medium, complete control can be effected only by monitoring and continuously correcting the drift as it occurs (Callow & Pirt, 1956). By using such a system of automatic regulation, incorporated into a chemostat, it is possible not only to control the pH closely (to within $\pm 0.05 \, \text{pH}$) but also to vary it at will without affecting simultaneously other parameters, such as the growth rate, while observing the physiological consequences and thereby assessing the
Table 1. Composition of media used to supply chemostat cultures

The media were made up from concentrated stock solutions of each component in distilled water. The 'trace salts' were mixed with the citric acid solution before adding to the bulk solution. The glucose solution (slightly acidified with HCl) was sterilized separately (121°C, 30 min) and added aseptically to the bulk solution which had been sterilized by autoclaving at 121°C for 30 min.

<table>
<thead>
<tr>
<th>Medium constituent</th>
<th>Mg²⁺-limited</th>
<th>SO₄²⁻-limited</th>
<th>NH₄⁺-limited</th>
<th>PO₄³⁻-limited</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH₄)_2HPO₄</td>
<td>17.5</td>
<td>17.5</td>
<td>17.5</td>
<td>2.0</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>7.5</td>
<td>2.0</td>
<td>0.5</td>
<td>5.0</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>2.0</td>
<td>10.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>K₂SO₄</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>(NH₄)_2SO₄</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citric acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.25</td>
<td>0.025</td>
<td>0.025</td>
<td>0.025</td>
</tr>
<tr>
<td>CaCl₂</td>
<td></td>
<td>0.025</td>
<td>0.025</td>
<td>0.025</td>
</tr>
<tr>
<td>FeCl₃</td>
<td></td>
<td>0.005</td>
<td>0.005</td>
<td>0.005</td>
</tr>
<tr>
<td>MnCl₂</td>
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<td>0.005</td>
<td>0.005</td>
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</tr>
<tr>
<td>ZnCl₂</td>
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<td>0.005</td>
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<tr>
<td>CuCl₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CoCl₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na₂MoO₄</td>
<td>0.005</td>
<td>0.005</td>
<td>0.005</td>
<td>0.005</td>
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<tr>
<td>Glucose</td>
<td>167</td>
<td>167</td>
<td>167</td>
<td>167</td>
</tr>
<tr>
<td>Titrant</td>
<td>4M-NH₂OH</td>
<td>4M-NH₄OH</td>
<td>2M-NaOH</td>
<td>4M-NH₂OH</td>
</tr>
</tbody>
</table>

functional significance of any change in cell composition. The results of such a study, on the wall teichoic acid content and composition of *Bacillus subtilis* organisms, are reported in this paper.

METHODS

Organisms. *Bacillus subtilis* var. niger (ATCC9372) and *B. subtilis* W23 (obtained from Dr S. C. Warren, Microbiology Unit, University of Oxford) were maintained by monthly subculture on tryptic meat digest agar slopes containing 0.2% (w/v) glucose.

Experimental. Continuous cultures of organisms were grown in 0.5 l chemostats of the type described by Herbert, Phipps & Tempest (1965). The temperature was controlled at 35°C, and the pH regulated automatically (to within ±0.05 pH unit of the desired value) using the method of Evans, Herbert & Tempest (1970). The dilution rate was fixed at 0.25 h⁻¹ unless specified otherwise. The components of the various media are listed in Table 1. Foaming of cultures was prevented by the periodic addition of small volumes (about 0.1 ml) of sterile antifoam (Silicone MS Antifoam Emulsion RD; Hopkins & Williams Ltd, Chadwell Heath, Essex). Small samples of culture (about 20 ml) were removed directly from the growth vessel, but larger volumes (1 to 2 l) were collected overnight via the overflow tube into an ice-cooled receiver. Organisms were harvested by centrifugation, washed once with distilled water, suspended in distilled water at a concentration of 10 g wet wt to 80 ml of water, and broken in a Braun MSK homogenizer with Ballotini grade 16 glass beads. Shaking at the fastest rate for 8 min generally effected a better than 99% disruption and the temperature could be maintained throughout at less than 5°C with occasional pulses of liquid CO₂. The homogenates, heated to 100°C for 10 min to prevent subsequent autolytic degradation, were separated into 'wall' and 'soluble' fractions by centrifugation at 20000 g for 30 min and the walls washed three times with 0.85% (w/v) NaCl and three times with water to remove traces of cytoplasm. Walls were then freeze-dried.
Effect of pH on B. subtilis walls

Table 2. Influence of the pH on the medium on the content and composition of Bacillus subtilis cell walls

Organisms were grown in chemostat cultures with the temperature regulated at 35°C and the dilution rate at 0.25 h⁻¹. Organisms were collected, fractionated and analysed as described in the Methods.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>pH 7.5</th>
<th>pH 6.5</th>
<th>pH 5.5</th>
<th>pH 4.8</th>
<th>pH 3.6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacillus subtilis w23</strong></td>
<td>24</td>
<td>25</td>
<td>22</td>
<td>24</td>
<td>Nil</td>
</tr>
<tr>
<td>(Mg²⁺-limited)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Bacillus subtilis var. niger</strong></td>
<td>20</td>
<td>22</td>
<td>22</td>
<td>20</td>
<td>Nil</td>
</tr>
<tr>
<td>(SO₄²⁻-limited)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Bacillus subtilis var. niger</strong></td>
<td>17</td>
<td>19</td>
<td>22</td>
<td>20</td>
<td>Nil</td>
</tr>
<tr>
<td>(NH₄⁺-limited)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* In order to obtain steady state conditions at pH 5.5, the dilution rate had to be decreased to about 0.1 h⁻¹.

Extraction of walls. Wall samples (100 mg) were suspended in 10% (w/v) trichloroacetic acid (10 ml) in small stoppered Erlenmeyer flasks and stirred slowly with a magnetic stirrer for 48 h in a cold room at 4°C. The temperature of the wall suspension did not rise above 8°C provided that the stirring was kept at a low rate. The wall residue was then removed by centrifugation (20000 g for 30 min) and washed twice with distilled water. The supernatant fluid fractions were combined, extracted five times with ether (5 ml) to remove the trichloroacetic acid, and freeze-dried.

Analytical methods. Phosphorus was determined by the method of Chen, Toribara & Warner (1956) and amino compounds by the method of Rosen (1957). Uronic acid was determined by reaction with concentrated H₂SO₄ and carbazole (Dische, 1947) and total carbohydrate by the procedure described by Dubois et al. (1956). Ester-bound alanine was determined by treating samples (0.5 ml of an 0.2%, w/v, aqueous solution of teichoic acid) with 1 M-NaOH (0.1 ml) for 2 h at 37°C. The sample was then neutralized with 0.1 M-HCl and the amino compounds determined as indicated above (Rosen, 1957).

Chromatographic methods. The polymer samples were hydrolysed by heating with 2 M-HCl (100°C, 3 h) in a sealed tube. The HCl was then removed by repeated evaporation in vacuo over NaOH pellets and P₂O₅. The hydrolysates were chromatographed on Whatman no. 1 paper with the solvents of Ellwood, Kelemen & Baddiley (1963) and those of Janczura, Perkins & Rogers (1961). Components were identified by comparison with authentic samples, whenever possible.

Mg²⁺-binding studies. To determine the affinity of binding of Mg²⁺ to bacteria or wall preparations the following procedure was adopted: 1 to 2 ml vol. of culture, each containing about 3 mg equivalent dry wt organisms (or about 5 mg purified walls), were centrifuged at 3000 g for 10 min. The supernatant fluid was carefully removed and the pellet dispersed in
RESULTS AND DISCUSSION

The walls of *Bacillus subtilis* var. *niger* varied markedly in response to changes in the ionic strength of the growth environment (Ellwood, 1971) and this variation correlated closely with changes in the affinity with which walls could bind Mg$^{2+}$ (Meers & Tempest, 1970). These observations suggested that teichoic acids possibly functioned, in some way, in regulating the movement of ions across the bacterial envelope (see also Archibald, Armstrong, Baddiley & Hay, 1961; Baddiley, 1964, 1970; Hughes, Stow, Hancock & Baddiley, 1971), and if this is so then it is reasonable to suppose that altering the medium $\text{H}^+$ concentration also should cause the wall anionic polymer content or composition or both to vary. Clearly (Table 2) the wall contents of variously limited *B. subtilis* var. *niger* organisms varied slightly (but in an irregular manner) with the pH, and so too did the teichoic acid contents of these walls. Furthermore, the teichoic acids varied qualitatively in that their contents of ester-bound alanine increased progressively as the pH of the culture medium decreased.

The walls of chemostat-grown *Bacillus subtilis* w23 organisms contained a poly(ribitol phosphate)teichoic acid (Fig. 1a) when the environment was not phosphate-limited. In this teichoic acid, the 4(0)-position of the ribitol moiety is glucosylated and the 2(0) or 3 position may be alanylated (Armstrong, Baddiley & Buchanan, 1961). With this structure,
Effect of pH on B. subtilis walls

Fig. 2. Influence of medium pH on the teichuronic acid and teichoic acid contents of walls of phosphate-limited Bacillus subtilis var. niger. Organisms were grown in a chemostat with the temperature regulated at 35°C and the dilution rate set at 0·3 h⁻¹. The medium pH was varied progressively from 8·0 to 5·0, and vice versa, by adjusting the controls on the automatic pH controller (see Evans, Herbert & Tempest, 1970). Wall content of: teichuronic acid, △-△; teichoic acid, ○-○.

in contrast to the poly(glycerol-phosphate)teichoic acid, there are always hydroxyl groups available to accept alanine; nevertheless, the walls of Mg²⁺-limited B. subtilis w23 organisms grown at pH above neutrality contained little ester-bound alanine (Table 2). With growth at successively lower pH, however, the amount of ester-bound alanine present in the wall teichoic acid increased progressively (up to 30% of the maximum possible value).

When grown in variously limited chemostat cultures, the walls of Bacillus subtilis var. niger contained a mixture of teichoic acids (Ellwood, 1970), the predominant one was a polyglucosyl-glycerol phosphate polymer (Fig. 1 b) similar to that in the walls of B. licheniformis ATCC 9945 (Burger & Glaser, 1966). Here again, hydroxyl groups are readily available to accept alanine, but, when organisms were grown at pH or more, little ester-bound alanine was associated with the wall teichoic acids (Table 2). Lowering the pH of the growth medium again caused the level of ester-bound alanine to increase, even when the organisms were NH₄⁺-limited when, presumably, only those N-containing components essential for growth would be synthesized. These findings suggest some important functional significance in the presence of ester-bound alanine in the walls of organisms grown at low pH, and adequately explain why stationary phase batch-grown B. subtilis organisms invariably had wall teichoic acids that were rich in ester-bound alanine.

It is possible, though unlikely, that organisms growing in alkaline environments do synthesize teichoic acids containing much ester-bound alanine, but that the ester bond is subsequently cleaved upon incorporation into the wall structure.

The walls of phosphate-limited Bacillus subtilis organisms (grown at \( D = 0·25 \) h⁻¹, 35°C, pH 7·0) contained teichuronic acid and no teichoic acid (Tempest, Dicks & Ellwood,
Fig. 3. Magnesium ion-binding affinity of Bacillus subtilis var. niger organisms that had been grown in simple salts media with the supply of either (a) sulphate, or (b) Mg$^{2+}$ limiting growth. The organisms were grown at a dilution rate of 0.25 h$^{-1}$ with the temperature controlled at 35°C and the pH at: 6.0, ○—○; 7.0, △—△; or 8.0, □—□. The experimental procedure is described in the Methods.
Effect of pH on B. subtilis walls

REFERENCES


