On the Mode of in Vivo Assembly of the Cell Wall of Bacillus subtilis*

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SUMMARY

During logarithmic growth Bacillus subtilis synthesizes teichoic acid, but not teichuronic acid. Under conditions of phosphate limitation, teichoic acid synthesis stops, and teichuronic acid synthesis is activated. Suitable radioactive labeling experiments have shown that both of these polymers are attached only to glycopeptide chains synthesized at the same time as these specialized polymers are made. An examination of the teichoic acid glycopeptide complex obtained by lysozyme digestion from cells labeled with D-[14C]glucosamine during a period of phosphate limitation, and with D-[14C]glucosamine after resumption of logarithmic growth, has shown that the glycan chains adjacent to those linked covalently to teichoic acid represent a nearly random selection of new and old chains. This last finding is interpreted to indicate that during cell wall growth there is a random intercalation of new and old glycopeptide chains.

In this paper, we first examine whether teichoic acid and teichuronic acid can be attached to pre-existing glycopeptide chains, or whether they can be attached only to glycopeptide chains synthesized at the same time as these polymers are made, and we then determine whether the glycopeptide chains to which teichoic acid is linked are surrounded by a random selection of new and old glycopeptide chains. These experiments have been made possible by the observation of Ellwood and Tempest (7) that when Bacillus subtilis is grown in a chemostat with phosphate as the limiting nutrient, the cell wall contains teichuronic acid, and not teichoic acid. Conversely, in the presence of excess phosphate, the cell walls contain teichoic acid and not teichuronic acid.

MATERIALS AND METHODS

Bacillus subtilis ATCC 3610 (Marburg) was grown at 37° in minimal medium (8) containing 10 mM P and 0 g per liter of glycerol. Where indicated the same medium was used without added P during periods of "phosphate limitation."

Cell walls were prepared as described previously (1). Concanavalin A was obtained from Sigma or Miles Laboratories, and was kept as a stock solution of 30 mg per ml in saturated NaCl. Concanavalin A will precipitate the glucosyl polyglycerophosphate polymer present in the cell wall of B. subtilis ATCC 3610 because it is a polymer of α-D-glucopyranosyl glycerol-P (9). The conditions were similar to that used for the precipitation of other carbohydrate polymers (10). In a typical experiment 2 ml of an enzymatic digest of B. subtilis walls containing 4 μmoles of glucose was diluted with 13 ml of 1 mM NaCl-0.018 M sodium phosphate, pH 7.2, containing 80 mg of Concanavalin A. This mixture was left at room temperature for 16 to 24 hours. The precipitate was collected by centrifugation and washed three times with 15 ml of 1 mM NaCl-0.018 M sodium phosphate, pH 7.2, and finally dissolved in 5 ml of 0.1 M α-methyl mannoside in 0.1 M sodium phosphate, pH 8.0. The dissolved polymer was freed of protein by phenol extraction and dialysis (11). The yield of glucose was 75 to 80% in all cases.

Isotopes were obtained from New England Nuclear. D- [PH]glucosamine was labeled at C-6 and d-[PC]glucosamine was uniformly labeled. Radioactivity was determined in a Packard liquid scintillation counter with absolute activity analyzer, using Aquasol (New England Nuclear) as the counting fluid. Amino sugars were determined after acid hydrolysis (6 N HCl for 12 hours) by the method of Levy and McAllan (12). In-

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individual amino sugars were determined in a Beckman amino acid analyzer at 46° to insure separation of muramic acid and glutamic acid. The amino acid analyzer was equipped with a stream splitter so that the radioactivity in individual amino sugars could be determined. Glucose and glycerol were determined after acid hydrolysis and phosphatase treatment (1). Uronic acid was determined by the method of Dische (13).

Preparation of N-Acetylmuramic Acid-7-Alanine Amidase—

The enzyme was prepared by a modification of the method of Brown, Fraser, and Young (14) to free the enzyme of amino sugar and teichoic acid. B. subtilis was grown in Difco Antibiotic Medium 3. Culture (3.6 liters) was harvested in mid-log phase, and cell walls were prepared by breaking cells with glass beads, followed by differential centrifugation. The walls were washed 10 times with cold distilled water, and suspended in 10 mM Tris-chloride, pH 9.2, 5 mM MgCl₂ and allowed to autolyze overnight. The enzyme was precipitated from the autolysate with ethanol (14), and dissolved in 5 ml of 10 mM Tris-chloride, pH 5.0, and placed on a column (2.5 x 17 cm) of DEAE-cellulose equilibrated with the same buffer. Approximately 50% of the enzyme activity is not retarded by the column and was used to hydrolyze the cell wall of B. subtilis. This enzyme preparation is essentially free of amino sugar and glycerol. Thus the quantity of enzyme that would solubilize 0.25 mg of wall per hour at 25° contained less than 0.05 μmole of each amino sugar or glycerol. In experiments in which this enzyme was used to degrade cell walls, less than 2% of the glycerol or amino sugar in the final reaction mixture could be derived from the enzyme. One unit of amidase hydrolyzes 1 mg of cell wall per hour in 10 mM Tris-chloride, pH 9.2, 5 mM MgCl₂ at 25°. The amidase is free of any measurable glycosidase activity (15).

To separate the products of cell wall lysis, whether by lysozyme or amidase, the lysate was chromatographed on a column (1.5 x 40 cm) of DEAE-cellulose (Whatman A-52) equilibrated with 0.01 M pyridine acetic acid, pH 5.1, and eluted with a 1.2 liter linear gradient to 3.0 M pyridine acetic acid, pH 5.1. The flow rate was 60 ml per hour and 5-ml fractions were collected (7).

To remove teichoic acid from cell walls, 1 ml of cell wall suspension in 0.1 M NaOH was put in a 10-ml ampoule; the ampoule was repeatedly evacuated and flushed with nitrogen, and finally sealed under nitrogen. The walls were incubated at 57° for 24 hours, collected by centrifugation, and washed twice with distilled water (16).

RESULTS

Teichoic and Teichuronic Acid Synthesis during Phosphate Starvation—Ellwood and Tempest (7) have shown that when B. subtilis is grown in a chemostat in which growth is limited by P₃, the cells contain teichuronic acid and not teichoic acid. We have shown¹ that when B. subtilis is allowed to reach stationary phase in medium in which growth is limited by phosphate (0.4 mM P₃), the cell wall contains predominantly teichuronic acid and not teichoic acid. In the following experiments we wanted to determine whether, during an abrupt shift of B. subtilis to

¹ For convenience, N-acetyl muramic acid-t-alanine amidase will be referred to as amidase in the remainder of the text. Glycan wall designate the linear N-acetyl muramic acid-A-acetyl glucosamine polymer obtained after amidase digestion of the cell wall glycopeptide. All sugars are of the D configuration.

² J. Mauck and L. Glaser, unpublished observations.

Table I

<table>
<thead>
<tr>
<th></th>
<th>% of original</th>
<th>% of original</th>
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<tr>
<td>Teichoic acid extractions</td>
<td>%H</td>
<td>%C</td>
</tr>
<tr>
<td>Experiment A NaOH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Walls</td>
<td>12,790</td>
<td>39,070</td>
</tr>
<tr>
<td>Extract</td>
<td>8,910</td>
<td>022</td>
</tr>
<tr>
<td>Amidase and Concanavalin A</td>
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<td></td>
</tr>
<tr>
<td>Walls</td>
<td>3,100</td>
<td>5,082</td>
</tr>
<tr>
<td>Extract</td>
<td>980</td>
<td>120</td>
</tr>
<tr>
<td>Experiment B NaOH</td>
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<tr>
<td>Walls</td>
<td>1.88 x 10⁴</td>
<td>1.0 x 10⁴</td>
</tr>
<tr>
<td>Extract</td>
<td>5.1 x 10⁵</td>
<td>1.2 x 10⁴</td>
</tr>
<tr>
<td>1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment C NaOH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Walls</td>
<td>1.64 x 10⁴</td>
<td>6.28 x 10⁴</td>
</tr>
<tr>
<td>Extract</td>
<td>3.55 x 10⁴</td>
<td>2.64 x 10⁴</td>
</tr>
</tbody>
</table>

P₃-free medium, teichoic acid synthesis would stop and teichuronic acid synthesis begin. The data in Table I show that no radioactive teichoic acid could be extracted from cells, labeled with a variety of radioactive precursors,² in the absence of P₃. Teichoic acid was either determined as alkali-extractable counts or as Concanavalin

² It should be noted that, although glucosamine labels predominantly amino sugars in the wall, it will also label teichoic and since considerable degradation of glucosamine to other metabolites occurs in these cells.
containing polymer which cochromatographs on DEAE-cellulose with teichuronic acid. No such polymer is present in cell alkaline extraction to remove teichoic acid), a galactosamine-containing polymer which cochromatographs on DEAE-cellulose with teichuronic acid. No such polymer is present in cell walls from logarithmically growing cells. This polymer of uronic acid and galactosamine is presumed to be similar to or identical with teichuronic acid (6, 7, 16, 18). It should be noted that cell walls obtained from B. subtilis ATCC 3610 in logarithmic growth phase contain galactosamine associated with the teichoic acid (19). This galactosamine is totally removed by alkaline extraction (16).

After incubation of B. subtilis in Pi-free medium for 75 min, the cells contained 35 µg of uronic acid per mg of cell weight. Under these conditions considerable glycopeptide synthesis also takes place. A rough estimate of the quantity of glycopeptides synthesized under these conditions can be obtained as follows. We consistently find that the incorporation of 14C- or 3H-labeled glucosamine, during phosphate limitation into cell wall muramic acid, glucosamine, and galactosamine, is in the ratio 1:1:2. Since teichuronic acid contains 1 galactosamine per uronic acid, there must be 0.18 µmole of amino acid sugar incorporated into glycopeptide per mg of cell, dry wt, per 75 min. This glycopeptide is not all associated with teichuronic acid, since the ratio of muramic acid to glucosamine to galactosamine in teichuronic acid glycan complex isolated after amidase digestion of cell walls from this organism is 1:1:6.

Can Teichoic Acid and Teichuronic Acid be Attached to Pre-existing Glycopeptide Chains?—To answer this question in the case of teichuronic acid, B. subtilis was labeled with [3H]glucosamine during logarithmic growth; the cells were then incubated in phosphate-free medium and labeled with [14C]glucosamine. The isolated cell walls were treated with NaOH to remove teichoic acid, digested with amidase, and chromatographed on DEAE-cellulose as shown in Fig. 2. As can be seen, essentially no 14C is present in the teichuronic acid peak. The radioactivity in muramic acid, glucosamine, and galactosamine in the cell wall glycan and in the teichuronic acid glycan complex, from this experiment, and from one in which the labels were reversed, is shown in Table II. We conclude that teichuronic acid cannot be attached to pre-existing glycopeptide chains.

In Table III is shown a similar experiment with teichoic acid. In this case the cell wall was labeled with [14C]glucosamine in Pi-free medium, and then transferred to phosphate-containing medium containing [3H]glucosamine. The glycan strands attached to teichoic acid were isolated after amidase digestion by precipitation with Concanavalin A. The data show that teichoic acid cannot be attached to pre-existing glycan strands, and can only be attached to glycan chains synthesized at the same time as the teichoic acid is synthesized. The galactosamine present in the teichoic acid glycan complex is part of the teichoic acid of this organism (19), perhaps associated with the teichoic and glycopeptide linkage region, and is not derived from teichuronic acid.

In both the teichoic and teichuronic acid experiments the corresponding polymer glycan complexes consistantly contain a small quantity of old wall. This could be due to some labeled metabolite accumulated in the cell, which contributes label to the polymer. It could also be due to incomplete amidase digestion. We have checked into the latter possibility by examining the muramic acid to diaminopimelic acid ratio in the isolated complex. In the experiment in Table III, 96% of the diaminopimelic acid had been removed by amidase digestion.

Nearest Neighbor Analyses of Teichoic Acid Chains—Fig. 3 shows a simplified model of the B. subtilis cell wall. It shows that after lysozyme digestion, Concanavalin A would precipitate

![Graph](http://example.com/graph.png)
Fig. 2. Attachment of teichoic acid to new glycan chains. B. subtilis was grown in 50 ml of minimal medium with 10 mM Pi. At an O.D.₆₆₀ = 0.1, 50 μCi of [¹⁴C]glucosamine (6 μmoles) were added. After two generations, the cells were collected by filtration and suspended in 50 ml of phosphate-free medium. After 15 min, 100 μCi of [¹³C]glucosamine (4 μmoles) were added. After 60 min, the cells were collected by centrifugation, washed with distilled H₂O, and diluted with carrier cells for preparation of cell walls. The walls were extracted with alkali and digested with amylase and chromatographed on a DEAE-cellulose. Peak I represents glycan strands and Peak II the teichoic acid glycan complex. The individual peaks shown were acid hydrolyzed and the radioactivity of individual amino sugars determined (Table II).

**Table II**

Attachment of teichoic acid to glycopeptide chains

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Glycan</th>
<th>Teichoic acid</th>
<th>Glycan complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycan</td>
<td>34</td>
<td>8</td>
<td>35.3</td>
</tr>
<tr>
<td>Teichoic acid</td>
<td>29</td>
<td>0.6</td>
<td>37.2</td>
</tr>
<tr>
<td>Experiment II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycan</td>
<td>114.0</td>
<td>16.2</td>
<td>176.0</td>
</tr>
<tr>
<td>Teichoic acid</td>
<td>1.1</td>
<td>1.29</td>
<td>0.3</td>
</tr>
</tbody>
</table>

**Table III**

Teichoic acid attachment to old and new glycopeptide

| B. subtilis was labeled with [³H]glucosamine in the absence of Pi, then transferred to conditioned medium (I). After 0.25 generations, [¹⁴C]glucosamine was added and incubation was continued for 0.5 generations before preparation of cell walls. The isolated cell walls were digested with amylase and the glycosylated teichoic acid was precipitated with Concanavalin A. The precipitate was dissolved in α-methyl mannoside and phenol extracted. Aliquots of whole cell walls and the teichoic acid glycan complex were acid hydrolyzed and the radioactivity of individual amino sugars determined.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Glycan</th>
<th>Teichoic acid</th>
<th>Glycan complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cell walls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muramic acid</td>
<td>13.9</td>
<td>5.97</td>
<td>2.34</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>10.8</td>
<td>1.00</td>
<td>1.34</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>23.6</td>
<td>4.50</td>
<td>5.25</td>
</tr>
<tr>
<td>Teichoic acid glycan complex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muramic acid</td>
<td>0.40</td>
<td>2.64</td>
<td>0.15</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>0.24</td>
<td>4.15</td>
<td>0.05</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>0.54</td>
<td>2.70</td>
<td>0.2</td>
</tr>
</tbody>
</table>

We have also obtained in separate experiments an estimate of the length of glycopeptide chain attached to teichoic acid after lysozyme digestion, followed by amylase digestion. The ratio of galactosamine to glucosamine in this teichoic acid complex is 15.6. If the chain length of teichoic acid is 30 disaccharide units (6), then, after enzymatic digestion, there remain 2 molecules of glucosamine and 2 molecules of muramic acid per chain.

A fragment of the cell wall containing not only the glycan chain to which teichoic acid is attached, but also neighboring chains. These two glycan chains will be referred in the remainder of this paper as main chain and side chain, respectively.

In Table IV are shown two experiments to determine the ratio of main chain and side chain in the teichoic acid glycopeptide complex obtained after two different times of lysozyme digestion. For this purpose the cell walls obtained from B. subtilis in logarithmic growth were digested with lysozyme and the teichoic acid glycopeptide complex isolated by column chromatography. The complex was then digested with amylase, and the teichoic acid glycan complex containing the main chain was isolated. Amino acid analyses of the samples indicated that the amylase digestion had proceeded to greater than 90% completion in all cases.

From the change in ratio of glycerol to amino sugar, before and after amylase digestion, the ratio of side chain to main
In Table V are shown data from two experiments in which B. subtilis cell walls were labeled with [3H]glucosamine in the absence of Pi (i.e. in the absence of teichoic acid synthesis) and then labeled with [14C]glucosamine for 0.5 generation during subsequent logarithmic growth in the presence of Pi. For each sample the teichoic acid glycane complex was isolated by amidase digestion and Concanavalin A precipitation and the teichoic acid glycopptide complex was isolated by lysozyme digestion followed by Concanavalin A precipitation.

It is clear that considerably more 3H is present in the glucosamine and muramic acid obtained after Concanavalin A precipitation of a lysozyme digest than in the corresponding precipitate obtained after amidase digestion. The Concanavalin A precipitate obtained after amidase digestion contains teichoic acid linked only to the main chain. The Concanavalin A precipitate after lysozyme digestion contains teichoic acid linked to the main chain plus side chain fragments. The presence of tritium in the latter fraction suggests that the teichoic acid chains have as neighbors chains synthesized during phosphate limitation which contain 3H.

In the last line of Table V are the calculated ratios of 3H:14C in the side chain, assuming the ratios of side chain to main chain obtained in Table IV under identical conditions of lysozyme digestion.

It was clearly of importance to obtain the ratio in the side chain directly. This was done in the experiment in Table VI, where cell walls prepared in the same way were first digested with lysozyme, and the teichoic and glycopeptide complex was isolated by Concanavalin A precipitation. This complex was cleaved with amidase to yield: (a) glycane chains, (b) glycane chains linked to teichuronic acid, and (c) teichoic acid glycane complex. The ratio of 3H:14C in the muramic acid and glucosamine of each of those compounds was determined, and is shown in Table VI. In this experiment also there is considerable 3H in the side chains. The ratio of 3H:14C in the unsubstituted side chains is 30% of that found in the whole cell wall. The total side chain that is unsubstituted plus teichuronic acid-containing side chains have 35% of the 3H:14C ratio of the whole

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Teichoic acid glycopeptide complex</th>
<th>Teichoic acid glycane complex</th>
<th>Ratio of side chain to main chain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glycosylated</td>
<td>Glucosamine</td>
<td>Amino sugar</td>
</tr>
<tr>
<td>A</td>
<td>4.52</td>
<td>1.46</td>
<td>3.53</td>
</tr>
<tr>
<td>B</td>
<td>9.0</td>
<td>1.72</td>
<td>4.1</td>
</tr>
</tbody>
</table>
The experimental procedure was that for Table III. Cell walls were obtained from cells which had been labeled with [3H]glucosamine in the absence of Pi, and with [14C]glucosamine after the cells were resuspended in minimal medium with 10 mM Pi. Experiment I is the same experiment as shown in Table III. In Experiment I, 5 mg of cell walls were digested with 2 mg of lysozyme at 37°C for 24 hours. In Experiment II, 5 mg of cell walls were digested with 0.4 mg of lysozyme at 37°C for 4 hours. In Experiment I the aliquot of cell wall digested with lysozyme contained 6.6 × 10^6 dpm of [3H] and 2.2 × 10^6 dpm of [14C], and yielded, after Concanavalin A precipitation, 3.5 × 10^5 dpm of [3H] and 7.7 × 10^4 dpm of [14C] in the teichoic acid glycopeptide complex. The [3H]:[14C] ratio in the side chain was calculated using the data in Table IV for the amino sugar ratio in the main chain and side chain. For example, in Experiment I, the ratio of amino sugar in the side chain to main chain is assumed to be 0.39. Therefore, [3H]:[14C] ratio in the side chain = 2.3. The value in parentheses represents the percentage of this calculated value relative to the value of the whole cell wall taken as 100%.

Thus, in the case of muramic acid, the total [3H] counts in side chains is 16.9 × 10^6 dpm and total [14C] is 15.8 × 10^6 dpm. The [3H]:[14C] ratio in the total side chain is 1.07 or 34.2% of the ratio observed in the whole cell wall.
teichoic acid main chain complex contained \(9.2 \times 10^6\) dpm of \(^{3}H\). After lysozyme and amidase the teichoic acid main chain complex contained \(0.5 \times 10^6\) dpm. Both of these experiments show that transglycosylation cannot account for the data in Tables V and VI.

**DISCUSSION**

The change in the pattern of cell wall polymer synthesis during phosphate starvation provides a unique opportunity to examine certain characteristics of cell wall synthesis. We could demonstrate that, within experimental error, both teichoic and teichuronic acid are attached only to glycopeptide chains synthesized at the same time as these polymers are made, and apparently cannot be attached to pre-existing glycopeptide chains.

This result is not entirely surprising, but this is the first time that this assumption could be tested. It implies that primary control of cell wall synthesis in these organisms need occur only at the level of glycopeptide synthesis, and that the availability of new glycopeptide chains can serve to control the rate of teichoic or teichuronic acid deposition in the cell wall.

The data obtained by nearest neighbor analysis are difficult to interpret in a quantitative way. For purposes of this discussion, we can consider the following model of cell wall growth. The septum, including the area where it connects to pre-existing wall, is totally new wall. Longitudinal growth occurs by random insertion of new glycopeptide chains in the pre-existing wall. In such a model, random insertion of glycopeptide chains in the side walls would yield, on nearest neighbor analysis, a random collection of old and new chains, if it were possible to analyze the side walls only. In the actual analyses the side wall and end walls are analyzed together. This limitation allows only a semiquantitative interpretation of the data. The data obtained suggest that the glycan chains adjacent to those linked to teichoic acid contain a large percentage of glycan strands synthesized before the teichoic acid chains were synthesized. Since this value clearly is greater than what would be expected for a single growth zone, where only the edges of new wall would be in contact with old wall, we interpret these data as indicating multiple sites of glycopeptide synthesis on the surface of *B. subtilis*.

In this connection it is worthwhile to emphasize the design of the experiments in Tables V and VI. After the cell wall was labeled with \(^{3}H\) during \(P_{1}\) limitation in the absence of teichoic acid synthesis, the cells were transferred to \(P_{1}\)-containing medium and allowed to grow for 0.25 generations before \([^{14}C]\)glucosamine was added.

Nevertheless, as pointed out before, there is always some \(^{3}H\) found in the main chain, which is puzzling. One possibility is that certain pools of intermediates are not exhausted during 0.25 generations in unlabeled medium. This possibility again emphasizes the difficulty in quantitative interpretation of the data but does not change the qualitative conclusions that have been derived from them.

It should be emphasized that the cell wall of *B. subtilis* turns over under these conditions, and that new cell wall is the sum of cell wall required for cell wall growth and cell wall required to replace material lost by turnover.

The observation that glycopeptide chains containing teichoic acid are surrounded by old and new glycopeptide chains should be considered in the light of two other recent observations.

(a) The wall of *B. subtilis* and *B. megarhizium* (as well as *L. acidophilus* (1-3)) can undergo extensive turnover during log-phase growth, and this turnover proceeds as a first order process for many generations (1). This observation implies that the cell wall structure is such that random erosion of cell wall material is possible over the whole cell surface without causing cell lysis. (b) We have determined the distribution of labeled cell wall material into daughter in *B. megarhizium* and found no segregation of the radioactivity in half-cells of the progeny after two generations (4). Both of these observations are in agreement with the existence of multiple sites of cell wall synthesis in these organisms.

The evidence for a single growth zone in gram-positive bacilli is not compelling (22-24). Fluorescent antibody experiments have not been carried out with single cell preparation, but, usually, with chains of cells, and it is not clear whether diffuse longitudinal growth could have been detected superimposed on the clearly visible new wall of the septum.

In the case of cocci, there is good evidence that growth can occur at a single point (25, 26), but that wall thickening can occur over the whole cell surface (25). It may well be that cell wall growth in cocci corresponds primarily to the mechanism of cell wall synthesis responsible for septum formation in bacilli, and that cell wall elongation, as such, does not take place in these organisms.

In general, it should be pointed out that deposition of cell wall material over the whole cell surface implies a much more complex regulatory system than would be envisaged by models of cell growth in which the cell surface (wall and membrane) is synthesized only at a single point on the cell surface (27).

In considering models for the arrangement of the glycopeptide on the cell surface which would allow random insertion of new cell wall polymers, as well as random extraction of cell wall material (i.e., turnover), we feel that a model in which the glycan strands are arranged tangentially to the cell surface, rather than in layers parallel to the cell surface, is compatible with these observations, and would allow cross-linking of nascent glycopeptide chains randomly to pre-existing chains, while the nascent

![Fig. 4. Hypothetical model of cell wall synthesis. Glycopeptide chains are assumed to be arranged tangentially to the cell surface. New chains are cross-linked to pre-existing glycopeptide as they emerge from the cell membrane, even before they are released from the membrane. Old glycan chains are shown as •, glycan strands in the process of synthesis as O, and peptide crossbridges as zigzag lines. The peptide crossbridges can assume different conformations so that the distance between glycan strands is variable. For cell growth some crossbridges must be broken to accommodate new strands. Cell wall thickening can occur in the absence of hydrolytic activity.](http://www.jbc.org/content/247/4/1186/F4.large.jpg)
glycopeptide is still attached to the lipid carrier on the membrane. A diagrammatic representation of this arrangement is illustrated in Fig. 4.

The known physical parameters of the cell wall are insufficient to either prove or disprove such an arrangement of the glycopeptide chains (28).

After completion of this work, Frehel, Beauvais, and Ryter (29) presented evidence based on electron microscopy that cell wall growth in *B. subtilis* and *B. megaterium* occurred by random insertion of new cell wall material.

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