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Cell Wall Assembly in *Bacillus subtilis*: Development of Bacteriophage-Binding Properties as a Result of the Pulsed Incorporation of Teichoic Acid

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Addition of a pulse of excess phosphate to a phosphate-limited culture of *Bacillus subtilis* W23 resulted in the synthesis and incorporation of wall material that contained teichoic acid. Consequently, the bacteria regained the ability to bind phage SP50 although maximum phage-binding properties did not develop until approximately half a generation time after incorporation of teichoic acid had ceased. The present findings strongly support our earlier suggestion that newly synthesized receptor material is incorporated at the inner surface of the wall and becomes exposed at the outer surface only during subsequent growth.

When grown under conditions of phosphate limitation, *Bacillus subtilis* W23 incorporates wall material that contains teichuronic acid instead of the teichoic acid that is incorporated when the bacteria are grown under potassium-limiting conditions. Walls of bacteria that are undergoing transition between phosphate and potassium limitations contain both teichoic and teichuronic acids, and we have shown (1) that phage SP50 can be used as a specific marker for the wall material that contains teichoic acid. In the earlier study (1) we found that bacteria harvested at late stages during transition from potassium to phosphate limitation had a much greater ability to bind phage than did bacteria containing similar small amounts of wall teichoic acid but that had been harvested at early stages during transition in the reverse direction. Apparently, therefore, recently incorporated wall material that contains teichoic acid is relatively ineffective in contributing to the phage-binding properties of the bacteria. Possible explanations for this observation were: (i) that wall material containing teichoic acid that is synthesized immediately after release of phosphate limitation differs structurally from that synthesized later, (ii) that the newly incorporated material has to undergo some structural modification before it can participate in phage binding, or (iii) that the newly incorporated material is unable to participate in phage binding because it is not present at the exterior surface of the cell. These possibilities are not mutually exclusive, but indirect evidence suggested that a major factor was that the newly synthesized material was incorporated first at the inner (cytoplasmic) surface of the wall. We now report further work that gives direct support for this explanation.

MATERIALS AND METHODS

Except as described below, all experimental procedures were as previously described (1).

Growth of *B. subtilis* W23. A phosphate-limited culture of *B. subtilis* W23 was established in a 3-liter chemostat under conditions similar to those described previously (1). After equilibration at a dilution rate (D) of 0.18 h^{-1} for 3 days, the addition of PO_4^{3-}-limiting medium was stopped and K^+-limiting medium was added at the same rate (D = 0.18 h^{-1}). After 105 min the addition of K^+-limiting medium was stopped and PO_4^{3-}-limiting medium was pumped in as before. Samples of bacteria were collected during 30-min intervals at the times shown in Table 1. Bacteria were collected and walls were isolated and analyzed as before.

Electron microscopy. Walls were incubated with an excess of phage particles as before (1), samples were fixed, embedded, sectioned, and stained with lead citrate as previously described (3), and sections were viewed on a Metropolitan-Vickers EM6 electron microscope.

RESULTS AND DISCUSSION

Walls of the phosphate-limited bacteria contained 0.21% P (dry weight of wall); these walls contained no detectable teichoic acid, and neither the walls nor the intact bacteria could bind phage SP50. The maximum content of wall teichoic acid was present in the bacteria harvested between 15 and 45 min after the addition of the pulse of K^+-limiting medium was stopped.

TABLE 1. *Alterations in phage adsorption efficiency and wall teichoic acid content of bacteria harvested at intervals after the addition of a pulse of potassium-limiting medium to a chemostat culture of B. subtilis W23 grown under phosphate-limiting conditions at D = 0.18 h^{-1}*[a]

Time after changeover to K+-limiting medium (h)	Wall-bound P (% dry wt of wall)	Phage adsorption efficiency of bacteria	Phage adsorption efficiency of isolated walls	Phage adsorption efficiency of walls/phage adsorption efficiency of bacteria
−1–0	0.21	<0.5	<1	
1.5–2.0	1.39	0.9	51.5	31
2.0–2.5	1.80	4.7	73	15.5
2.5–3.0	1.60	16.4	210	12.8
3.0–3.5	1.37	40.2	397	9.9
4.0–4.5	0.95	52.0	ND[b]	
6.0–6.5	0.68	49.5	110	2.2
24–25	0.21	<0.5	ND	

[a] Addition of phosphate-limiting medium was discontinued at zero time; potassium-limiting medium was then pumped into the chemostat at the same rate for 105 min and the addition of phosphate-limiting medium was resumed. Samples were harvested during the times shown.

[b] ND, Not determined.

These bacteria contained approximately 55% as much wall teichoic acid as was present in K+-limited bacteria, but their phage adsorption efficiency (1) was less than 1/10 that of the latter. This is in accord with our earlier report that recently incorporated teichoic acid material is relatively ineffective in restoring the phage-binding properties of the bacteria. Diminished amounts of wall teichoic acid were present in bacteria harvested during subsequent intervals, indicating that incorporation of wall teichoic acid had ceased within about 45 min after the addition of K+-limited medium was stopped. Although the bacteria harvested after this time contained less wall teichoic acid, their ability to bind phage increased greatly, the maximum phage adsorption efficiency being shown by the sample collected 2 h (i.e., half a generation time) later than that which contained the maximum amount of wall teichoic acid (Fig. 1). Since the receptor for phage SP50 is wall material that contains teichoic acid (1), the finding that the phage adsorption efficiency of bacteria increased after teichoic acid synthesis had stopped shows directly that recently incorporated wall material containing teichoic acid undergoes some subsequent modification which increases its ability to participate in phage binding. That this modification involves, or at least includes, a change in location within the wall was shown by electron microscopy.

Walls of bacteria that had been grown under potassium limitation adsorbed phage particles at both surfaces (Fig. 2A). Examination of the orientation of the phage particles showed that they were specifically bound by their tails to the inner surface and not simply trapped inside the wall. Many of the apparently more intact walls were devoid of phage particles at their inner surface although their exterior surfaces were completely covered with phages. However, fragmented walls clearly showed phages adsorbed to both surfaces. In contrast, walls of the bacteria harvested soon after incorporation of teichoic acid had begun bound phage particles almost exclusively at only one surface and this was clearly the inner surface (Fig. 2B and C). Walls of bacteria harvested after incorporation of teichoic acid had ceased also bound phage particles almost exclusively at only one surface, but in this case the receptor was located at the outer surface (Fig. 2D). The small number of phage particles that did bind to the inner surface of these walls did so almost exclusively at cell poles remote from the sites of septation; this is consistent with our earlier report that incorporation of new wall material

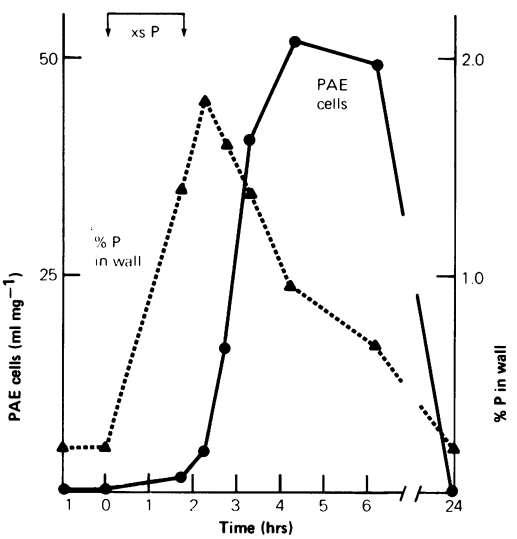

FIG. 1. *Phage adsorption efficiencies and wall teichoic acid contents of bacteria harvested during the pulsed release of phosphate limitation. K+-limiting medium was pumped into a chemostat culture of phosphate-limited bacteria so as to provide an excess of phosphate over the interval shown. Wall teichoic acid contents were determined by phosphate analysis of isolated walls (▲), and phage adsorption efficiency values (●) were determined as described in the text.*

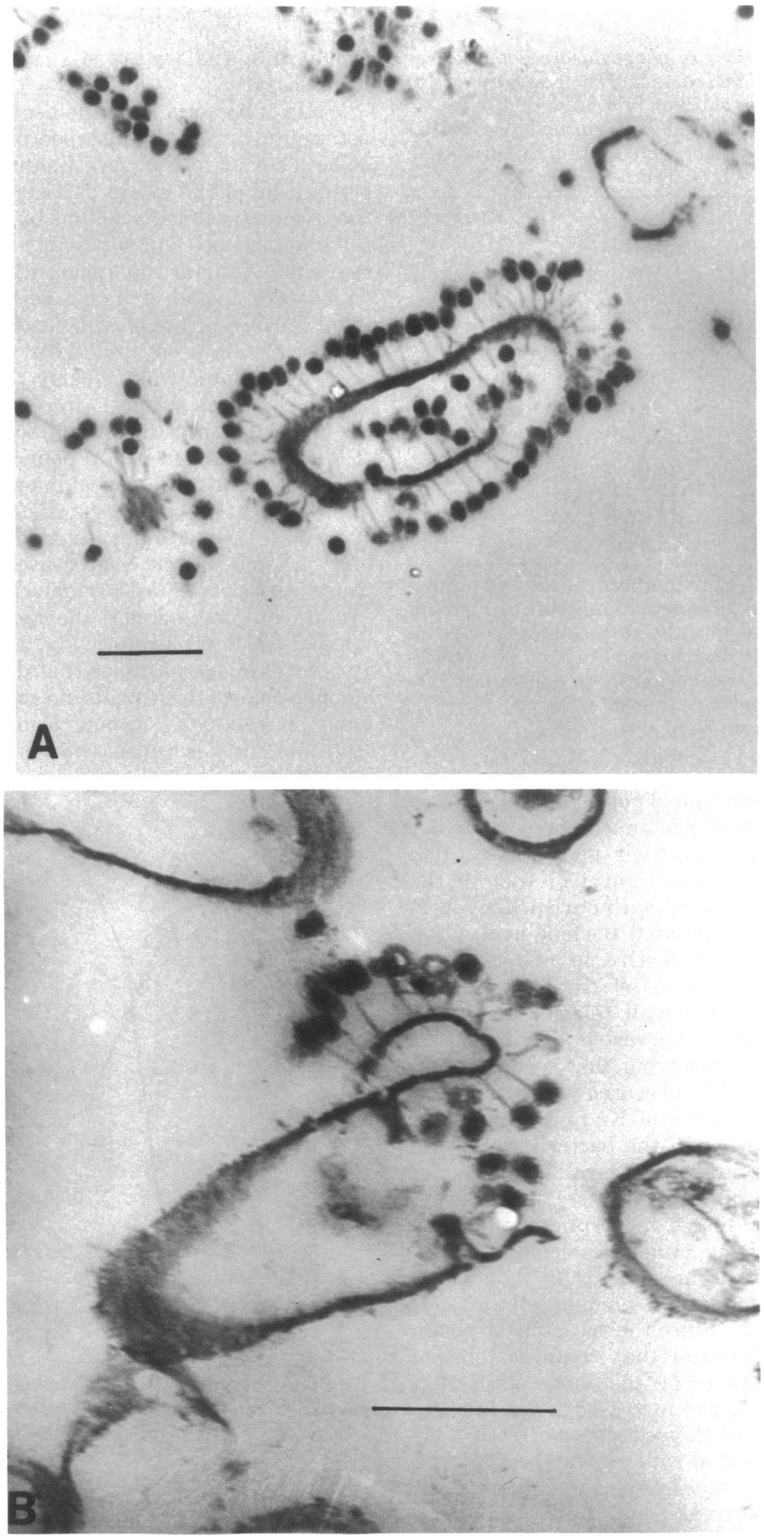

FIG. 2. *Electron micrographs showing location of phage particles adsorbed to walls isolated from K^+-limited bacteria (A) and from bacteria harvested between 60 and 90 min after the addition of the pulse of K^+-limiting medium was begun (B and C) and between 135 and 165 min after the addition of K^+-limiting medium was stopped (D). Bar represents 0.5 μm.*

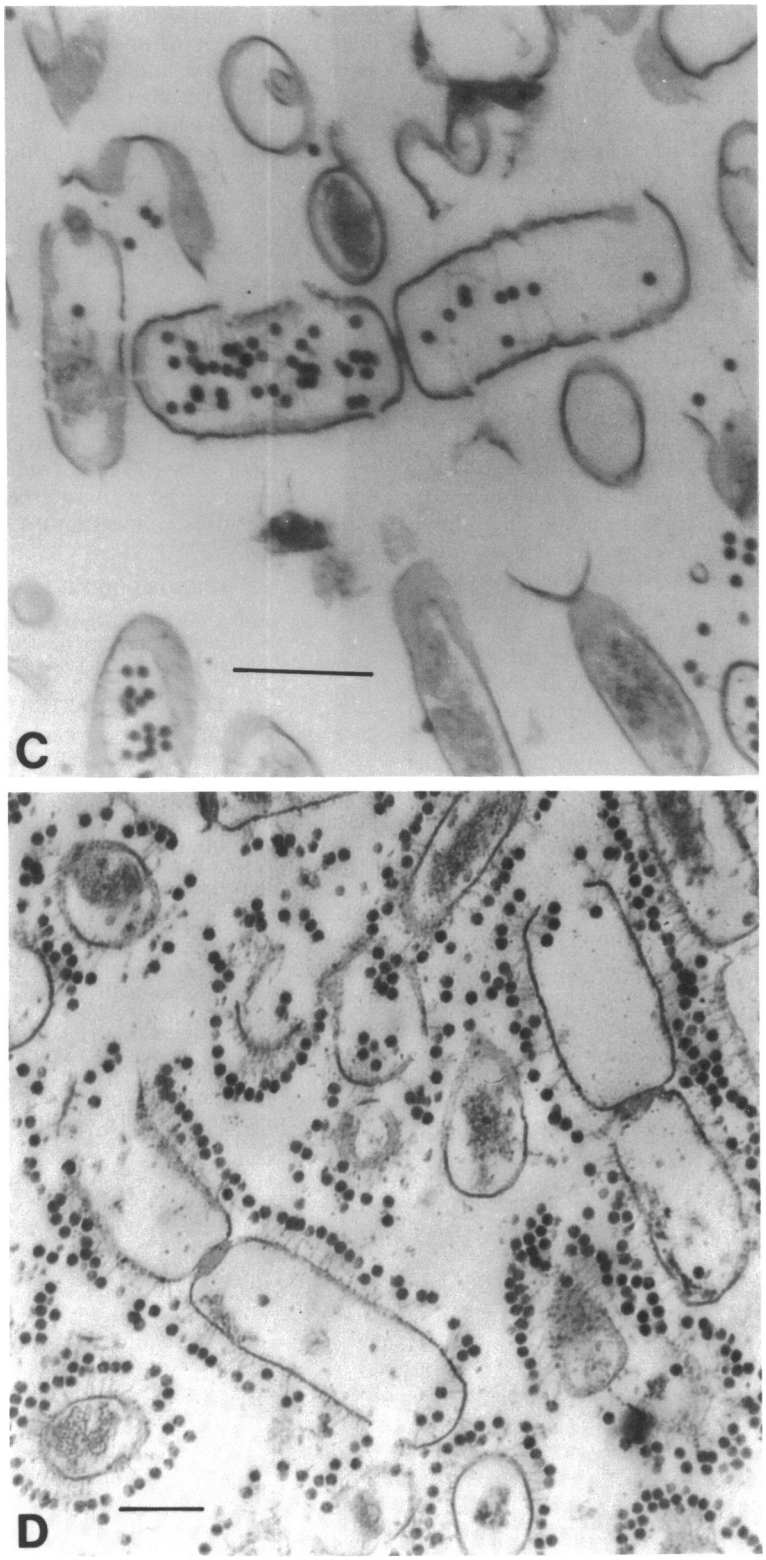

FIG. 2C and D

into existing cell poles takes place more slowly than elsewhere.

The phage adsorption efficiencies of the wall samples were also compatible with movement of receptor to the outer surface during growth. Thus, walls isolated from bacteria that were harvested soon after release of phosphate limitation are very much more efficient in phage binding, relative to the whole bacteria, than are walls isolated from bacteria harvested after synthesis of teichoic acid had stopped (Table 1). These findings must be interpreted with caution since disruption of the bacteria might alter the composition of the wall, but the results obtained (Table 1) are those that would be expected on the basis that recently incorporated receptor is located at the inner surface of the wall. In this location it is unable to contribute to the phage-binding properties of the intact bacteria but is accessible to phage in isolated walls. The latter, therefore, have very much greater phage adsorption efficiencies than do the bacteria from which they were derived. This consequence would ensue even if the ability of receptor material to bind phage were much less when the material is located on the inner surface of the wall. In samples in which the receptor material is present at only the outer surface of the wall, disruption of the cells would not expose previously inaccessible material and, indeed, disruption might lead to a loss of receptor material since, in at least certain bacteria, external layers of the wall seem to be partly removed during disruption of the bacteria (3).

The present results strongly support our earlier proposal that, at least during the transitions we have examined, newly synthesised wall material is incorporated first at the inner surface of the wall and then moves to the outer surface during subsequent growth. A similar process for wall assembly in *B. subtilis* 168 has been proposed by Fan et al. (2), who carried out an electron microscope study of bacteria that had resumed synthesis of wall after having been plasmolyzed, and by Pooley (5, 6), who has made a detailed study of autolysis and turnover of pulse-labeled wall material in that organism.

Our results show that in strain W23, growing under the conditions we have studied, recently synthesised wall material is not exposed at the surface of the cell until between half and one generation time after it is incorporated at the inner surface of the wall. Mauck et al. (4) have previously demonstrated that in this same organism recently synthesised wall material does not become available for turnover until between half and one generation time after its incorporation. This finding can readily be explained on the basis that turnover involves removal of wall material only from the external surface layers of the wall whereas newly synthesised material is incorporated at its inner surface.

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