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Bacteriophage SP50 as a Marker for Cell Wall Growth in *Bacillus subtilis*

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When grown under conditions of phosphate limitation, *Bacillis subtilis* W23 lacked wall teichoic acid and did not adsorb phage SP50. During transition from growth under conditions of phosphate limitation to those of potassium limitation, the bacteria developed an ability to adsorb phage which increased exponentially in relation to their content of wall teichoic acid. During transition in the reverse direction, the bacteria retained near-maximum phage-binding properties until their content of wall teichoic acid had fallen to a fairly low level. These observations suggest that newly incorporated wall material does not immediately appear at the cell surface in a structure to which phage can adsorb. Examination of the location of adsorbed phage particles showed that recently incorporated receptor material appeared at the cell surface first along the length of the cylindrical portion of the cell. The results are consistent with models of wall assembly in which newly synthesized wall material is intercalated at a large number of sites that are distributed along the length of the cell. This newly incorporated material may be located initially at a level underlying the surface of the cell and may become exposed at the surface only during subsequent growth. Incorporation of new material may also proceed rapidly into the developing septa, but new wall material is incorporated into existing polar caps more slowly, or perhaps not at all.

The chemical structures of the principal components of the cell walls of gram-positive bacteria have been studied in some detail (2, 32), and it is known that the composition and structure of bacterial walls is subject to considerable phenotypic variation (12). Although a great deal of information has been gained concerning the enzymatic mechanisms for the biosynthesis of the component polymers of walls, the way in which wall synthesis is regulated, in respect to variations in its composition and structure and in relation to other processes that occur during growth of the cell, is less well understood (21, 31). Information concerning the growth and assembly of walls of bacilli has been obtained in studies using fluorescent antisera (9, 10, 24), by autoradiography (8, 27), by electron microscopic examination, particularly of mutants or of bacteria grown in the presence of antibiotics or under conditions of nutritional deprivation (14, 17, 18, 22, 23, 25, 29), and by biochemical analysis (6, 26, 28, 30). Certain of these studies have indicated that growth proceeds by the incorporation of new wall material at multiple sites that are distributed over the entire surface (27, 28) or along the cylindrical portion (18, 23) of the wall. Other work has indicated growth from one or a few zones situated at or near the positions of incipient cross wall formation and cell division (9, 10, 24).

In *Bacillus subtilis* W23, the receptor for phage SP50 consists of wall material that contains teichoic acid. The integrity of both the teichoic acid and peptidoglycan components of the wall is necessary for adsorption of phage, and so it seems likely that the receptor is an organized structure involving both of these polymers. When the bacteria are grown under conditions of phosphate limitation, the walls contain teichuronic acid and no teichoic acid (D. C. Ellwood and D. W. Tempest, Biochem. J. 108:40P, 1968). Such bacteria do not bind (3), and are not infected by, phage SP50. However, the incorporation of wall teichoic acid that occurs when excess of phosphate is added to a phosphate-limited culture of the bacteria is accompanied by the restoration of their ability to bind and be infected by the phage. We have now examined the relationship between the incorporation of teichoic acid into the wall and its appearance at the cell surface in a structure to which the phage can adsorb. The relative pro-

portion of the surface at which phage receptor material (i.e., wall that contains teichoic acid) is exposed has been determined by quantitative assay of the phage-binding properties of bacteria harvested during transition from growth under conditions of phosphate limitation to those of potassium limitation and vice versa. The location of this receptor material has been determined by electron microscopic examination of bacteria to which phage had adsorbed. The results are discussed in relation to previous studies on the manner of assembly and growth of the cell wall.

MATERIALS AND METHODS

Organisms. *B. subtilis* W23 was maintained on nutrient agar. Chemostat cultures were grown as described below. For propagation of bacteriophage SP50, cultures were grown in medium (16) containing tryptone (Difco), 10 g; yeast extract (Difco), 5 g; NaCl, 5 g; 0.1 M $MnSO_4$, 1 ml; 1.0 M $MgSO_4$, 10 ml; and distilled water, 1 liter. A flask (250 ml) containing this medium (60 ml) was inoculated with a sample (0.10 ml) of a culture of *B. subtilis* W23 that had been grown in similar medium at 37 C for 16 h. After incubation at 37 C with shaking for 2.5 h, the culture reached a density of 10^7 bacteria/ml. Four fresh plaques of SP50 were then added and incubation was continued for a further 2.5 to 3 h, when lysis occurred. The lysate was passed through a 0.45-μm membrane filter (Millipore Corp.) and added to a flask containing a 600-ml culture at a density of 10^8 bacteria/ml. Lysis occurred after incubation for a further 2.5 h. Bacterial debris was removed by centrifugation for 10 min at 15,000 $\times$ *g*. Phage particles were then sedimented by centrifugation at 25,000 $\times$ *g* for 2 h; they were suspended in 10 ml of 2.5% nutrient broth containing 0.01 M $MgSO_4$ (NB-Mg medium) and filtered into a sterile screw-cap bottle through a 0.45-μm membrane filter. Such phage suspensions contained approximately 2×10^{12} plaque-forming units (PFU) per ml and were stable for several weeks on storage at 2 C. Phages were assayed in soft-agar layers as previously described (4), but using as indicator exponential-phase cultures of *B. subtilis* W23 that had been grown at 37 C for 5 h in medium containing: nutrient broth (Oxoid), 25 g; glucose, 10 g; 1.0 M $MgSO_4$, 10 ml; and water, 1 liter.

Phage-binding measurements. Binding studies were carried out in NB-Mg medium. Stock phage suspension was diluted into this medium immediately before use. Control phage suspensions were completely stable during the incubations described below. Before incubation with phage, suspensions of bacteria and of walls were first heated at 100 C for 10 min so as to prevent lysis. All bacterial and wall concentrations quoted are expressed in terms of dry weight per volume.

Standard phage-binding ability (PBA) was determined as follows. A suspension (0.2 ml) of bacteria (100 μg/ml) was added to diluted phage (0.2 ml, 4×10^5 PFU/ml) in a sterile plugged tube and incubated for 1 h at 37 C. Duplicate samples (0.1 ml) were then removed and assayed for unadsorbed phage. Results are expressed as the percentage of phage bound.

For determination of phage-binding capacity (PBC), a suspension (0.10 ml) of bacteria at a concentration sufficient to adsorb roughly half of the added phage (see Table 1) was mixed with 0.30 ml of phage suspension (containing approximately 3.3×10^{11} PFU/ml) and incubated at 37 C for 3 h. After suitable dilution, so that approximatey 200 plaques were present on each plate, samples were assayed in triplicate for unbound phage. Preliminary experiments showed that binding was complete after 1 h.

The phage adsorption efficiency (PAE) is here defined as the reciprocal of the concentration (in milligrams per milliliter) of bacteria which bind 50% of the phage particles during incubation for 1 h in a mixture containing an initial phage concentration of 2×10^5 PFU/ml. Samples (0.1, 0.2, and 0.4 ml) of a suspension of bacteria were separately mixed with phage (2×10^5 PFU) in a total volume of 1 ml of NB-Mg medium in sterile plugged tubes. After incubation for 1 h, samples (0.1 ml) of each mixture were removed and mixed with NB-Mg medium (10 ml), and duplicate samples (0.1 ml) were assayed for unbound phage. Graphs of the logarithm of the free phage concentration against the bacterial concentration gave straight lines (e.g., Fig. 2) from which the concentration (Ph_{50}) of bacteria binding 50% of the phage was determined. Preliminary results showed that the results obtained on incubation at 0 C were more reproducible than those obtained at 37 C. The reasons for this are not yet clear, but values quoted here were all obtained from mixtures incubated at 0 C.

In making the above measurements, our objective was to determine the relative proportions of the surfaces of the various bacterial samples that are capable of binding phages. It should be noted that although PBC values will depend on the proportion of the surface to which phage can adsorb, the values may also be affected by steric considerations. Thus the observed maximum PBC value corresponds to the adsorption of approximately 730 phage particles per bacterium, and consideration of the total surface area of the cell and of the size of the phage suggests that this represents a fairly tight packing of phage particles over the surface. This is also shown by electron microscopic examination, and it is likely that values of PBC are limited in part by steric crowding of adsorbed phage particles rather than solely by the density of receptor material present at the surface.

PAE values are determined under conditions of incubation where the number of bacteria greatly exceeds that of phage particles. Steric crowding of adsorbed phage particles thus cannot arise. However, PAE values can be affected by differences in affinity of potential receptor material. The adsorption of phage particles follows the equation

$$-\frac{d\phi}{dt} = k\phi B$$

$$\text{i.e., } \log(\phi_o/\phi_t) = \frac{kBt}{2.303}$$

where ϕ_o and ϕ_t are the concentrations of unadsorbed phage particles present initially and at time t, B is the concentration of bacteria, and k is the rate constant for the reaction (19). By definition, $Ph_{50} = B$ when $\phi_0/\phi_t = 2$ and $t = 1$ h. Hence, $PAE = 1/Ph_{50} = \text{constant} \times k$. The value of k, and hence of PAE, depends on the chance that collision between a phage particle and a bacterium leads to adsorption. This will depend on the chance that the collision involves a potential receptor, i.e., on the proportion of the bacterial surface able to absorb phage, and on the affinity of that potential receptor for the phage. The PBA value is also determined by the rate constant of the binding reaction but, at least as determined in the present study, such values are substantially less accurate than the PAE values.

Continuous culture experiments. Preliminary experiments were carried out in the 0.5-liter Porton-type chemostat that we used previously (3). The studies reported here were carried out on bacteria that had been grown in a 3-liter chemostat (L. H. Engineering Co. Ltd., Stoke Poges, England) of similar design. The PO_4^{3-}-limiting medium was that described by Tempest et al. (33) modified so that it contained 1.44 mM PO_4^{3-} and 4.3 mM K^+; the K^+-limiting medium was similar but contained 4.81 mM PO_4^{3-} and 3.02 mM K^+. Approximately 75% of the nonlimiting component in each medium was utilized under the growth conditions used. Continuous cultures were established by inoculating PO_4^{3-}-limiting medium in the growth chamber with 300 ml of an exponential-phase culture of *B. subtilis* W23 grown in nutrient broth. The dilution rate *(D)* was set to 0.18/h, the air flow was 3 liters/min, the pH was maintained at 7.0 by the automatic addition of sterile 2 M NH_4OH, the temperature was maintained at 37 C, and the foaming was suppressed by the addition of sterile polypropylene glycol 2025 (B.D.H. Ltd., England). A stable turbidity was reached within 24 h, and changeover experiments were started after equilibration for a total of 72 h. Samples were collected via the effluent culture line into flasks surrounded by ice. For changeover between PO_4^{3-} and K^+ limitations, a sample was collected for 1 h before change of medium. Samples were then collected at 30-min intervals up to 3 h and at hourly intervals between 3 and 9 h, and a final sample was collected between 24 and 25 h after the change of medium. After equilibration for a further 48 h, PO_4^{3-}-limiting medium was passed into the chemostat and samples were collected as before at 2-hourly intervals up to 4 h, at hourly intervals between 4 and 15 h, and finally between 24 and 25 h after changeover. Bacteria were collected by centrifugation at 2 C, washed twice with cold distilled water, and then freeze dried. The yields of bacteria obtained throughout these experiments were approximately 3.5 mg/ml.

Chemostat theory. As described by Ellwood and Tempest (11), the theoretical curve for the washout of any component is given by the equation $x_t/x_o = e^{-Dt}$ where x_0 and x_t are the concentrations of the component in samples taken initially and after time t when D is the dilution rate. If, during transition from K^+ to PO_4^{3-} limitation, the bacterial density remains constant and the incorporation of wall teichoic acid proceeds at a steady rate until phosphate becomes limiting and then ceases completely at that time, the above equation will describe the decrease in the teichoic acid content of the wall provided that teichoic acid is not removed, for example, by turnover of wall material. During this transition the concentration of potassium in the medium will increase but remain limiting until the concentration of phosphate falls to a limiting value; thereafter the concentration of phosphate will fall until it reaches the equilibrium value. Consequent deviations from the above theory are minimized by the media used in this study since the nonlimiting substrates are present in only small excess; substantial deviations from the above theoretical values are therefore likely to be significant. Similarly, it is likely that substantial deviations from the theoretical curve for the increase in wall teichoic acid during transition in the reverse direction will be significant. If, at the moment of changeover, synthesis of wall teichoic acid starts and continues at a constant rate equal to the overall rate of biomass synthesis, its concentration will increase according to the equation $Z_t/Z_s = 1 - e^{-Dt}$, where Z_t is the concentration of the component at time t, and Z_s is its final steady-state concentration.

Isolation and analysis of cell walls. Bacteria were disrupted by shaking with glass beads and walls were isolated and incubated with trypsin as previously described (5). The phosphate content of wall samples was determined by the procedure of Chen et al. (7). Walls were examined chemically by using procedures described previously (1).

Electron microscopy. Suspensions (0.05 ml) of heat-killed bacteria or walls at concentrations of 2 mg/ml in NB-Mg medium were mixed with phage (0.25 ml, 5×10^{10} PFU) in the same medium. The mixtures were incubated for 2 h at 37 C, diluted to 1 ml with 0.01 M $MgSO_4$, and then collected by centrifugation. The precipitate was suspended in 0.01 M $MgSO_4$ (0.05 ml), mixed with 1% formaldehyde (0.05 ml), and incubated at 22 C for 5 min. After the addition of 0.1 M $MgSO_4$ (1 ml), the sample was again collected by centrifugation and suspended in water (0.1 ml), and then a drop was applied to a carbon-coated Formvar grid. When dry, the grid was floated for 2 h on 1% uranyl acetate in water, washed with water, and then examined in a Metropolitan-Vickers EM6 electron microscope.

RESULTS

Adsorption of phage to K^+-limited bacteria and their walls. The adsorption of phage SP50 to heat-killed K^+-limited bacteria followed first-order kinetics at both 0 and 37 C (Fig. 1). Removal of bacteria from the incubation mixtures by filtration before dilution and assay of unadsorbed phage made no difference to the results obtained under any of the incubation conditions used, so that adsorption was irreversible under all of these conditions. On incubation with excess of phage at 37 C, the bacte-

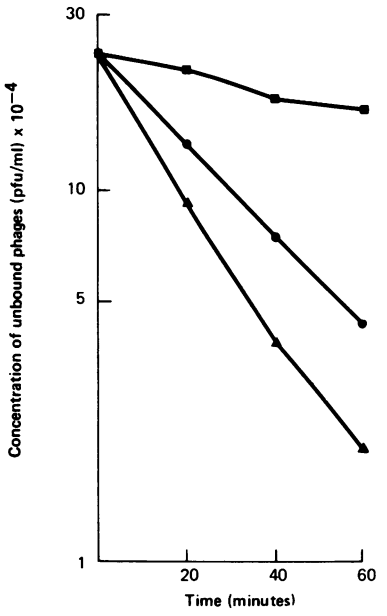

FIG. 1. *Binding of phage (2.33 × 10⁵ PFU/ml) by heat-killed K⁺-limited bacteria (24 μg/ml) at 37 C (▲) and 0 C (●), and at 0 C in the presence of 2.5 mg of teichoic acid-glycopeptide complex per ml (■).*

TABLE 1. *Changes in the phage-binding capacity of bacteria harvested during transition from growth under conditions of PO_4^{3-} limitation to those of K^+ limitation and vice versa at D = 0.18/h*

Time after change-over (h)	Concn of bacteria in adsorption mixture (μg/ml)	Initial phage concn [(PFU/ml) × 10⁻⁹]	Phage concentration after incubation for 3 h at 37 C [(PFU/ml) × 10⁻⁹]	No. of phages bound/pg of bacteria (PBC)
PO_4^{3-} limitation → K^+ limitation				
0	2,264	246 ± 9	236 ± 9	0
1.0–1.5	4,030	242 ± 8	208 ± 6	8
1.5–2.0	3,812	242 ± 8	129 ± 5	30
2.0–2.5	1,220	242 ± 8	58 ± 3	151
2.5–3.0	400	246 ± 9	92 ± 4	385
3.0–4.0	325	242 ± 8	76 ± 4	511
24–25	215	258 ± 10	147 ± 5	516
K^+ limitation → PO_4^{3-} limitation				
4.0–5.0	273	242 ± 8	108 ± 4	490
6.0–7.0	400	258 ± 10	129 ± 5	322
10–11	604	258 ± 10	146 ± 5	186

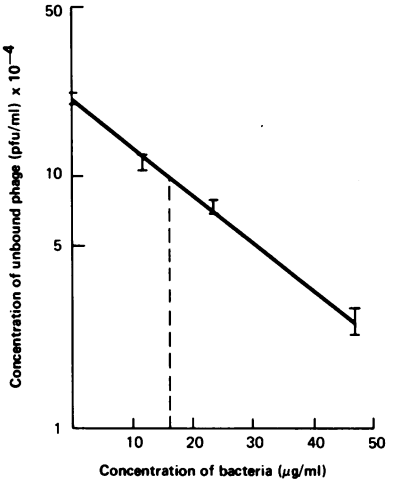

FIG. 2. *Binding of phage by heat-killed K^+-limited bacteria under standard conditions used for determination of PAE.*

ria adsorbed 520 phage particles per pg of bacteria (Table 1), corresponding to approximately 730 phages adsorbed per cell. The PBC of isolated walls was 3,000 PFU/pg; on the basis that the wall comprises roughly 20% of the dry weight of the cell, this corresponds to approximately 840 phages adsorbed per wall. Thus the receptor material in heat-killed bacteria is present in the walls. The PBC of bacteria that had not been killed by heat could not be determined because they lysed on incubation with phage. On incubation of heat-killed bacteria with phage at 0 C under the conditions used for determination of PAE, the logarithm of the concentration of unbound phage was linearly related to the concentration of bacteria in the incubation mixture (Fig. 2), as would be expected of a reaction that follows first-order kinetics. Closely similar results were obtained with bacteria that had not been heat killed. Therefore the phage receptor material in K^+-limited bacteria is stable to heat and is present in the wall. The ability of isolated walls to bind phage was not affected by heat, by digestion with trypsin, or by treatment with detergent so that the receptor is part of the covalently linked wall substance. However, the peptidoglycan fractions obtained after treatment (1) of walls with trichloroacetic acid, with dilute NaOH, or by oxidation with aqueous $NaIO_4$ did not adsorb any phage even when incubated at concentrations of 2 mg/ml under the conditions used for determination of PAE. Walls of PO_4^{3-}-limited bacteria, which contain teichuronic acid and not teichoic acid, were also unable to bind phage. Isolated teichoic acid did not inactivate phage, nor did a teichoic acid-glycan complex isolated after digestion of walls with lysozyme. At high concentrations, however, the latter complex did inhibit binding of phage to the bacteria (Fig. 1). These observations show that the receptor for phage SP50, like those for various other phages that infect *B. subtilis* (20, 34),

contains, or is closely associated with, both the teichoic acid and peptidoglycan components of the wall. As yet the detailed structural features of the receptor material are obscure, as, indeed, is the structural organization of the component polymers in the wall. However, phage SP50 does not bind to wall material synthesized under conditions of PO_4^{3-} limitation, though it does bind to wall material synthesized by *B. subtilis* growing under conditions of K^+ limitation. The phage can therefore be used as a marker for this latter wall material. Since the syntheses of teichoic acid and peptidoglycan are apparently coordinated in *B. subtilis* (25, 28), measurement of the teichoic acid content of walls of the bacteria harvested during transition between growth under K^+-limiting and PO_4^{3-}-limiting conditions could be used to determine the proportion of the wall substance that had been synthesized under each limitation.

Wall composition and phage binding properties of bacteria harvested during transition. Walls of the K^+-limited bacteria contained 3.3% P, and an acid hydrolysis gave products characteristic of glucosylated poly(ribitol phosphate) teichoic acid (1) together with components derived from the peptidoglycan. Walls of the PO_4^{3-}-limited bacteria contained 0.2% P; acid hydrolysis of these walls showed that they were devoid of teichoic acid but contained a teichuronic acid, composed of galactosamine and glucuronic acid, in addition to peptidoglycan. Walls of bacteria harvested during transition between PO_4^{3-} and K^+ limitations contained intermediate amounts of phosphate (Table 2). Hydrolysis of these walls gave components derived from teichoic acid and teichuronic acid in proportions that were consistent with the conclusion that differences in the phosphate contents of the walls were due to differences in their contents of teichoic acid.

Incorporation of wall teichoic acid occurred approximately 1 h after K^+-limiting medium was pumped into the chemostat containing PO_4^{3-}-limited bacteria. Thereafter the amount of wall teichoic acid, determined by analysis of the phosphate content of isolated walls, increased rapidly (Table 2, Fig. 3), as did the PBA values of the bacteria (Table 2, Fig. 4). During changeover in the reverse direction ($K^+ \rightarrow PO_4^{3-}$), the disappearance of wall teichoic acid proceeded rapidly (Table 2, Fig. 5), although the PBA values of the bacteria decreased more slowly (Table 2, Fig. 6). Since PBA values, although easily determined and useful for the detection of alterations in surface chemistry, are of limited value in accurate assessment of phage-binding properties of bacteria that differ greatly in this respect, the PAE and PBC values of the samples were determined. Under the conditions used for the determination of PAE, PO_4^{3-}-limited bacteria at a concentration of 4.0 mg/ml bound less that 5% of the added phage, whereas bacteria harvested between 1 and 1.5 h after changeover to K^+-limiting medium had a Ph_{50} value of 2.63 mg/ml and the Ph_{50} value of K^+-limited bacteria was 16 μg/ml. During transition from PO_4^{3-} to K^+ limitation, the PAE and PBC values of the bacteria increased rapidly (Tables 1 and 2, Fig. 7). The PAE value reached a maximum in samples harvested between 7 and 8 h after changeover. These bacteria contained 80% of the maximum content of wall teichoic acid, and this is presumably sufficient to insure the maximum probability that collision with phage leads to adsorption. Maximum PBC values were given by samples harvested between 3 and 4 h after changeover. As already discussed, this limiting

TABLE 2. *Changes in the phage-binding ability, phage adsorption efficiency, and content of wall-bound phosphorus of bacteria harvested during transition from growth under conditions of PO_4^{3-} limitation to those of K^+ limitation and vice versa at D = 0.18/h*

Time after changeover (h)	Wall-bound P (% dry wt of wall)	Phage-binding ability[a]	Phage adsorption efficiency[b]
PO_4^{3-} limitation → K^+ limitation			
0	0.21	<5	<0.3
0–0.5	0.21	<5	<0.3
0.5–1.0	0.29	<5	<0.3
1.0–1.5	0.39	<5	0.38
1.5–2.0	0.86	<5	1.3
2.0–2.5	1.52	23	8.3
2.5–3.0	1.79	62	22
3–4	2.14	98	40
4–5	2.53	98	59
7–8	2.68		67
24–25	3.30	98	63
K^+ limitation → PO_4^{3-} limitation			
0	3.30	98	62
0–2	2.30	96	
2–4	1.46	94	53
4–5	0.93	90	59
5–6	0.74	88	
6–7	0.61	78	48
9–10	0.41	76	33
10–11	0.34	63	13
12–13	0.34	68	6.7
14–15	0.34	43	6.9
24–25	0.21	<5	0.6

[a] Percentage of phage bound under standard conditions by bacteria at 50 μg/ml.

[b] Reciprocal of concentration (in milligrams per milliliter) of bacteria binding 50% of phage under standard conditions.

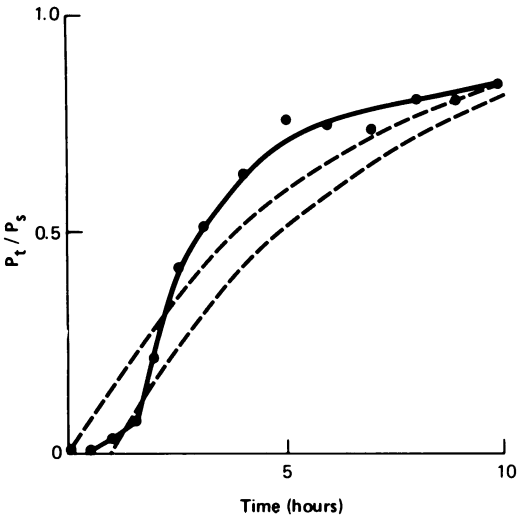

FIG. 3. *Increase of teichoic acid content (●) of walls of bacteria harvested during transition from PO_4^{3-}- to K^+-limited growth in chemostat culture at D = 0.18/h. Theoretical rate of increase (- - -) is shown assuming initiation of teichoic acid synthesis at the time of changeover or 1 h later. P_t is the phosphate content (percent dry weight of wall) of walls of bacteria harvested at time t after changeover, and P_s is the phosphate content of walls of bacteria harvested after equilibration under K^+-limiting conditions.*

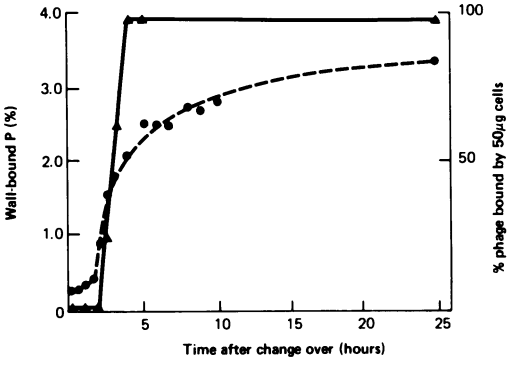

FIG. 4. *Increase in wall teichoic acid content (●) and PBA (▲) of bacteria harvested during transition from PO_4^{3-}- to K^+-limited growth in chemostat culture at D = 0.18/h. Wall teichoic acid content was determined by measurement of the phosphate content (percent dry weight of wall) of isolated walls.*

value may be imposed in part by steric crowding of adsorbed phage particles so that it may be reached before the maximum proportion of potential receptor material is present at the surface. In earlier samples PBC values were directly proportional to PAE values (Fig. 8); since PBC values are not affected by variations in the affinity of potential receptor material and since PAE values cannot be affected by steric crowding of adsorbed phage particles, the observed proportionality between these values suggests that both are directly related to the proportion of the surface at which potential receptor material is exposed, although PBC values are eventually limited because of crowding.

Although in intermediate samples the phage-binding properties of the bacteria were directly proportional to their content of wall teichoic acid, in early samples this relationship was exponential (Fig. 7). Thus wall material containing teichoic acid that was incorporated at early stages during transition from PO_4^{3-} to K^+ limitations was relatively ineffective in restoring the phage-binding properties of the bacteria. However, the phage-binding properties of

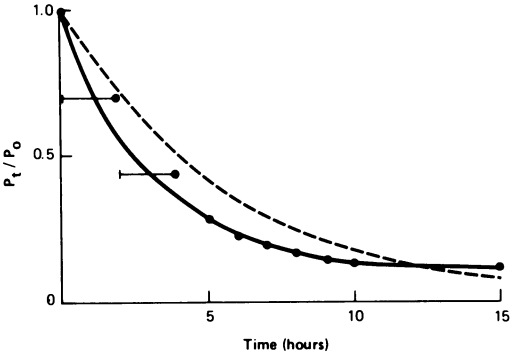

FIG. 5. *Decrease in wall teichoic acid content (●) of bacteria harvested during transition from K^+- to PO_4^{3-}-limited growth in chemostat culture at D = 0.18 h. Theoretical rate of dilution out (- - -) is shown assuming synthesis of teichoic acid stops at the moment of changeover. Wall teichoic acid content was determined by measurement of the phosphate content (percent dry weight of wall) of isolated walls.*

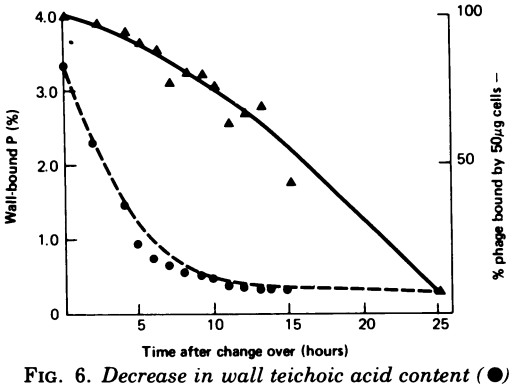

FIG. 6. *Decrease in wall teichoic acid content (●) and PBA (▲) of bacteria harvested during transition from K^+- to PO_4^{3-}-limited growth in chemostat culture at D = 0.18/h.*

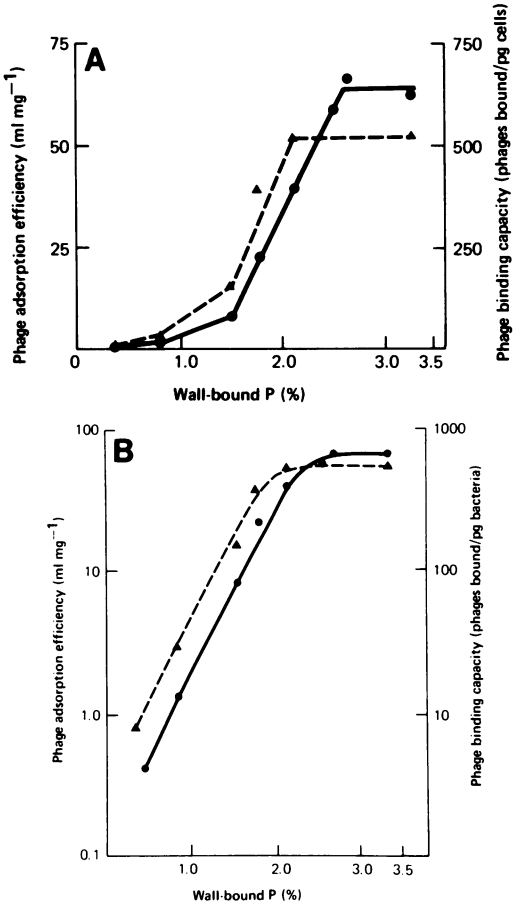

FIG. 7. *Phage-binding properties and wall teichoic acid content of bacteria harvested during transition from PO_4^{3-}- to K^+-limited growth in chemostat culture. Symbols: ●, PAE; ▲, PBC.*

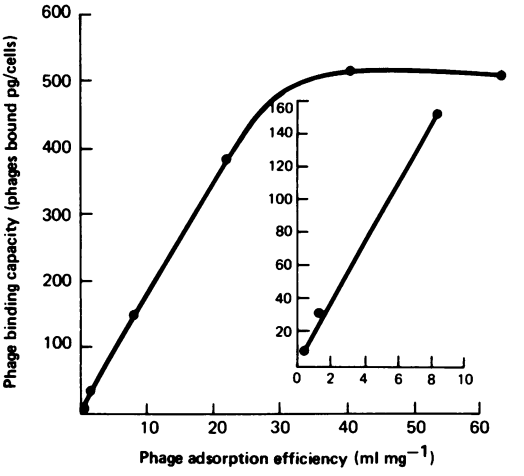

FIG. 8. *Relationship between PAE and PBC values of bacteria harvested during transition from PO_4^{3-}- to K^+-limited growth in chemostat culture.*

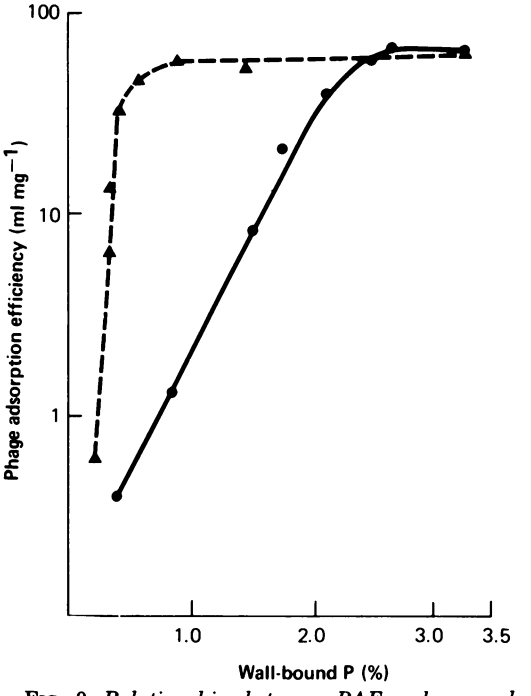

FIG. 9. *Relationship between PAE values and wall teichoic acid content of bacteria harvested during transition from PO_4^{3-}- to K^+-limited growth (●) and from K^+- to PO_4^{3-}-limited growth (▲) in chemostat culture.*

bacteria harvested during transition from K^+ to PO_4^{3-} limitation remained high until their content of wall teichoic acid had fallen to a low level (Tables 1 and 2, Fig. 9). Thus bacteria that contained approximately 20% of the maximum content of teichoic acid and that were harvested during transition from K^+ to PO_4^{3-} limitation had a PAE value approximately 40 times greater than that of bacteria that contained a similar amount of wall teichoic acid but were harvested during transition in the reverse direction. This did not appear to be due to major differences in the structures of the teichoic acids present in these bacteria since both gave identical hydrolysis products characteristic of glucosylated poly(ribitol phosphate). Since receptor material contains both teichoic acid and peptidoglycan, it is possible that teichoic acid-glycopeptide complexes are incorporated in such a way that they are not initially present in an appropriate local concentration or in an appropriately organized structure and that they can contribute to phage-binding properties only

after some modification of the wall material that takes place subsequently.

The rate at which wall teichoic acid was lost from chemostat cultures of *B. subtilis* W23 during transition from growth under conditions of potassium limitation to those of phosphate limitation was approximately twice what would be expected on the basis of a cessation of teichoic acid synthesis and dilution out of existing wall material. Similarly, incorporation of wall teichoic acid during transition in the reverse direction proceeded approximately twice as fast as the net rate of biomass synthesis. Similar observations have been made with other bacilli (11, 17) and are consistent with the occurrence of turnover of wall material during growth (26). During transition from phosphate to potassium limitation, incorporation of receptor material into the wall will thus take place as a result of turnover as well as of growth. Similarly, during transition in the opposite direction, existing receptor material will be displaced by turnover.

Location of receptor material. In samples harvested at early stages during transition from PO_4^{3-} to K^+ limitation, phage particles adsorbed to the cylindrical portions of the cells, in some cases mainly in regions where cross wall formation might be expected to occur (Fig. 10). However, phage particles were present along the entire length of the cylindrical portion of most of the bacteria harvested between 2.5 and 3.0 h after changeover.

Complementary results were obtained with bacteria harvested during transition from K^+ to PO_4^{3-} limitation. Almost all of the bacteria harvested between 4 and 5 h after changeover in this direction were completely covered with phage particles, though a small number (less than 1%) of bacteria were seen that adsorbed phage particles mainly at the poles (Fig. 11). Increasing numbers of such cells were seen in later samples, and increasing numbers of bacteria were seen that adsorbed phage mainly at one pole (Fig. 11). In samples harvested between 12 and 13 h after changeover, approximately 90% of the bacteria did not show any adsorbed phage particles, whereas about 10% of the bacteria adsorbed phage particles only at one pole. A small number (less than 0.5%) of bacteria were seen that adsorbed phage particles over their entire surface; these may be metabolically inactive bacteria that originated during growth under K^+ limitation, though some might be mutants that lack the ability to synthesize teichuronic acid.

DISCUSSION

The addition of excess phosphate to phosphate-limited cultures of *B. subtilis* W23 results in the synthesis and incorporation of wall material that contains teichoic acid. The conditions necessary for the incorporation of this material can readily be studied since its presence in the wall results in the restoration of the ability of the bacteria to bind phage SP50. We have found (unpublished observations) that the restoration of phage-binding properties that follows such release of phosphate limitation is blocked by the presence of antibiotics that inhibit the synthesis of ribonucleic acid or protein. Presumably, therefore, the synthesis of appropriate enzymes is necessary for the resumption, by the previously phosphate-limited bacteria, of the synthesis and incorporation of wall material that contains teichoic acid.

The phage-binding properties of bacteria harvested at early stages during transition from phosphate- to potassium-limited growth conditions increased exponentially in relation to their increasing content of wall teichoic acid. However, although wall material containing teichoic acid that is incorporated into the wall soon after the release of phosphate limitation is relatively ineffective in increasing the phage-binding properties of the bacteria, the phage-binding properties of bacteria harvested during transition from potassium to phosphate limitations remain high until their content of wall teichoic acid, and therefore of "old" wall material, has fallen to a fairly low level. Little is yet known about the structural basis of the recognition between phages and walls that contain teichoic acid, although in several cases the presence of a sugar substituent has been shown to be required (20, 32). Teichoic acid incorporated into walls of *B. subtilis* W23 soon after release of phosphate limitation appears to be similar in composition to that present in potassium-limited bacteria, but its relative inefficiency in contributing to phage binding might be due to some subtle difference in its structure or in the way in which it is oriented in relation to the rest of the wall structure. Thus we have found that the integrity of both the teichoic acid and peptidoglycan components of the wall is necessary for inactivation of the phage. This may result from a requirement for a certain minimum size or for a particular organization of the component polymers that is imposed or maintained by the wall structure. It is possible that such organization takes place at some time after the newly synthesized wall material is incorporated and that this explains the relative inefficiency of newly incorporated wall material in binding phage. Alternatively, the newly incorporated wall material may be unable to participate effectively in phage binding because it is not initially present at the cell surface. Since the components of the cell wall are

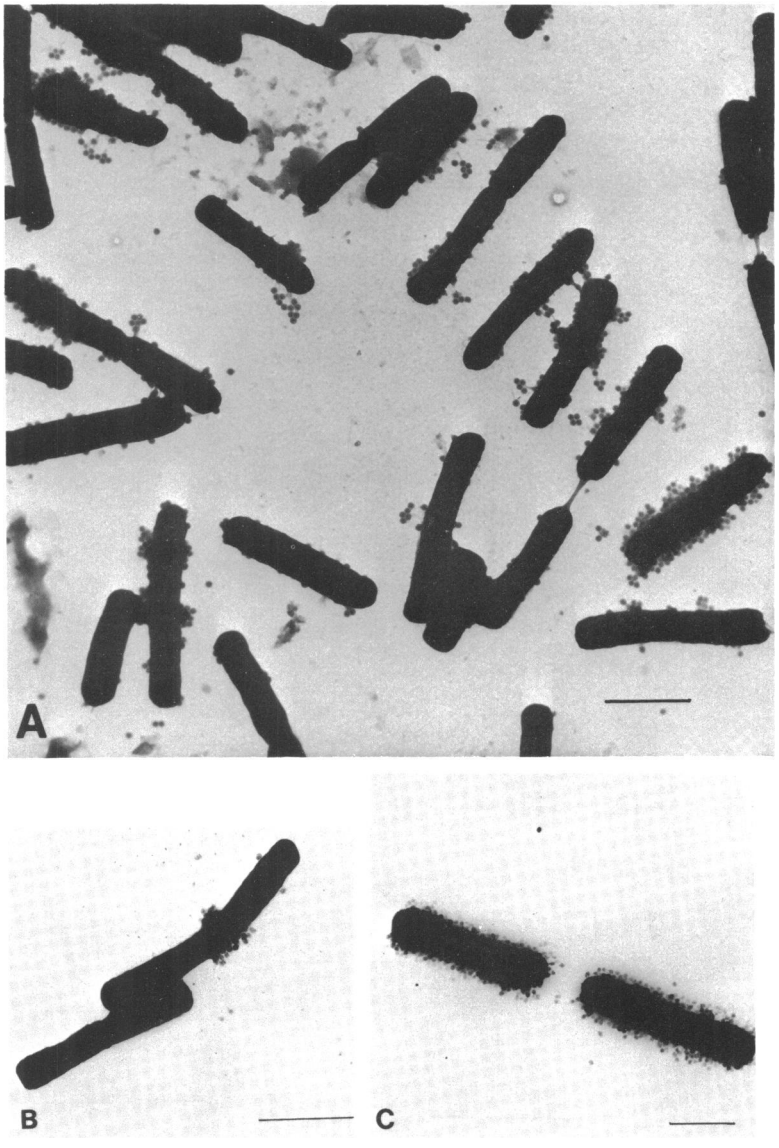

FIG. 10. *Electron micrographs showing location of phage particles adsorbed to bacteria harvested during transition from PO_4^{3-}- to K^+-limited growth. (A and B) Bacteria harvested between 1.5 and 2 h after changeover; (C) bacteria harvested between 2 and 2.5 h after changeover. Bar represents 1 μm.*

synthesized by enzyme systems that are located in the cytoplasmic membrane, it seems likely that such components are incorporated first at the inner (cytoplasmic) surface of the wall. Deposition of newly synthesized wall material at the inner surface of the wall has been proposed by Pooley (30) to account for his observations concerning turnover of pulse-labeled peptidoglycan in *B. subtilis* 168; this might also partly explain the observation (26) that recently synthesized wall material in *B. subtilis* W23 does not become available for turnover until about half to one generation time after its incorporation. Fan and his colleagues (14) have shown that in lysin-deficient mutants of *B. subtilis*, newly incorporated wall material is located at the inner surface of the wall. Since the wall is a thick structure, material incorporated at its inner surface might not extend to the outer surface and might become exposed only during subsequent growth and turnover of the wall. Detection of newly incorporated material by phage or by antibodies might therefore give results differing from those ob-

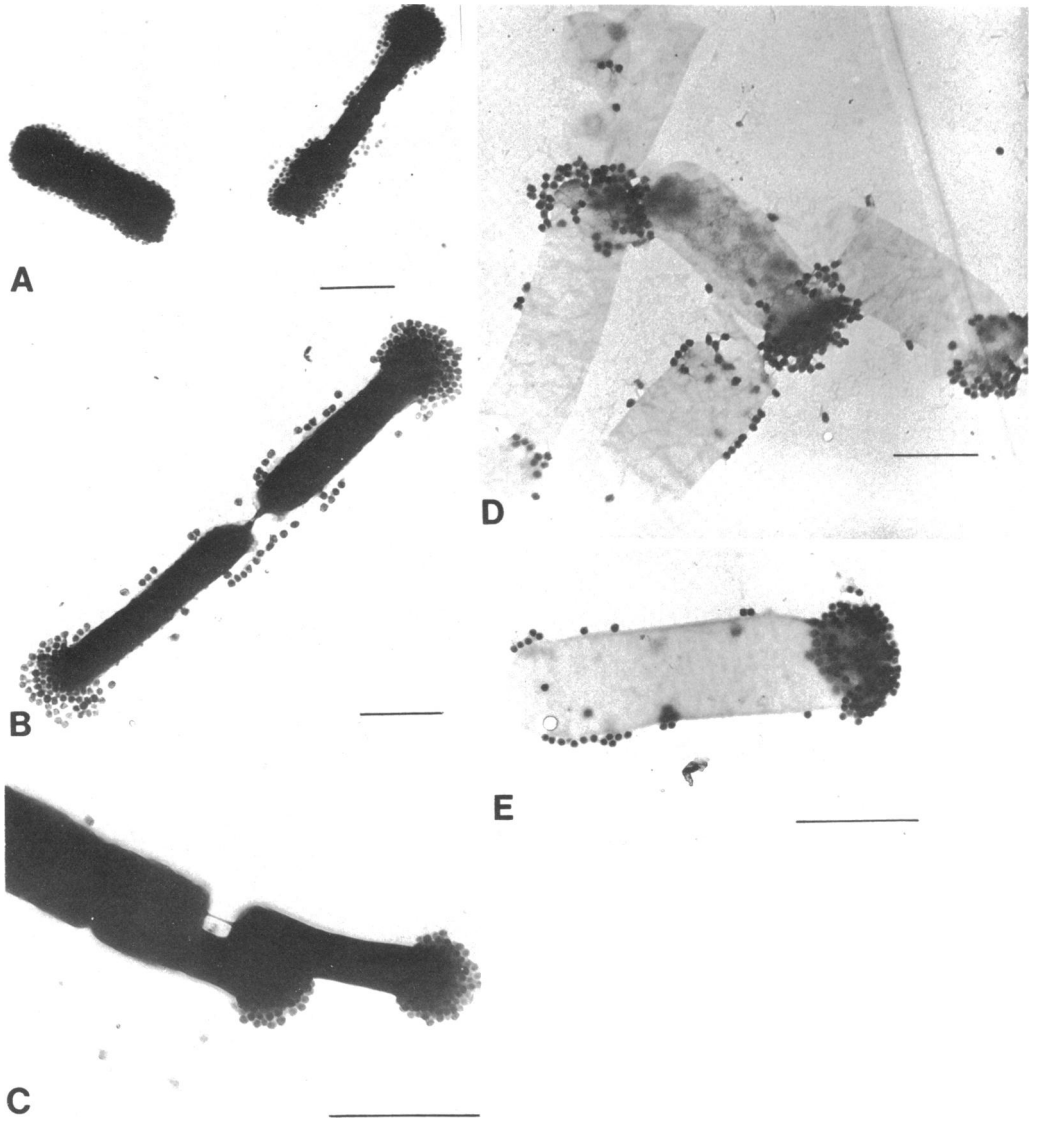

FIG. 11. *Electron micrographs showing location of phage particles adsorbed to bacteria harvested during transition from K^+- to PO_4^{3-}-limited growth. Samples harvested between (A) 4 and 5 h, (B) 6 and 7 h, and (C) 12 and 13 h after changeover. (D and E) Walls isolated from samples harvested between, respectively, 6 and 7 h and 10 and 11 h after changeover. Bar represents 1 μm.*

tained by procedures such as autoradiography or biochemical analysis, which do not depend on the presence of the newly incorporated material at the cell surface.

During transition, new wall material is incorporated as a result of turnover as well as growth. Consequently, new material must be incorporated throughout a large proportion of the wall and not just from one, or a few, growth zones. This conclusion is supported by the observation that phage particles could bind over the entire surface of almost all of the bacteria present in the sample harvested between 4 and 5 h after changeover from potassium to phosphate limitation. These bacteria contained approximately 23% of the amount of wall teichoic acid present in potassium-limited bacteria. Therefore, approximately 77% of the wall material in these bacteria had been synthesized under phosphate-limiting conditions and so was of

inappropriate composition to participate in phage binding. Had this material been incorporated from a single growth zone, it would have been present as a continuous area constituting the major part of the cell surface. That it is not present in this manner is shown both by the results of quantitative measurement and by electron microscopic examination of adsorbed phage particles. We conclude that incorporation of new material takes place at multiple sites over the wall. This agrees with the finding (28) that teichoic acid-glycopeptide complexes recently incorporated into walls of *B. subtilis* W23 are linked randomly to old and new glycopeptide chains.

However, incorporation of newly synthesized material into existing polar caps appears to proceed less rapidly than does incorporation into the cylindrical portion of the wall. Thus, in samples harvested at later stages of transition between potassium and phosphate limitations, increasing numbers of bacteria were observed that adsorbed phage particles at the ends but not along the length of the cell. Bacteria were also seen in which phage particles adsorbed only to one pole, and in the sample harvested between 12 and 13 h after changeover the only bacteria that adsorbed phage did so exclusively at one pole. Clearly the incorporation of new material into existing polar caps must proceed more slowly than does incorporation into the rest of the wall. It is interesting that the polar caps of walls of *B. subtilis* differ from the rest of the wall in that they are resistant to autolytic amidases (13, 15). This may account for the observation (18) that during resumption of growth after the initiation of protein synthesis in bacilli that had been incubated with chloramphenicol, the outer portion of the thickened cell wall fragments into small pieces along the cylindrical part of the cell whereas the wall remains thick at polar regions for at least two generations. Highton and Hobbs (23) have proposed that their observations of the effect of penicillin on wall synthesis in *B. cereus* can best be explained by a model in which longitudinal extension of the wall occurs by addition of material to a large number of growing points that are uniformly distributed over the cylindrical surface of the cell; addition occurs only in the lingitudinal direction so that the cell diameter remains constant and the ends are conserved. The present results are consistent with such a model or with a modification in which the newly incorporated material is intercalated at a large number of points along the inner surface of the wall and reaches the exterior surface only as a consequence of further growth and turnover.

During transition from phosphate to potassium limitation, newly incorporated receptor material becomes exposed at the surface first along the length of the cell, as would be expected on the results obtained with bacteria undergoing transition in the opposite direction. However, in early samples the receptor material appears to be concentrated at sites where cross wall formation would be expected to occur. Therefore, although incorporation of new material takes place at a large number of sites along the length of the cell, it appears that material incorporated in the regions of the developing septa is first to appear at the surface of the cell. This may partly explain certain earlier observations (9, 10, 24) showing that new wall material, detected by labeled antibody preparations, was produced from only one or a few growth zones in various bacilli.

A model of cell wall assembly that satisfactorily explains the present results, and that has several features in common with earlier proposals, is one in which new wall material is incorporated at a large number of sites, or in a layer, along the inner surface of the cylindrical portion of the wall. This material becomes exposed at the cell surface only during subsequent growth, but incorporation of new material may also proceed rapidly into the developing septa and such material may more quickly become exposed at the surface. However, incorporation of new material into existing polar caps proceeds more slowly: the polar caps may be conserved or they may undergo turnover that involves incorporation of new material at a rate slower than that which occurs in other regions of the wall.

Since the phage-binding properties of small quantities of whole bacteria can readily be measured, and since the location of adsorbed phage particles can readily be determined by electron microscopy, the procedures described in this paper should facilitate the study of a number of factors that influence the synthesis and incorporation of cell wall material.

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