Cell Wall Assembly in *Bacillus subtilis*: Visualization of Old and New Wall Material by Electron Microscopic Examination of Samples Stained Selectively for Teichoic Acid and Teichuronic Acid

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Uranyl acetate staining of thin sections allowed a distinction to be made between cell wall material that contains teichoic acid and that which contains teichuronic acid. The stain was used to study the pattern of wall assembly in *Bacillus subtilis* undergoing transitions between growth conditions leading to incorporation of the different anionic polymers. The results showed that new material is incorporated along the inner surface of the cylindrical region of the wall confirming, by a more direct method, results obtained earlier with teichoic acid specific phages. New material appears to be evenly distributed along the inner surface and no evidence was obtained for the presence of specific zones of incorporation.

INTRODUCTION

The mode of cell wall assembly in *Bacillus subtilis* was first studied by Chung *et al.* (1964) who concluded that growth proceeded by incorporation of new material from a narrow annular growth zone located half way along the cell. This was supported by fluorescent antibody studies of wall assembly in *Bacillus licheniformis* (Hughes & Stokes, 1971). The idea that the bacterial surface expands from a single zone is in accord with the replicon hypothesis (Jacob *et al.*, 1963) in which genome segregation is accomplished through attachment to the envelope: this intellectually attractive hypothesis has had a major influence on both the design of experiments and the interpretation of their results. Nevertheless, this idea is contradicted by an increasing body of evidence showing that wall assembly in *B. subtilis* reflects two distinguishable processes. Septa, and hence the cell poles, are formed by incorporation of new wall material from an annular growth zone but cylindrical wall is formed by the incorporation of new material at its inner surface, at many sites or in a layer, and such material is gradually pushed to the outer region of the cylinder by the continual incorporation of newer wall material during growth (Anderson *et al.*, 1978; Mobley *et al.*, 1984).

Evidence for an 'inside-to-outside' assembly process for cylindrical wall was obtained first in the electron microscopical study of Fan *et al.* (1975) who noted that bacilli that had earlier been plasmolysed subsequently deposited a densely staining layer of material at the inner wall surface and that this appeared to move through the wall during continued growth. Pooley (1976*a, b*) suggested that the delay observed (Mauck & Glaser, 1972; Mauck *et al.*, 1971) between incorporation of new material into the cell wall and its eventual appearance in the culture fluid as a degraded 'turnover' product could be explained on the basis that new material is

Abbreviations: TA, teichoic acid; TU, teichuronic acid; TA or TU walls, cell wall material containing predominantly TA or TU as the anionic polymer, respectively.

incorporated at the inner surface of the wall but shed only after it has moved to the outer surface. Pulsed incorporation of radiolabelled wall material supported this interpretation: newly incorporated material was not solubilized by lytic enzymes added to a suspension of whole bacteria but was attacked by enzymes added to a suspension of isolated walls in which the inner surfaces were, of course, accessible to the lysin.

Direct evidence for the initial incorporation of new material at the inner surface of the wall was obtained by using phages and lectins to locate teichoic acid (TA), an integral wall polymer, incorporated during pulsed release of phosphate limitation in chemostat cultures of *B. subtilis* (Archibald, 1976; Anderson *et al.*, 1978). These studies showed that the newly incorporated material was not detectable at the outer surface of the cylinder but was detected and located at the inner surface by phages specific for TA. Consideration of both the quantitative binding data and the electron microscopic distribution of phages led to the conclusion that new material was incorporated all along the inner surface of the cylinder at many sites or in a layer. This conclusion has been refuted by Schlaeppi *et al.* (1982) who have concluded that wall is assembled from a limited number of incorporation zones, though Mobley *et al.* (1984) have also concluded that incorporation proceeds at many sites. The experimental approaches used by these latter two groups, autoradiography and lectin adsorption to whole bacteria, do not give direct information on the pattern of incorporation at the inner surface. The relationship between the location of sites at which material is incorporated into the inner surface of the cell and of sites at which it becomes exposed at the cell surface may not be simple (Anderson *et al.*, 1978), and it is therefore necessary to look directly at sites of incorporation at the inner surface in order to distinguish between multiple and few sites of incorporation.

A difficulty with the use of phages is that the maximum packing density is limited by the size of the phage heads (phage SP50 has a head diameter of 80 nm) rather than by the density of receptor material in the wall; also, since even small amounts of TA can ensure phage binding (Givan *et al.*, 1982), the technique does not discriminate between regions of the wall which have incorporated large and small amounts of new material. For example, phages might not reveal a specific growth zone if, simultaneously, material lost by turnover was replaced by sufficient material at other locations along the cylinder to permit phage binding. A further disadvantage of the technique is that phages can bind to the inner surface of the wall only after the bacteria have been disrupted. While the technique has provided the most direct and convincing evidence for the incorporation of new material along the inner surface of the wall, its disadvantages have precluded its use for a more definitive assessment of the possibility that incorporation into the inner wall surface proceeded from specific zones. In spite of the continuing experimental and theoretical attention being given to wall assembly and growth, no other direct study of the pattern of assembly at the inner wall surface has appeared since the work with phages (Archibald, 1976).

We now show that under the staining conditions used for electron microscopy, wall material that contains TA binds more uranium than does an equal weight of wall material containing a second type of anionic polymer, teichuronic acid (TU). This differential staining enables changes at the inner surface of the wall to be studied directly in bacilli undergoing transition between growth conditions resulting in incorporation of either TA or TU. The results confirm and extend earlier observations obtained with phages, and show that new wall material is inserted all along the inner surface of the cell cylinder. No evidence was found for specific zones of incorporation.

METHODS

Organism and maintenance. Our laboratory strain of *B. subtilis* 168 (CH724) is a prototrophic derivative of that used by Coley *et al.* (1975). Stock cultures were stored at -20°C in TYE broth containing 15% (v/v) glycerol, and maintained for short periods on TYE medium solidified with 1.5% (w/v) agar (Difco). TYE medium contained (l^{-1}) 16 g Bacto-tryptone (Difco), 10 g yeast extract (Difco) and 5 g NaCl. Chemostat cultures were always initiated from the glycerol-preserved stock.

Chemostat culture. Continuous cultures, under magnesium or phosphate limitation, were grown in a chemostat (L H Engineering) (Lang *et al.*, 1982) with automatic pH control (by addition of aqueous $3\text{M-NH}_4\text{OH}$) and

antifoam addition (Dow Corning X2-3240). The total culture volume was 1 litre and the dilution rate 0.2 h^{-1} . Temperature was maintained at 37°C and pH at 7.0. The culture was aerated at 2 l min^{-1} and stirred at 750 r.p.m.

The defined medium was that described by Lang *et al.* (1982). Phosphate-limiting medium contained 0.25 mM inorganic phosphate and magnesium-limiting medium 5 mM inorganic phosphate; the magnesium concentration for both limitations was $75 \mu\text{M}$ and the carbon source was glycerol (30 mM), autoclaved in the medium. Tryptophan, separately filter-sterilized, was added to 1 mM .

Continuous cultures were initiated by addition of 50 ml of a late-exponential culture in TYE broth to 950 ml magnesium-limiting medium in the chemostat. After culturing for 12 h without dilution, medium flow was commenced and continued for at least 10 generation times to ensure steady state. Culture density was monitored by measurement of optical density in samples diluted to give readings in the range of 0.2 – 0.6 at 600 nm . The dilution rate was calculated from the measured medium flow rate and the volume of culture in the vessel, measured at the end of the experiment.

Collection and treatment of samples from the chemostat culture. In balanced growth and during the changes between limitations samples (12 ml) were collected from the overflow of the chemostat into a vessel at 0°C . A portion (1 ml) of each sample was immediately centrifuged at 10000 g for 2 min and the supernatant stored at -20°C for subsequent estimation of inorganic phosphorus. A portion (10 ml) of each sample was also added to 0.43 ml 25% (v/v) glutaraldehyde (Polaron) and incubated with gentle shaking at 37°C for 2 h . Glutaraldehyde-fixed bacteria were recovered by centrifugation at 15000 g for 10 min at room temperature and suspended in 1 ml 1% (v/v) glutaraldehyde at room temperature. These samples were embedded and dehydrated for electron microscopy within 36 h of collection.

Electron microscopy. Fixed bacteria were recovered from 1% glutaraldehyde by brief centrifugation (1400 g for 5 min) and taken up into agar (2% , w/v, in water) blocks as described previously (Hobot *et al.*, 1984). Samples were completely dehydrated in a graded series of increasing ethanol concentration followed by infiltration and embedding in the acrylic resin LR White (London Resin Co.) following the procedure of Newman & Hobot (1987). However, an extra step of an overnight infiltration with pure resin, then two further changes of 30 min were added. Samples were polymerized chemically at 0°C for 24 h , then hardened for 2 h at 50°C (Newman & Hobot, 1987).

Thin sections were cut on an LKB Ultramicrotome III with glass knives and stained with 2% (w/v) or 0.2% aqueous uranyl acetate for 1 min with or without subsequent treatment with the lead acetate of Millonig (1961) for 0.5 – 1 min . Sections were examined in a Philips 300 electron microscope operating at 80 kV .

Relative densities of staining of different parts of the cell wall were measured on electron micrograph prints using the Gemini image analysis system (Joyce Loebel).

Phosphate estimation. Total phosphorus in culture supernatants was measured by the method of Chen *et al.* (1956), after digestion of the samples with ethanolic $\text{Mg}(\text{NO}_3)_2$ (Ames, 1966).

Uronic acid estimation. Glucuronic acid in cell wall TUs was measured by the method of Blumenkrantz & Asboe-Hansen (1973).

Metal binding to cell walls. Cell walls from *B. subtilis* 168 grown in chemostat culture under phosphate limitation (feed P_i concentration 0.35 mM) and under magnesium limitation (feed P_i concentration 5 mM) were prepared as described by Lang *et al.* (1982). Samples (1 mg) were treated with glutaraldehyde, uranyl acetate and alkaline lead acetate following the protocol described for staining thin sections for electron microscopy. Treated walls were washed with distilled water and the absorbed metals were then extracted into a mixture of concentrated nitric acid/ 60% (w/v) perchloric acid ($6:1$, v/v) under reflux for 20 min . Metal concentrations were measured in the extract by atomic absorption spectrometry for lead (Pye Unicam SP9 atomic absorption spectrometer), or by the arsenazo III colorimetric method for uranium (Zosim *et al.*, 1983).

Chemicals. Chemicals were AnalaR grade (BDH) unless otherwise stated.

RESULTS

Differential staining of anionic polymers in the B. subtilis cell wall

Cells of *B. subtilis* 168 grown in chemostat culture under phosphate-replete conditions (magnesium limitation) synthesize and incorporate TA into their walls. Electron micrographs of thin sections of such cells show the wall and associated cytoplasmic membrane, together referred to as the cell envelope, to contain bands of markedly different electron density (Figs 1*a* and 2*a*). Immediately outside the electron-translucent inner layer of the cytoplasmic membrane, seen only rarely as a double-track in this type of preparation (Hobot *et al.*, 1984), is a very electron-opaque band, the 'A' layer (Fig. 2*a*), which appears to consist of the outer leaf of the cytoplasmic membrane and a layer possibly of an anionic polymer like membrane TA (see Discussion).

Fig. 1. Deposition of new cell envelope material in *B. subtilis* 168 during transition from magnesium to phosphate limitation (a-c) and during transition from phosphate to magnesium limitation (d). Samples were collected (a) 5 h, (b) 10 h, (c) 10 h and (d) 1.5 h after changeover. Bar marker, 0.5 μ m.

Towards the outside of the cell at least two other layers are discernible: a thinner less electron-opaque inner 'B' layer and an outer thick electron-opaque but heterogeneous 'C' layer. The ragged appearance of the latter layer is presumably due to the activity of autolysins which are responsible for cleaving material from the outer surfaces of the cylindrical regions of the cell wall (Frehel *et al.*, 1971). It is notable that the poles are comparatively smooth, suggesting a reduced level of autolytic activity or a different mechanism of wall growth and turnover (see, for example, Fig. 1a).

Fig. 3 shows the rate of depletion of phosphorus from the culture medium during the period following the change to the phosphate-limiting medium. The phosphate concentration falls to

Fig. 2. Deposition of new cell envelope material in *B. subtilis* 168 during transition from magnesium to phosphate limitation. Bacteria were equilibrated under magnesium limitation at a dilution rate of 0.2 h^{-1} and the inflowing medium was changed to effect phosphate limitation. Samples were collected (a) 5 h, (b) 8.5 h, (c) 10 h, (d) 10 h, (e) 11.5 h and (f) 13 h after changeover. Bar marker, $0.2 \mu\text{m}$. Layers A, B and C, and the feature indicated by the arrowheads, are discussed in the text.

Fig. 3. Total phosphate in the culture during transition from magnesium to phosphate limitation. Bacteria were equilibrated under magnesium limitation at a dilution rate of 0.2 h^{-1} and the inflowing medium was changed to effect phosphate limitation.

about 0.65 mm over a period of 7 h. During this time the rate of TU synthesis is increased and that of TA is decreased. The first changes in cell envelope structure which accompany the transition from phosphate-replete to phosphate-limiting medium are observed between 7 and 8.5 h after medium changeover (Fig. 2*b*). The 'C' layer remains electron opaque at the periphery of the cell wall, but appears to become thinner as the electron-translucent region adjacent to the 'B' layer expands. In samples taken at 10 h (Fig. 2*c*) there is even less of the electron-opaque 'C' layer as the inner wall layer increases in thickness and the 'A' layer begins to reduce in electron density. In samples taken at 10 h (Figs 1*b* and 2*d*) more than half of the electron-opaque 'C' layer has disappeared and areas of the ragged, outermost part of this layer are starting to be replaced by less densely staining material so that in places sections of the wall consist only of less dense material. In samples taken at 11.5 h discrete patches of darkly staining material are present and these are found only at the very outer edge of the cell wall (Fig. 2*e*, arrowed). At 13 h darkly staining material has been completely removed from the cylinder of the cells (Fig. 2*f*). At that time the electron density of the 'A' layer is much reduced, as compared to the densely stained material observed under phosphate-replete conditions (Fig. 2*a*), and has a double-tracked appearance (see Discussion).

Cells grown for about 5 generation times under conditions of phosphate limitation have weakly staining walls with no division of the wall into 'B' and 'C' layers (Fig. 4*a*). The 'A' layer has a double-tracked appearance. One hour after changing over to phosphate-replete (magnesium-limiting) medium, the innermost 'A' layer is seen to increase in electron opacity (Fig. 4*b*), and at 1.5 h its double-tracked appearance is lost (Fig. 4*c*). At the latter time, however, a layer of increased electron opacity is observed in the inner part of the wall just outside the 'A' layer (Fig. 4*c*, arrowed). The outermost, ragged region of the wall remains weakly stained (Figs 1*d* and 4*c*) and the inner electron-opaque layer increases in width (Fig. 4*d*). At 2.5 h the 'C' layer is uniformly electron dense except for patches of lightly stained material at the outer surface of the cell (Fig. 4*e*, arrowed), but by this time most of the walls have completely regained their multilayered appearance (compare Fig. 4*f* with Fig. 2*a*). These patches of wall material are occasionally seen for up to 3 h after changeover to phosphate-replete conditions (not shown).

Previous experiments have shown that when *B. subtilis* growing under phosphate-replete conditions is changed to phosphate-limited conditions, TA synthesis stops and this polymer is replaced by newly synthesized TU (Ellwood & Tempest, 1969). The reverse occurs during transition in the opposite direction. We interpret the electron microscope results as showing differential staining of these anionic wall polymers and their location in the wall.

Quantitative analysis of the staining procedure for electron microscopy

Results presented above suggest that a larger amount of one or both of the heavy metal ions used in the staining procedure, lead or uranium, was bound to TA than to TU. The stoichiometry of lead and uranium binding to isolated cell walls stained by the electron microscopy procedure was measured using walls containing TA or TU as predominant anionic polymer, before and after treatment with glutaraldehyde. Table 1 shows the amount of lead and uranium bound by the walls. The only significant difference in metal content between the two types of wall was between the amounts of uranium present, TA-containing walls binding 1.7 times as much uranium as the TU-containing ones. Treatment of the walls with uranyl acetate alone, under the same conditions, led to the same difference in uranium content; the subsequent treatment with lead acetate slightly reduced the amount of uranium bound. Thin sections treated with 0.2% instead of 2% uranyl acetate showed the same differential staining (compare Fig. 5*a* with Fig. 2*b*). The metal-binding results suggested that staining with uranyl acetate alone should provide as much contrast between the different kinds of wall material as obtained with uranium plus lead, though the overall density would be reduced. This was confirmed (compare Fig. 5*b* with Fig. 2*d*).

The relative densities of stain (uranium plus lead) in strongly stained and weakly stained layers within the thickness of a single cell wall section (see for example Figs. 2*b* and 4*b*) were measured in micrographs of single cells using a video-scanning image analysis system. In a sample in transition from phosphate to magnesium limitation, the ratio of optical densities of

Fig. 4. Deposition of new cell envelope material in *B. subtilis* 168 during transition from phosphate to magnesium limitation. Bacteria were equilibrated under phosphate limitation at a dilution rate of 0.2 h^{-1} and the inflowing medium was changed to effect magnesium limitation. Samples were collected (a) 0 h, (b) 1 h, (c) 1.5 h, (d) 1.5 h, (e) 2.5 h and (f) 2.5 h after changeover. Bar marker, $0.2 \mu\text{m}$. Layer A, and the feature indicated by the arrowheads, are discussed in the text.

Fig. 5. Deposition of new cell envelope material in *B. subtilis* 168 during transition from magnesium to phosphate limitation. (a) Sample collected after 8.5 h and stained with 0.2% uranyl acetate and lead acetate; (b) sample collected after 10 h and stained with 2% (w/v) uranyl acetate in the absence of lead acetate. Bar marker, $0.25 \mu\text{m}$.

Table 1. *Quantification of metal binding to cell walls in the staining procedure*

The figures are means of triplicate measurements of samples from duplicate experiments. Measurements varied from the mean by less than 1%, and the results of duplicate experiments differed from the mean by less than 2%.

Staining procedure	Bound metal [$\mu\text{g (mg wall)}^{-1}$]			
	TA-containing wall		TU-containing wall	
	Uranium	Lead	Uranium	Lead
Without glutaraldehyde fixation	490	516	290	497
With glutaraldehyde fixation	493	529	304	463

the two types of wall, at a site of contact between the two within the cylindrical part of the wall, was 1.9.

DISCUSSION

Wall material that contains TA can be distinguished from wall material that contains TU by direct electron microscopic examination of sections of chemostat-grown and glutaraldehyde-fixed cells of *B. subtilis*, embedded in an acrylic resin and stained with uranyl and lead acetate. The discrimination is due to a difference in uranium uptake, and the difference in staining intensity, measured on the electron micrographs, is similar to the difference in the amounts of uranium bound by wall samples containing the different anionic polymers. This affords a new and more direct technique for examining the location of old and new material throughout the entire thickness of walls of bacilli undergoing transitions between growth conditions that change the anionic polymer being synthesized.

The ability of walls of Gram-positive bacteria to bind cations is well documented (Hancock, 1986) and is due primarily to the anionic polymers TA and TU. Under some conditions (Heckels *et al.*, 1977) metals are bound equally well by walls containing either of the anionic polymers but, as shown here, under the conditions used for electron microscopy staining, uranium cations are bound more extensively by walls of *B. subtilis* that contain TA. Isolated cell walls fixed and stained by the procedure used for thin sections bound 1.2–1.3 µg-atoms uranium per microequivalent anionic group (TA walls) or 0.8 µg-atoms uranium per microequivalent anionic group (TU walls). No attempt was made to achieve equilibrium binding and there was no evidence of the substantial precipitation of insoluble uranium hydroxides that occurs on prolonged exposure of the cell walls to uranyl salts (Beveridge, 1978). Substantial binding of lead, which must have occupied the same and/or additional, unknown, sites in the walls, was unnecessary for the differential staining of TA- and TU-containing walls (see Fig. 5*b*).

We examined the cell wall of *B. subtilis* 168 undergoing transitions between phosphate limitation, when TU is the major anionic polymer present, and magnesium limitation, when TA is present instead. Under the particular growth conditions used here, walls of the phosphate-limited bacteria contain 1.44 mmol TU carboxyl and 0.06 mmol TA phosphate per gram of wall whereas those of magnesium-limited bacteria contain 1.61 mmol TA phosphate and less than 0.01 mmol TU carboxyl per gram of wall. No information is available on the differences in contents of membrane TA and phospholipids in the bacteria under the particular conditions studied here. However, phosphate-limited *B. licheniformis* was shown to contain only one-tenth as much membrane TA (Button *et al.*, 1975) and only half as much phospholipid (Minnikin *et al.*, 1972) as do the bacteria grown under conditions where phosphate is freely available.

Changes in staining are seen throughout the envelope during the growth transitions and appear to involve the outer leaf of the cytoplasmic membrane as well as the cell wall. Magnesium-limited bacteria show a narrow, intensely staining layer (the 'A' layer) which shows changes in staining intensity that appear independent of the changes in the overlying wall layers. For example, in bacteria undergoing transition to phosphate limitation, less densely staining material becomes incorporated into the inner 'B' wall layer before any change is seen in the staining of the innermost intensely staining 'A' layer. The sharply defined appearance of the 'A' layer and its possible relation with the outer leaflet of the cytoplasmic membrane, together with the consideration that new material is seen in outer wall layers before it is seen in the 'A' layer, suggests that this layer is associated with membrane rather than being an innermost layer of the wall. Although direct experimental evidence is not available the chemical similarity of membrane and wall TAs suggests that they will have similar uranium binding properties and it seems possible that the intense staining of the 'A' layer is due to membrane TA. The change of the 'A' layer to a less electron-opaque appearance in phosphate-limited bacilli is consistent with its diminished content of membrane TA (Button *et al.*, 1975).

During transition from limiting to excess phosphate, walls acquire densely staining material at the inner region and the amount of this material increases as the outer, less intensely staining

material decreases. During transition in the reverse direction walls acquire lightly staining material and the intensely stained material becomes increasingly confined to only the outermost region of the wall. These results show that differently staining material incorporated following a change in growth conditions appears first in the inner region of the wall while the existing, or older, material is gradually displaced so that it is found only in the outer fringes of the wall. This occurs both during transition to phosphate limitation, when the new material (TU-peptidoglycan complex) is that which stains less intensely and during transition from phosphate limitation, when the new material (TA-peptidoglycan complex) is that which stains more intensely. Our micrographs provide the clearest evidence to date that new wall is incorporated at the inner surface of the cylindrical wall in *B. subtilis* and moves outward during growth, and corroborate the conclusions of Archibald & Coapes (1976) and Pooley (1976*a, b*). In the accompanying paper (Clarke-Sturman *et al.*, 1989), the same differential staining technique is used to investigate the incorporation of new material into polar caps.

As well as confirming the inside-to-outside direction of cylindrical wall assembly, the micrographs provide information relating to the existence of growth zones. The original evidence for specific growth zones came from work using fluorescent antibody techniques. The interpretation of these results was based on the premise, subsequently shown to be incorrect (Archibald & Coapes, 1976), that new wall material is detectable immediately at the bacterial surface. More recently Schlaeppli *et al.* (1982) have concluded that their autoradiographic experiments show that the wall in *B. subtilis* is incorporated in a small number of specific zones whereas Mobley *et al.* (1984), partly on the basis of similar autoradiographic results, have concluded that incorporation takes place at many sites. In both of these studies conclusions have been based on the distribution of residual radiolabelled wall several generation times after its incorporation. That approach is questionable firstly because an increasingly large proportion of the total radioactivity present in samples collected after a chase may be due to protein rather than to cell wall (Clarke-Sturman & Archibald, 1982; Mobley *et al.*, 1984) and secondly (Anderson *et al.*, 1978) because the relation between the sites at which material is incorporated into the wall and those to which it moves later is not known. This latter reservation applies equally to our observations (Anderson *et al.*, 1978) on the locations at which phages bind to whole cells of *B. subtilis* following transition from phosphate limitation and to the similar observations made by Mobley *et al.* (1984) on the locations at which fluorescent concanavalin A bound to mutants following transition to and from conditions permissive for synthesis of α -glucosylated TA.

Though experimental and theoretical studies of wall assembly in bacilli have been recently pursued (see Koch & Doyle, 1986; Doyle & Koch, 1987), no direct evidence relating to the location or number of sites at which new material is incorporated into the inner wall surface has appeared since Archibald (1976) showed that phage SP50 bound along the inner surface of walls isolated from bacteria collected during release of phosphate limitation. As mentioned above, the phage technique does not discriminate between regions of the wall which have incorporated substantial amounts of new material and those in which much smaller amounts are present, so specific 'growth zones' could be obscured by a secondary incorporation related to wall turnover.

The selective staining procedure described here avoids that difficulty and shows the relative proportions of different materials as well as their locations. The micrographs show that the envelope contains bands that stain at markedly different intensities, particularly in bacteria grown under phosphate-replete conditions.

During transition to phosphate limitation changes are seen in all of the envelope layers. In all cases the changes appear uniformly along the length of the wall cylinder (Fig. 1) and there is no indication of any localized zone of thicker material. Changes seen in envelope layers during transition from phosphate limitation mirror those seen in the opposite direction and again no zones are seen where the newer or older wall material is of differing thickness.

We conclude from these observations that new material is incorporated uniformly along the length of the wall cylinder. As mentioned previously (Anderson *et al.*, 1978) this does not necessarily imply a uniform distribution of synthetic sites in the membrane, and it does not exclude the possibility that there might be a limited number of such sites. Their detection would

depend, however, upon how much of the total wall material must be present as 'new' wall of altered composition before it can be visualized by the present technique. Our results could therefore be consistent with suggestions made by Mendelson (Mendelson, 1976, 1985; Mendelson *et al.*, 1984) that wall is laid down from a limited number of synthetic sites that move round the membrane. Provided that such sites fully traverse the wall cylinder in the time taken to incorporate the minimum of wall material necessary for visualization, a markedly asymmetric mode of incorporation could nevertheless give an apparently uniform distribution. While such asymmetric incorporation cannot be demonstrated directly, at least by currently available techniques, Mendelson's studies clearly show anisotropy in the wall (Mendelson *et al.*, 1984) that must reflect some aspects of the structure, arrangement and assembly that have so far been exposed only in studies of macrofibre formation.

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