

Cellulose biosynthesis in *Acetobacter xylinum*: Visualization of the site of synthesis and direct measurement of the *in vivo* process*

(darkfield microscopy/electron microscopy/bacterial envelope/microfibril/enzyme complex)

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ABSTRACT *In vivo* synthesis of cellulose by *Acetobacter xylinum* was monitored by darkfield light microscopy. Cellulose is synthesized in the form of a ribbon projecting from the pole of the bacterial rod. The ribbon elongates at a rate of $2 \mu\text{m min}^{-1}$. The ribbon consists of approximately 46 microfibrils which average $1.6 \times 5.8 \text{ nm}$ in cross section. The observed microfibrillar elongation rate corresponds to 470 amol of glucose/cell per hr assimilated into cellulose. Electron microscopy of the process using negative staining, sectioning, and freeze-etching indicated the presence of approximately 50 individual synthetic sites organized in a row along the longitudinal axis of the bacterial rod and in close association with the outer envelope. The process of cellulose synthesis in *Acetobacter* is compared with that in eukaryotic plant cells.

Cellulose biosynthesis by *Acetobacter xylinum* has been widely investigated (1-6). It is generally believed that the synthesis of microfibrils occurs by an extracellular process involving the sequential addition of glucose residues to the growing tips at a distance from the cell (5-7). The biochemical reactions of cellulose synthesis have been extensively documented, the intermediate products being hexose phosphates (8), sugar nucleotides (9), and perhaps glycolipids (6). However, recent findings from kinetic data (1) and biochemical incorporation studies using defined cell wall extracts (2-4) indicate a closer relationship than previously expected between the bacterial envelope and the cellulose polymer.

The purpose of this investigation is to clarify and lay groundwork on the cytology of cellulose formation in *Acetobacter xylinum*. In order to achieve this, we have carefully observed the dynamics of the *in vivo* process, using darkfield and Nomarski interference microscopy. We have also made use of electron microscopic techniques of thin-sectioning, negative staining, and freeze etching. From these approaches has emerged a coherent picture of cellulose microfibril assembly—one which should be of benefit to future biochemical, cytochemical, and physicochemical studies of the process.

METHODS

Growth Conditions. *Acetobacter xylinum* strain ATCC 23769 from the American Type Culture Collection was maintained on mannitol agar slants. Pellicles were produced at liquid-air interfaces of standing cultures in Schramm and Hestrin's glucose medium (10) at 25° . Pellicles aged 24-48 hr were used.

Isolation and Culture of Cells. The pellicles described above were transferred to a flask of deionized water and shaken vigorously by hand in order to separate the cellulose-synthesizing bacteria. The pellicle retains its integrity during this process, and the bacteria are readily poured off. The separated bacteria were centrifuged at approximately $1400 \times g$ for 5 min. The

supernatant was discarded, and the bacterial pellet was resuspended in deionized water. This suspension was immediately cooled to 5° to attenuate cellulose synthesis. These T_0 cells were cultured in deionized water at 25° for specified periods (indicated by T_5 , T_{10} , etc., where the subscripts refer to minutes) and observed as described below.

Light Microscopy. *In vivo* cellulose synthesis was observed by Nomarski interference and darkfield light microscopy using a Zeiss Photoscope equipped with a $100\times$ Planachromatic oil immersion objective with variable aperture, numerical aperture = 1.25. Rates of microfibrillar growth were measured with an ocular micrometer calibrated with a stage micrometer.

Electron Microscopy. Cell suspensions were negatively stained with 2% aqueous uranyl acetate, using bacitracin as the spreading agent (11). Cells were prepared for freeze etching as follows: Material was placed onto gold specimen carriers and immediately frozen in Freon 22. The specimens were transferred into liquid nitrogen until used. Specimens were etched at -100° for 40 sec, and the temperature was lowered to -108° before replication in a Balzers BA 360 freeze etch instrument. Platinum-carbon replicas were cleaned by flotation on Clorox bleach for 1 hr, followed by 75% H_2SO_4 for 5 hr. The specimens were mounted on formvar-coated grids.

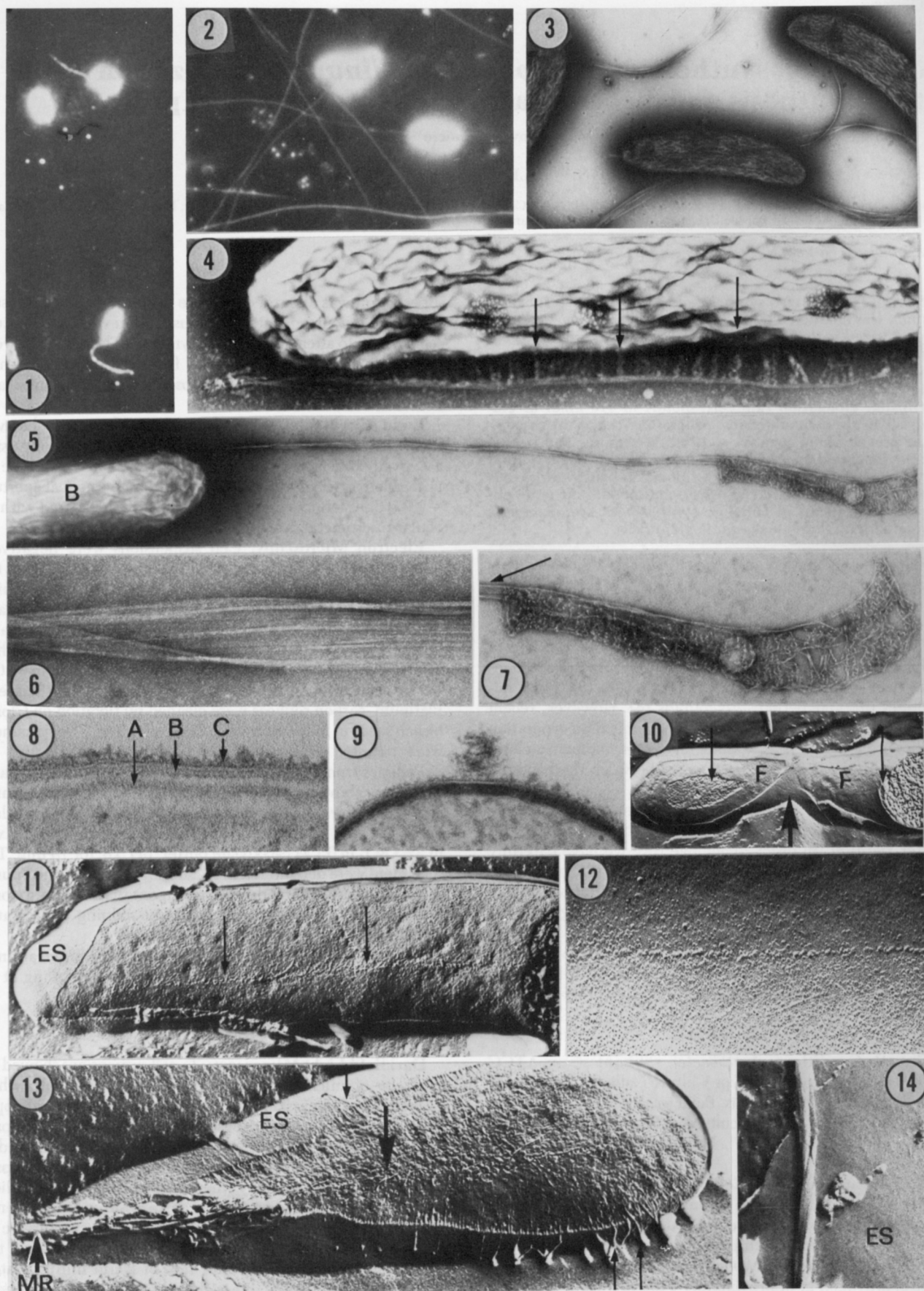
Pellicles from the T_{10} standing cultures in deionized water described above were carefully transferred to a 2% tannic acid-2% glutaraldehyde fixative in 0.05 M sodium cacodylate buffer, pH 5.8, for 90 min at 25° . The material was post-fixed in 2% OsO_4 in 0.1 M cacodylate buffer, pH 5.8, for 75 min. The specimens were dehydrated in ethanol and embedded in Spurr's resin. Sections were made with a Dupont diamond knife on a Reichert OM U2 ultramicrotome. Post-staining with uranyl acetate and lead citrate was routinely used.

For electron microscopy, all preparations were examined with a Hitachi HU 11E electron microscope operating at 75 kV.

RESULTS

Darkfield Light Microscopy. T_0 cell suspensions showed no evidence of microfibrillar projections from the cells when viewed with Nomarski interference or darkfield microscopy; however, the T_5 preparations revealed numerous short projections extending from the bacteria. These projections extended from one pole of each bacterial rod (Fig. 1). The ribbon projections exhibited anisotropy when examined with crossed Nicol prisms under darkfield conditions and a positive birefringence with the first-order red compensator, both characteristic of cellulose. The projections extended continuously during the following 15 min (T_5 - T_{20}) at a rate of $2 \mu\text{m min}^{-1}$. Later, growth ceased, probably due to the anaerobic conditions under the cover slip. The control suspensions continued to make microfibrillar projections at the air-liquid interface for several

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FIGS. 1-14. (Legend appears at bottom of the following page.)

hours, giving rise to an intermeshing network, familiar as the cellulosic pellicle (Fig. 2).

Microfibrillar growth was determined to originate at the bacterial surface rather than at tip regions distinctly separated from this organism as follows. Once the microfibrillar ribbons had lengthened sufficiently for them to stick to the cover slip, it was possible to use the ocular micrometer as a reference marker. A distinctive tip was placed to coincide with one end of the micrometer scale, and the bacterium was observed at a given value at the other end of the scale. It was found that the position of the attached tip did not change, but that the bacterium moved at a steady rate of $2 \mu\text{m min}^{-1}$, producing a continuous, elongating projection.

Negative Staining. Having established with living cells that the microfibrillar ribbons constituting the cellulosic pellicles are attached to the bacteria and elongate only at the end associated with the cell, we followed the process more carefully at the ultrastructural level with negative staining (Fig. 3).

In a few fortuitous instances in T_0 preparations, remnants of the microfibrillar ribbon were observed along the longitudinal axes of bacteria (Fig. 4). Approximately 50 fine projections, which appear wavy and may be helical, emerge from the cell surface. The projections appear to be integrated into an accumulating ribbon, running parallel with the bacterial axis. These projections pass towards one pole. In the case illustrated, it seems probable that the microfibrillar ribbon had been broken close to the polar region during the separation process described above. We suppose that the lateral stress that broke the microfibrillar ribbon placed stress on the attachment sites along the longitudinal axis, resulting in a tearing from these sites of the forming (nascent) microfibrils that constitute the ribbon.

From electron micrographs of negatively stained ribbons in various states of disaggregation, it was deduced that intact ribbons are composed of approximately 46 microfibrils, each about $1.6 \times 5.8 \mu\text{m}$ and associated in two layers such that the narrow axes of the microfibrils lie parallel with the ribbon thin axis (Fig. 6). All ribbons examined were of a constant uncoiled width of $133 \mu\text{m}$. In the cases illustrated, there is close correspondence between the numbers of projections from the bacterial surface (50) and the number of microfibrils making up the ribbon (46). Large bacterial cells about to divide and with an incipient furrow had two ribbons extending towards one pole.

In T_5 cultures, fractured attachment sites illustrated in Fig. 4 were not found. Instead, masses of coiled lateral projections were commonly found at the termini of ribbons, at a distance from the bacteria to which the growing ribbons were attached (Figs. 5 and 7). Those parts of ribbons associated with these

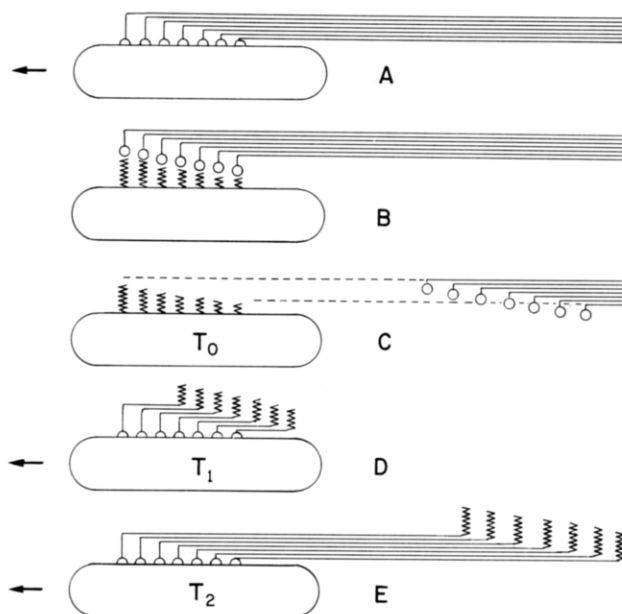


FIG. 15. Schematic diagram of the supposed events occurring during isolation of bacteria from the pellicle and subsequent regrowth of cellulose. (A) Bacterium attached to microfibrillar ribbon in pellicle. (B) Shearing of bacterium from pellicle results in tearing of elements of synthetic sites, forming coiled lateral projections. (C) Separation of pellicular ribbon from lateral projections. (D and E) Regrowth of the ribbon results in coiled lateral projections' being separated from bacterial surface.

lateral projections always tapered to points (Fig. 7), and the lengths of the lateral projections (and therefore the tapers) were always similar to the pattern observed along the lengths of the bacterium shown in Fig. 4. The ends of ribbons usually tapered in cases where lateral projections were absent. We interpret these results as indicated in the diagram (Fig. 15).

Freeze Etching. Having determined, using negative staining, that the ribbon is subdivided into microfibrils which have independent termini along the length of the bacterial envelope, we looked for further structural information using freeze etching of never-dried, untreated cells. Fractures usually occurred within the outer lipopolysaccharide membrane, as demonstrated by rare cases in which the inner plasma membrane was also fractured (Fig. 10). In many cases, distinctive rows of particles extending most of the length of the bacterium were found on both EF and PF (see ref. 12 for freeze-etching nomenclature) fracture faces of the outer membrane (Figs. 11 and 12). In cases in which a microfibrillar ribbon was associated

FIGS. 1–14 (on preceding page). Fig. 1. Darkfield light micrograph of T_5 aqueous culture showing growing microfibrillar ribbons attached to bacteria, $\times 1250$. Fig. 2. Darkfield light micrograph of T_{15} aqueous culture demonstrating a forming pellicle. Microfibrillar ribbons are attached to two of the visible bacteria, $\times 2000$. Fig. 3. Negatively stained bacterial cells which have recently formed a pellicle, $\times 9300$. Fig. 4. T_0 aqueous culture, showing an unusual example of a partially sheared ribbon and points of microfibrillar attachment to the bacterial envelope (arrows). Uranyl acetate negative stain, $\times 38,100$. Fig. 5. T_5 aqueous culture. Growth of the microfibrillar ribbon has separated the coiled lateral projections formed by shearing (right) from the bacterium (B). Uranyl acetate negative stain, $\times 18,000$. Fig. 6. Negatively stained microfibrillar ribbon. Note twisting of the ribbon (left) and sub-division into microfibrils in the untwisted section (right), $\times 81,100$. Fig. 7. Wavy lateral projections of Fig. 5. Note that the ribbon (arrow, left) thins progressively towards the far tip, $\times 30,900$. Fig. 8. Glutaraldehyde/tannic acid-fixed thin section of the bacterial envelope, showing plasma membrane (A), outer lipopolysaccharide membrane (B), and surface layer (C), $\times 153,750$. Fig. 9. Glutaraldehyde/tannic acid-fixed thin transverse section showing the close relationship between the ribbon (center) and the surface layer of the envelope, $\times 111,000$. Fig. 10. Freeze-etch micrograph showing external surface revealed by etching (large arrow), fracture face of outer lipopolysaccharide membrane (F), and fracture face of plasma membrane (small arrow), $\times 18,000$. Fig. 11. Freeze-etch micrograph showing typical row of particles on "PF" fracture face of outer lipopolysaccharide membrane (arrows) and outer surface of bacterium revealed by etching (ES), $\times 39,600$. Fig. 12. Fractured EF face of outer lipopolysaccharide membrane showing distinctive row of particles, $\times 91,600$. Fig. 13. Freeze-etch micrographs showing PF fracture face of outer lipopolysaccharide membrane, and demonstrating the linear relationship between the microfibrillar ribbon (MR) and the row of particles on the outer membrane fracture face (large arrow). Note possible plastic deformation of particles within the row and of other particles within the membrane (small arrows), $\times 46,900$. Fig. 14. Microfibrillar ribbon lying on etched surface (ES) of a bacterium. Note the tendency of coiling of the rolled ribbon, $\times 79,600$.

with the etched, outer surface of this membrane, the linear axes of the rows of particles and ribbons coincided (Fig. 13). Ribbons revealed by ice-etching (Fig. 14) were equivalent to those observed in negatively stained preparations. Frequently, the appearance of both the particles constituting the axial row and other particles in the outer membrane suggested plastic deformation (Fig. 13).

Thin Sections of Fixed Cells. Sectioned material fixed with tannic acid to improve the preservation of soluble proteins (13) revealed that the cell envelope consists of an electron-transparent periplasm, an electron-dense lipopolysaccharide layer, and an encompassing electron-dense granular layer (Fig. 8 and 9). Cross sections through the rod show the microfibrillar ribbon to be tightly associated with the outer granular layer (Fig. 9).

DISCUSSION

It is clear from these investigations that: (i) growth of microfibrils occurs at the cell surface; (ii) there is one line of structures, axially arranged, within each bacterial envelope which must be associated with the forming ends of microfibrils; (iii) 50 or so microfibrils generated in a row by each bacterium associate close to the surface of the bacterial envelope to constitute a ribbon which extends from the cell; and, (iv) the ribbon grows at a rate of $2 \mu\text{m min}^{-1}$ for an indeterminate length to form the familiar pellicle at the air-liquid interface.

Cooper and Manley (2-4) have shown that several of the enzymes necessary for the synthesis of the uridine diphosphoglucose (UDPG) precursors of cellulose lie within the bacterial envelope, and they suggest that a carrier molecule is synthesized there. The structures that we have observed may represent these enzymes. That there is a direct relationship between these structural features and the microfibrils constituting the cellulose ribbon implies that the final stages of microfibril synthesis occur very close to these intermediate enzymes. We suggest that the enzymes are locked together to form an enzyme complex.

Our interpretation of the results, although indicated by recent studies (1-4), is at variance with the interpretations of several preceding studies [see reviews by Colvin (5, 6)] that cellulose microfibril synthesis occurs at a distance from the bacterium. It seems possible that ethanol extraction (7, 14) removed precursors from their *in vivo* sites, leading to the conclusion that the experimental conditions are equivalent to *in vivo* conditions. The results are also at variance with the electron microscopic observations of Millman and Colvin (15). We suggest that the collodion solvent used in making the pseudo-replicas used in their study may have produced unanticipated changes. It seems clear from our studies that the sites of attachment of the microfibrillar ribbons to bacteria are readily damaged. Remarkably, some of our observations had been made before, but without their significance having been realized. For instance, the coiled lateral projections produced by tearing microfibrils from the bacterial surface are clear in earlier micrographs (5, 15, 16). The substructure of the bacterial ribbon observed in this study is very similar to that described by Ohad and coworkers (16).

Interesting comparisons of the rate of cellulose synthesis can be made. On the basis of the data of Cooper and Manley (3), we calculated the rate of glucose incorporation into cellulose produced by intact cells (based on radioactive tracers) to be 2.7 amol/hr per cell for their experiments. Calculations from our direct measurements of microfibrillar growth indicate 472 amol glucose converted/hr per cell, a value approximately 174 times greater. Since this report provides *in vivo* data based on direct

observation of the amount of *product* formed, it seems logical to assume that the methods of measurement based on glucose uptake and conversion into cellulose would yield lower values, particularly if the metabolism has a capacity to shunt some of the feeder glucose into intermediates and compounds other than cellulose (17). Surprisingly, the early studies on rates of cellulose synthesis by Hestrin and Schramm (18) indicated rates of synthesis comparable to our data (250 amol glucose assimilated into cellulose/hr per cell) even though these data were based on incorporation of radioactive glucose into cellulose. The discrepancy between the results of Cooper and Manley (3) and Hestrin and Schramm (18) cannot be elucidated at present, but our rate of glucose assimilation into cellulose represents a conservative estimate of the highest reported rate for this organism.

In a recent work on the basis of freeze-etch ultrastructure of never-dried cells, Leppard and co-workers (19) suggested that there is an amorphous sheath around forming microfibrils. We found no such sheath and suggest that the influence of the carbon backing film and of undissolved organic material in their replicas, as discussed at length in similar studies (20, 21), may have been ignored by these workers. Consequently, there can be reasonable doubt that these investigators were observing nascent stages of cellulose biosynthesis in the form of an intermediate polymer; however, we do not rule out the possibility that such a polymer, or at least a modified form of cellulose, can be synthesized by *Acetobacter xylinum*. Preliminary studies in our laboratory have confirmed the presence of band-like material synthesized radially from the longitudinal complex under semi-anaerobic conditions when glucose was supplied in place of the deionized incubation medium. It would be interesting to characterize this product more fully and compare it with the ribbon of cellulose microfibrils which we believe is the *in vivo* product of unaltered aerobic metabolism leading to the surface pellicle.

One of the most interesting cytological observations is the mode of ribbon production at cell division. Preliminary observations suggest that the long row of microfibril-synthesizing sites is longitudinally duplicated prior to division, and that both parent and daughter sites are active just before division. The two sites are intercepted at cell fission, and each daughter cell receives a comparable set of synthesizing complexes which does not extend in length and which synthesizes a microfibrillar ribbon of constant dimensions.

In conclusion, it seems worthwhile to compare the process of cellulose biosynthesis in *Acetobacter xylinum*, a prokaryote, with protistan and eukaryotic cell types. In the unicellular algae *Oocystis* (22) and *Glaucozystis* (23), terminal linear complexes synthesize cellulose microfibrils through the complexes' mobility within the plane of the fluid plasma membrane. A similar situation exists for higher plant cells, as recently demonstrated in corn root by Mueller *et al.* (24), and most probably in growing cotton fibers (25). An extreme case exists in which cellulose is synthesized within the restricted space of differentiating Golgi cisternae prior to exocytosis (26, 27). In the bacteria (as exemplified by *Acetobacter xylinum* in the present study) the synthesizing site appears to be *external* to the plasma membrane, and it is *stationary* with respect to the cell surface. Consequently, the propulsive force of the observed cell movement is created by the directed polymerization and crystallization of the ribbon of cellulose microfibrils along the longitudinal axis of the cell; the resulting propulsion is akin to unilateral cell movement caused by unidirectional slime secretion in some algal cells, for instance, *Micrasterias* and *Hormotillopsis*.

From the scant evidence presently available, it is interesting to speculate on the evolution of the systems necessary for the synthesis of cellulose. The systems may have begun extracellularly, subsequently locating in enclosed space exterior to the plasma membrane (the bacterial envelope), then moving to the plasmalemma of eukaryotes with specialization of orienting and mobilizing processes, and eventually residing in the endomembrane system, where highly specialized unit structures (scales) can be fabricated.

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