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THE BIOSYNTHESIS OF CELLULOSE

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ABSTRACT

Cellulose is one of the major commercial products of Sweden and constitutes the most abundant of the natural polymer systems. Thus, it is of interest to review the molecular design and architecture of cellulose with particular reference to the controls of its biosynthesis. The bioassembly process is highly ordered and structured, reflecting the intricate series of events which must occur to generate a thermodynamically metastable crystalline submicroscopic, ribbonlike structure. The plant cell wall is an extremely complex composite of many different polymers. Cellulose is the "reinforcing rod" component of the wall. True architectural design demands a polymer which can withstand great flexing and torsional strain. Using comparative Hydrophobic Cluster Analysis of a bacterial cellulose synthase and other glycosyl transferases, the multidomain architecture of glycosyl transferases has been analyzed. All polymerization reactions which are processive require at least three catalytic sites located on two different domains. In contrast, retaining reactions with glycosyl transferases require only a single domain and two sites. Cellulose synthase appears to have evolved a mechanism to simultaneously bind at least three UDP-glucoses and to polymerize, by double addition, two UDP-glucoses in such a manner that the 2-fold screw axis of the β-1,4-glucan chain is maintained. Thus, no primer is required as the glucose monomers are added two-by-two to the growing chain. At the next higher level of assembly, the catalytic sites simultaneously polymerize parallel glucan chain polymers in close proximity so that they will favorably associate to crystallize into the metastable cellulose I allomorph. Recent energy analysis suggests that the first stage of this associ-

ation is the formation of a minisheet through van der Waals forces, followed by layering of these minisheets to form the crystalline microfibril. In native cellulose biogenesis, the microfibril shape and size appear to be determined by a multimeric enzyme complex (TC) which resides in the plasma membrane. This complex, known as a terminal complex, was discovered through electron microscopy of freeze fracture replicas. The entire complex moves in the plane of the fluid plasma membrane as the result of polymerization/crystallization reactions. The assembly stages for native cellulose I are coordinated on a spatial/temporal scale, and they are under the genetic control of the organism. This might lead one to conclude that cellulose I could only be assembled with Nature's indigenous machinery; however, this is not the case. Recently, in collaboration with Professor Kobayashi and his colleagues in Sendai and Tokyo, we have synthesized cellulose I abiotically under conditions very different from those in the living cell or from isolated cell components. Purification of an endoglucanase from *Trichoderma* which serves as the catalyst and the addition of β-cellobiosyl fluoride as the substrate in acetonitrile/acetate buffer has led to the assembly of synthetic cellulose I. Although natural and synthetic assembly pathways are very different, there are similar, underlying fundamental mechanisms common to both. These mechanisms will be discussed in relation to the more thermodynamically stable allomorph of cellulose (cellulose II) first demonstrated by Professor Rånby in 1952. The evolution of cellulose biosynthesis will be summarized in terms of the demands for maintaining optimal cellular environments to generate the complex macromolecular assemblies for cell wall biogenesis. Nature provides an exceptional model for cellulose biosynthesis that will lead us toward the biotechnological production of improved natural cellulose as well as synthetic cellulose and its derivatives.

INTRODUCTION

We have gathered in Stockholm to honor Professor Bengt Rånby, a colleague whose long and productive career has traversed the domain of polymer science. Professor Rånby's significant contributions in the field of polymer chemistry and more specifically his work on the structure of cellulose, support the idea that a topic covering the biosynthesis of cellulose is appropriate for the theme of this symposium, "Nature as a Model of Molecular Design of the Polymeric Materials of Tomorrow." In terms of the massive quantity of natural polymer biosynthesis, we know that *Nature is alive and well* with respect to cellulose. This biopolymer is the most abundant macromolecule on earth and is synthesized by plants, fungi, algae, bacteria, and several animals (Table 1).

Although cellulose is polymerized from a simple sugar, glucose, its biosynthesis is complex and only incompletely understood. In this presentation the molecular architecture of cellulose will be introduced, followed by an update on the details of its natural biosynthesis. A comparison of native and synthetic cellulose production will be made, followed by a discussion of evolutionary implications and recent

TABLE 1. Cellulose Found among Living Organisms. Several Representative Genera Which Have Been Studied for Cellulose Biosynthesis are Included

Prokaryotic organisms:
- Gram-positive anaerobic bacteria *Sarcina*
- Purple bacteria *Acetobacter*, *Rhizobium*, *Alcaligenes*, *Agrobacterium*

Eukaryotic organisms:

A. Photosynthetic organisms [66]
- Chlorophyta (green algae) – *Oocystis apiculata*, *Valonia*, *Boergesenia*, *Chara*, *Mougeotia*, *Coleochaete*
- Charophyta (stoneworts) – *Chara*, *Nitella*
- Phaeophyta (brown algae) – *Pelvetia*
- Chrysophyta (yellow-green, golden-brown algae, and diatoms) – *Vaucheria*, *Pleurochrysis*
- Rhodophyta (red algae) – *Erythrocladia*
- Vascular plants:
  - Mosses, liverworts, ferns, angiosperms, gymnosperms, etc. – *Funaria*, *Arabidopsis*, *Zea mays*, *Gossypium*, *Pinus*, *Phaseolus*

B. Nonphotosynthetic organisms:
- Protists – *Dictyostelium discoideum*
- Fungi – *Saprolegnia*, *Allomyces*, *Achlya*
- Animals:
  - Tunicates – *Metandrocarpa*, *Hyalocynthia* [67]
  - Humans – (associated with the disease scleroderma) [68]

molecular genetics research, ending with perspectives on future research directions in the field of cellulose research.

THE MOLECULAR ARCHITECTURE OF CELLULOSE

What is "cellulose?" In the past, confusion and misunderstanding have prevailed when the definition of "cellulose" was considered. In 1839, Anselme Payen, a French industrialist and chemist [1], coined the term "cellulose" to describe an acid-resistant substance obtained by treating wood with nitric acid. Payen found that irrespective of its origin, cellulose had the same chemical composition ($C_6H_{10}O_5$). The modern definition of cellulose must take into account not only the *composition* of the polymer but also its *linkage, molecular weight, and crystalline arrangement* of the individual polymer chains. The chemical composition of cellulose is simple: It is a homopolymer consisting of glucose monomers linked β-1,4 (Fig. 1).

To be defined as cellulose, the molecular weight of the glucan chains must be at least 30–40 kDa (i.e., the polymer must contain at least several hundred glucose residues). β-1,4-Glucans with a degree of polymerization greater than 6–8 will not remain in solution. The mechanism of glucan chain aggregation into the "insoluble" product is of great importance in understanding the diverse physical properties of cellulose. On one extreme, glucan chain aggregations result in a highly crystalline

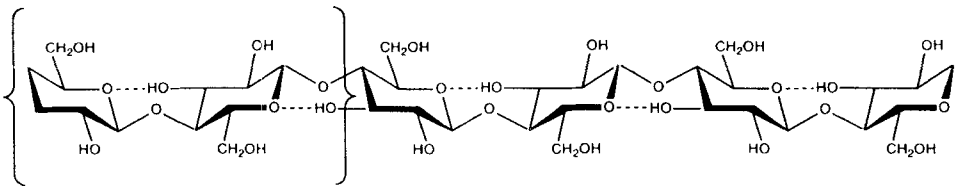

FIG. 1. Structural formula for cellulose, showing the β-1,4 linkages. Note that cellobiose (bracketed) is the basic repeating unit. The polyglucan chain exhibits a 2-fold screw chain axis, with 180° rotations for each monomer. (Courtesy Dr. Susan Cousins.)

product, and at the other, a rather disordered, "amorphous" product [2]. The percent crystallinity is an index of the quantity of crystalline components within the insoluble cellulose.

Native cellulose, representative of more than 99% of all living organisms which synthesize it, exists as a crystalline allomorph designated *cellulose I*. This allomorph is the thermodynamically metastable form of cellulose, first demonstrated by Professor Rånby in 1952 [3–5]. It is important to note that the term "metastable" is being considered not in the enzymatic sense or in relation to truly short-term transition states, but rather in a global, thermodynamic perception. Obviously, cellulose I is quite "stable" in the ordinary environment; however, it can be converted to cellulose II. No matter how cellulose II is treated (even through its complete oxidation to CO_2 and H_2O), it can never be converted into any other allomorph. It is in this concept that the term "metastable" will be employed to describe cellulose I. Cellulose I consists of extended parallel glucan chains [6], and these have been imaged using the transmission electron microscope (Fig. 2).

Two suballomorphs of cellulose I have been described [7] (Fig. 3). Cellulose Iα has a triclinic unit cell with a single chain, whereas cellulose Iβ has a 2-chain monoclinic unit cell [8]. Ordinarily, cellulose I has a high molecular weight, in some cases approaching a DP_w of 23,000 for the alga *Boergesenia forbesii* [9].

Morphologically, cellulose I exists in the form of submicroscopic rods known as "microfibrils" (Fig. 4). Note in Fig. 2 that the ordered arrangement of the glucan chains occurs throughout the entire structure. Presumably, the microfibril is free from surface imperfections, paracrystalline regions, or a crystalline core as suggested by the fringe micelle concept. Microfibrils often can be tens of micrometers in length, and their shape and size appear to be governed by the genetic makeup of the organisms which synthesize them. Cellulose microfibrils are the major "reinforcing rods" of the cell wall (Fig. 4), and the "glue" which provides further strength and plasticity comes from xyloglucans, pectins, proteins, and lignin. Microfibrils often are deposited in a highly oriented pattern. Microfibril orientation is a major controlling factor in determining cell shape and differential growth [10].

Cellulose II (Fig. 3), on the other hand, is synthesized naturally by only a few organisms such as the gametophyte cells of the marine alga *Halicystis* [11], the gram positive bacterium *Sarcina* [12], or mutants of the gram negative bacterium *Acetobacter xylinum* [13]. The glucan chains in cellulose II are antiparallel, and the presence of an extensive intersheet H-bonding confers the greatest thermodynamic stability for this allomorph.

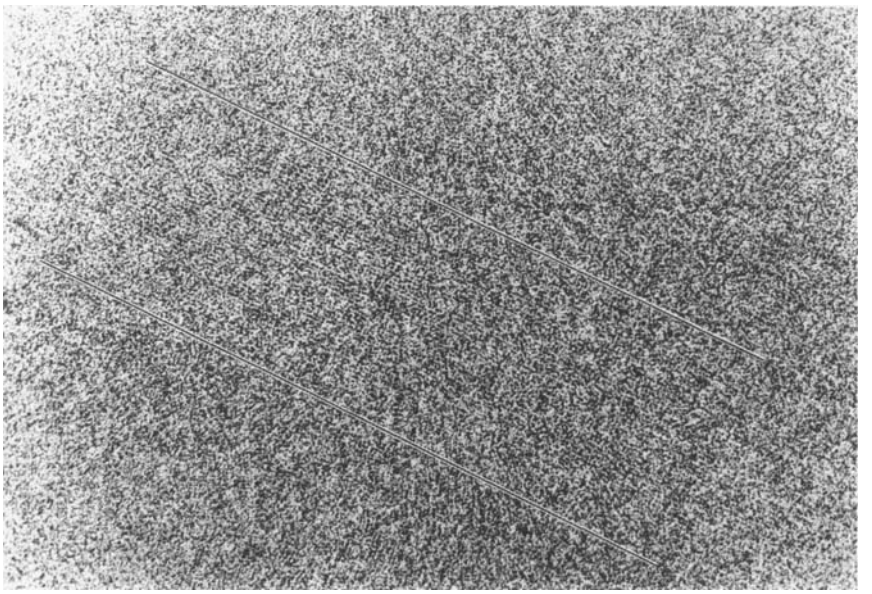

FIG. 2. Lattice image of cellulose I microfibril of the alga, *Boergesenia*. This microfibril is rectangular in cross section, thus its wide axis is shown binding to the substrate surface of the TEM grid. This same orientation occurs on the cell surface. Note the 0.53 nm spacings of the glucan chains in this projection. The boundary of the microfibril is indicated. Note the perfect crystalline arrangement throughout the entire microfibril. (Courtesy Dr. Shigenori Kuga.)

Cellulose II is mostly widely known as a synthetic product made from native cellulose I and is frequently called regenerated or hydrate cellulose. The native cellulose I allomorph can be dissolved and reprecipitated by a process known as mercerization [14]. During this process the cellulose crystallizes into the more thermodynamically stable allomorph known as cellulose II [3–5]. Common commercial products of cellulose II include cellophane and rayon. Cellulose II is characterized by a lower molecular weight (degree of polymerization of several hundred) and a greater affinity for dyes. One of the major goals in the industrial application of dissolved celluloses is to reproduce natural cellulose I of Nature or its equivalent physical properties. New approaches in the use of cellulose solvents and wet-spinning have led to a number of remarkable synthetic fibers with many exciting and interesting properties [15].

We have defined cellulose, and from this diverse and oftentimes seemingly complicated delimitation it is evident that a variety of methods are required to properly identify this biopolymer. These include: (a) complete hydrolysis yielding only glucose; (b) insolubility in NaOH; (c) methylation analysis verifying 1,4 linkage; (d) enzymatic degradation by cellulases; (e) electron microscopic observation of microfibrils and rodlets; (f) NMR, Raman, and IR spectroscopy; and (g) x-ray and electron diffraction analysis. We have also briefly reviewed the various architectural forms of cellulose. How does Nature assemble these nanostructures?

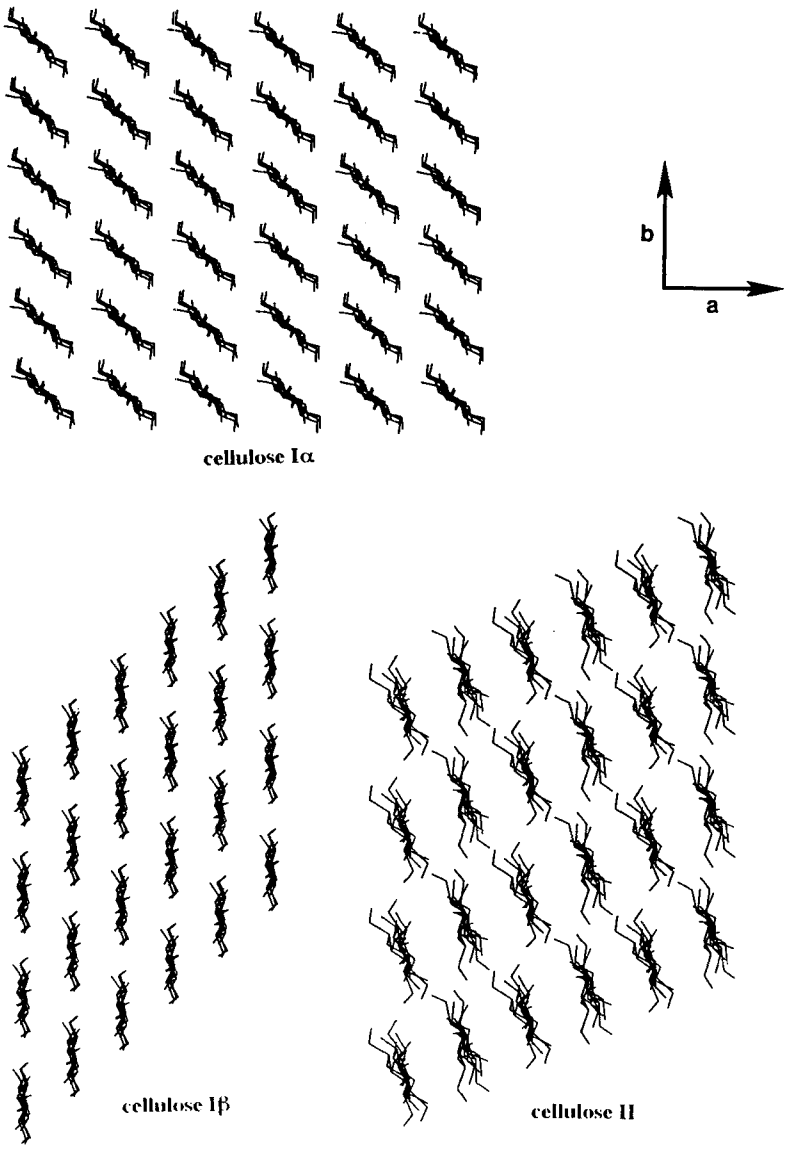

FIG. 3. Crystal packing of cellulose Iα, cellulose Iβ, and cellulose II allomorphs, showing arbitrary face planes. Cellulose Iα follows the Aabloo and French model [63] which uses the indexing of Sugiyama et al. [25]. Cellulose Iβ follows the model of Woodcock and Sarko [64], and cellulose II follows the Stipanovic and Sarko model [65]. (Courtesy Dr. Susan Cousins.)

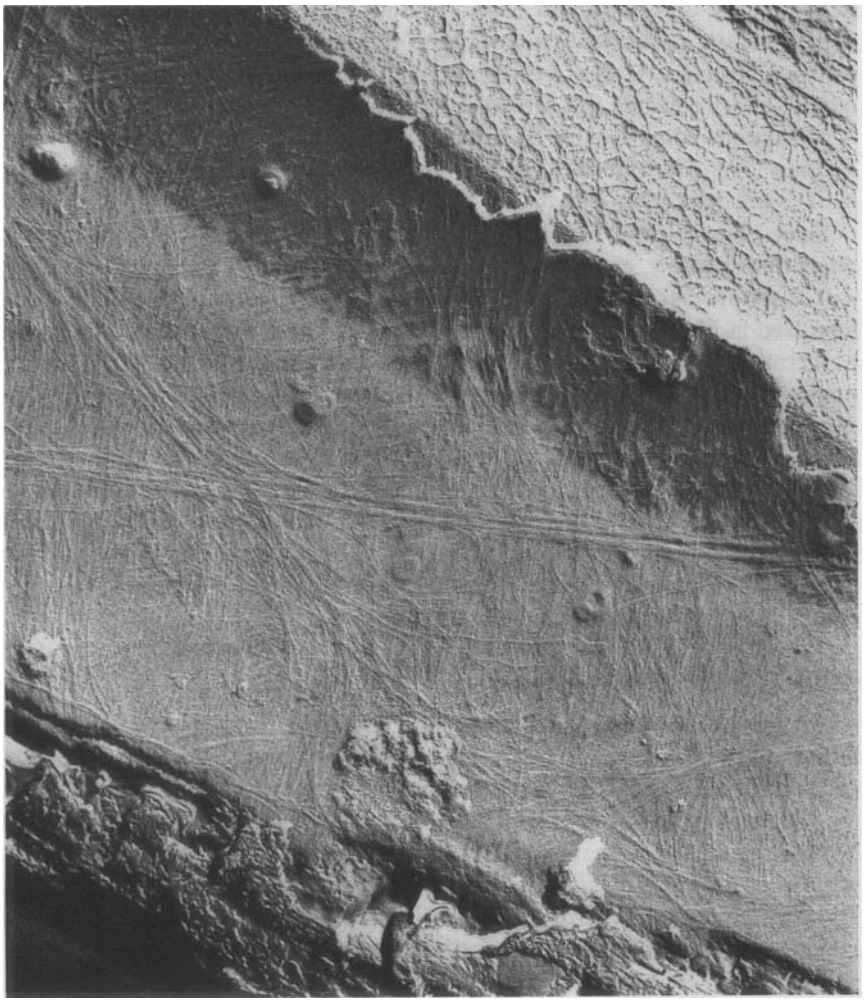

FIG. 4. Freeze fracture of a cell wall from a growing root of radish (*Rhapanus sitava*). Note the prominent twisting of microfibrils, characteristic of cellulose synthesized by land plants. (Courtesy Kay Cooper.)

THE BIOSYNTHESIS OF CELLULOSE I BY THE TERMINAL COMPLEX—NATURE'S ASSEMBLY MACHINE

In 1958 Roelofsen [16] first proposed that the assembly of a cellulose microfibril must be under the precise control of an enzyme complex located at the growing tip of the microfibril. In 1964 Preston [17] proposed that this structure must have a high degree of organization because he had observed highly ordered membrane-associated structures with freeze fracture. On this basis and even though Preston had no direct evidence for any ordered structures in cellulose synthesis, he proposed the *Ordered Granule Hypothesis* which stated that the intrinsic order of the subunits within the complex should dictate the assembly and orientation of cellulose microfi-

brils. It was not until 1976 that Brown and Montezinos first observed this highly ordered structure using the freeze-fracture technique. We found a highly linear, rod-shaped structure at the growing tip of the microfibril in the alga *Oocystis apiculata*, and we named this structure a "terminal complex" or "TC" [18]. Three rows of subunits comprise the TC in *Oocystis*. Other similar TCs have been found in *Valonia* [19], *Boergesenia* [20], and *Glaucocystis* [21]. Several organisms with linear TCs are depicted in Fig. 5.

Since 1976, research has shown TCs of differing morphologies to be present in a variety of organisms, and a wealth of information about TC geometry and microfibril architecture has become available [22–26]. The known TC morphologies are summarized in Fig. 6, the fundamental ones being *linear* and *rosette*. The relationships between TC geometry and microfibril architecture will now be explored through a discussion of selected organisms. Understanding this relationship requires a knowledge of how the number and proximity of catalytic sites on the TC subunit relate to minisheet formation, the first stage of cellulose crystallization. In the following examples, relationships between catalytic site density and minisheet formation will be presented, elements of which are conjectural; however, from the dimensions of a microfibril, one can obtain a reasonably close estimate of the number of glucan chains present. Then the task becomes to determine how many glucan chains form a minisheet.

Specific TC Examples: *Vaucheria*

An unusual linear TC found in the alga *Vaucheria* consists of rows of subunits arranged diagonally (Fig. 5B) [27, 28]. The offset of the microfibril axis with respect to the TC strongly suggests that primary aggregation of the glucan chains occurs via the diagonal rows of subunits (Fig. 7). Each subunit is postulated to have only one catalytic site. Groups of three subunits in a diagonal row participate in the formation of a minisheet, and four minisheets from a single diagonal row associate to form a minicrystal. Microfibril width is governed by the number of diagonal rows, each row contributing one minicrystal to the microfibril.

Specific TC Examples: *Erythrocladia*

One of the most exceptional examples of the relationship of TC geometry to the architecture of the cellulose synthesized is from the exquisitely organized TCs of the rhodophycean marine alga *Erythrocladia* [29]. In these TCs there is a precise arrangement of four rows of subunits (Fig. 5C). As with *Vaucheria*, each subunit is postulated to have only a single catalytic subunit. The microfibril synthesized by this TC is very thin (only about 1.5–2.0 nm) Microfibril thickness is constant, but microfibril width is variable. This variability correlates with change in TC length. In *Erythrocladia* the glucan chain minisheet is first assembled as a product of the four subunits of the transverse row (Fig. 8). As each row of transverse subunits adds minisheets to the microfibril, its width is increased.

Specific TC Examples: *Valonia*

Unlike *Vaucheria* and *Erythrocladia*, each TC subunit of *Valonia* is conjectured to have as many as 10–12 catalytic subunits (Fig. 9). The linear TC of *Valonia* with its three rows of subunits has the same plan for microfibril assembly as *Eryth-*

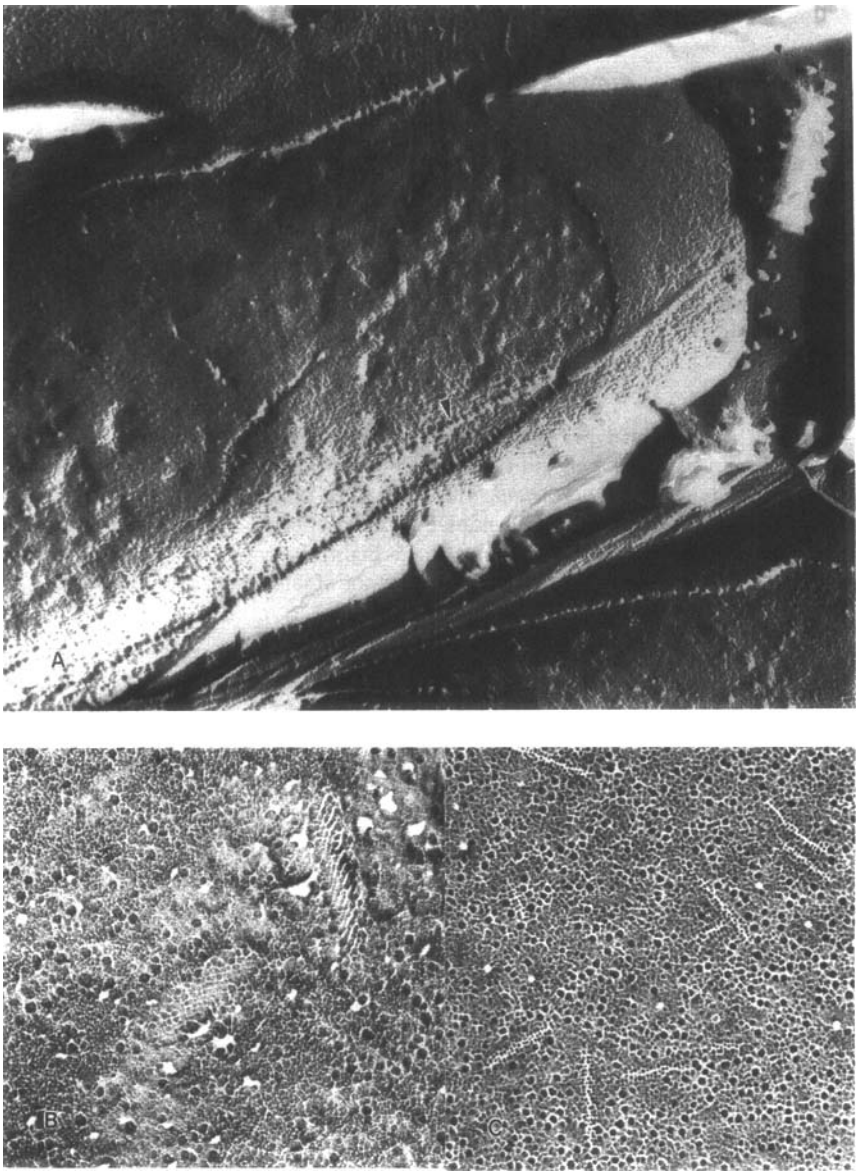

FIG. 5. Freeze fracture replicas of TCs from three different organisms. (A) an *Acetobacter* cell showing a ribbon of microfibrils on the cell surface and a fracture through the outer membrane revealing the underlying linear TCs arranged in an orientation parallel to the cell's longitudinal axis (courtesy Professor Takao Itoh); (B) two TCs from *Vaucheria* showing diagonal rows of subunits (courtesy Professor Shun Mizuta); (C) TCs from the rhodophycean alga, *Erythrocladia*, showing the transverse rows, each with 4 subunits. (Courtesy Dr. Kazuo Okuda.)

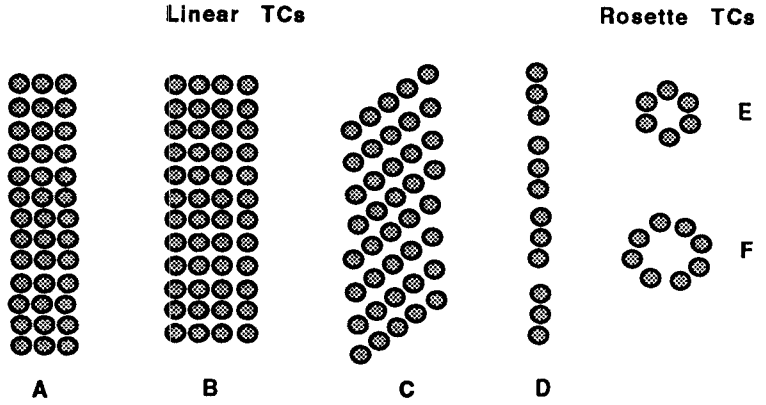

FIG. 6. The major types of TCs found in living organisms. Classes A–D indicate the linear TC. Transverse rows typical of *Valonia*, *Boergesenia*, and *Oocystis* are shown in A. Transverse rows typical of *Erythrocladia* are shown in B. Diagonal rows typical of *Vaucheria* are shown in C. Classes E–F are representative of the rosette TC. Sixfold symmetry typical of all major land plant rosette TCs is shown in E. Eightfold symmetry characteristic for an advanced alga, *Coleochaete*, believed to be an ancestor of land plants, is shown in F.

rocladia; however, the product of a transverse row of three subunits is a massive minisheet of more than 30 glucan chains. It is surmised that the three glucan chain minisheets do not aggregate to form a minicrystal in this early stage, but instead these minisheets become arranged end-to-end to form a single, giant minisheet. As in *Erythrocladia*, each succeeding transverse row of three subunits adds another giant minisheet. The result is a highly crystalline microfibril with more than 1000 glucan chains [30]. It should also be noted that the isolated *Valonia* microfibril does not undergo twisting as is typical for the microfibrils of *Vaucheria* and *Erythrocladia*.

Variations on this theme are found in the related alga, *Boergesenia*. Here the TC subunit is hypothesized to contain fewer catalytic sites, and the TC is longer. These alterations result in a microfibril which is rectangular in cross section, and one which is thinner than the microfibril synthesized by *Valonia* [31].

Specific TC Examples: *Acetobacter*

Unlike the TCs described above from eukaryotes, the TCs of the gram negative bacterium *Acetobacter* are fixed with respect to the cell surface. They consist of massive basket-shaped subunits which traverse the cell membrane, periplasmic space, and outer membrane (Fig. 15). A single cell of *Acetobacter* synthesizes a ribbon of 10–100 microfibrils (Fig. 5A). The ribbon is spun out into the culture medium, and the cellulose products of the entire culture combine to form a gelatinous membrane. Each TC subunit is basket-shaped, and when isolated from the cell, this structure can assemble cellulose I in vitro [32]. *Acetobacter* has been the most widely investigated model system for cellulose microfibril assembly [33–45]. Recent investigations of the effects of an optical brightener, Tinopal, on the crystal-

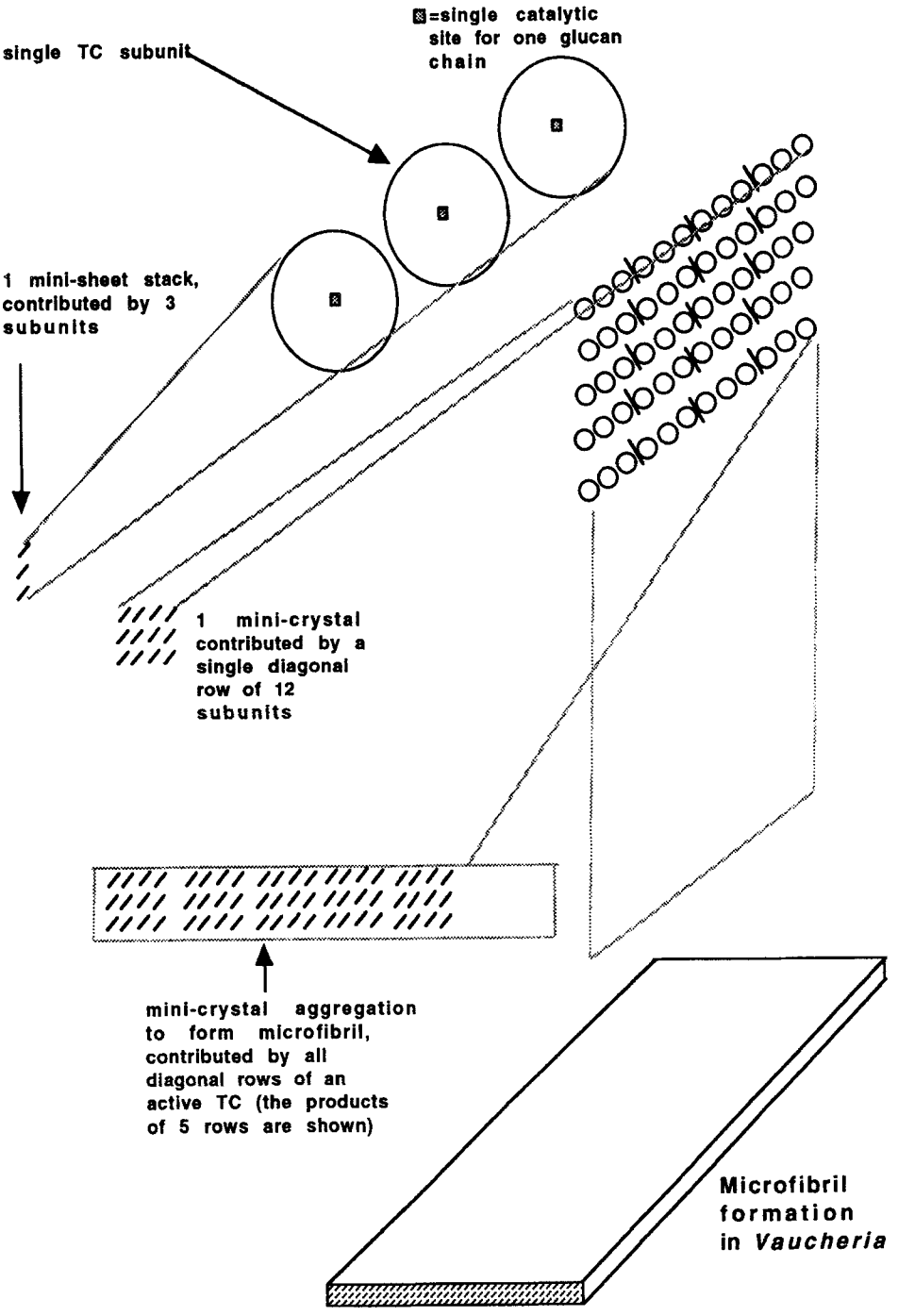

FIG. 7. Schematic representation of the geometry of the TC in relation to the size and shape of microfibril synthesized. In *Vaucheria* (the only known example of this type), a quasi-linear TC is found with unique diagonal rows of subunits. Each subunit is postulated to contain only one catalytic site for glucan chain polymerization. Within the diagonal row, three subunits contribute to a thin mini-sheet as depicted. Four sets of three subunits in one diagonal row are required to generate a mini-crystal. Mini-crystal addition is limited to and based on the number of diagonal rows of the TC.

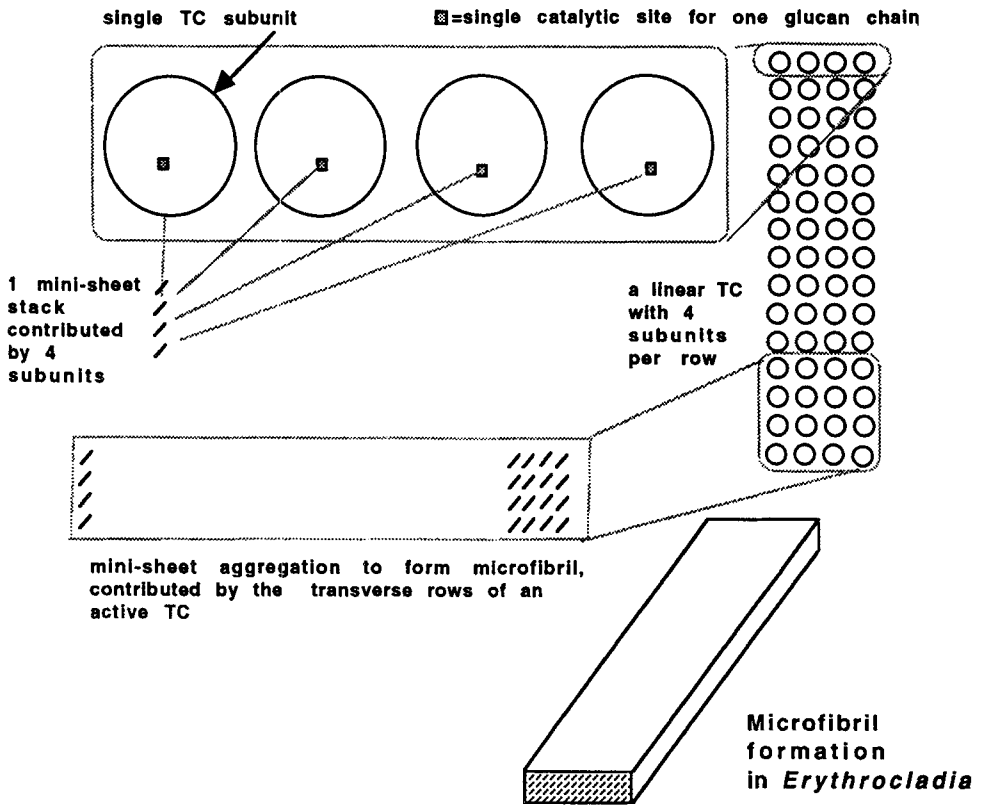

FIG. 8. Schematic representation of the geometry of the TC in relation to the size and shape of microfibril synthesized. In *Erythrocladia* a linear TC is found with transverse rows of subunits. Each subunit is postulated to contain only one catalytic site for glucan chain polymerization. Within the transverse row, four subunits contribute to a thin minisheet as depicted. The microfibril is generated by additions of minisheets based on succeeding transverse rows of subunits. Microfibril width is somewhat variable and is limited to and based on the number of diagonal rows of the TC.

lization patterns of cellulose have provided new clues to the stages of crystallization leading to a cellulose I microfibril [46]. When grown in the presence of high concentrations of the dye, *Acetobacter* synthesizes only glucan chain sheets [38]. Using molecular modeling, Cousins and Brown have shown that the initial stage of cellulose I crystallization occurs through van der Waals forces to generate a glucan chain sheet [47]. The molecular modeling conclusions are substantiated by observations of glucan chain sheets and associated Tinopal molecules. How does this relate to the model presented in Fig. 10? The catalytic sites within the basket-shaped structure assemble four minisheets which then emerge from the pore as a minicrystal. Minicrystals from adjacent TC subunits associate to form the metastable cellulose I microfibril. Within this context, a single TC of *Acetobacter* would consist of a minimum of three subunit baskets. The linear arrangement of the subunits parallel to the longitudinal axis of the bacterium insures not only minicrystal assembly

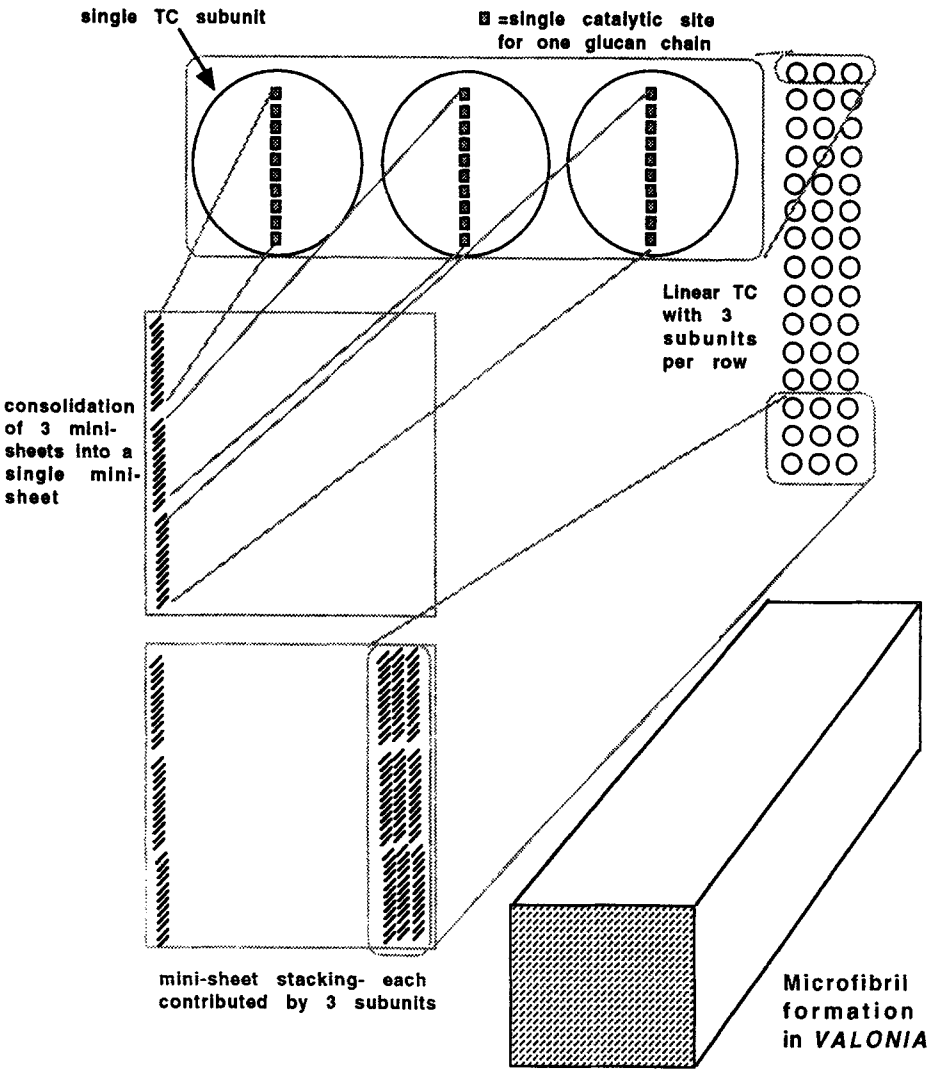

FIG. 9. Schematic representation of the geometry of the TC in relation to the size and shape of microfibril synthesized. In *Valonia* a linear TC is found with three transverse rows of subunits. Each subunit is postulated to contain multiple catalytic sites for glucan chain polymerization. Within the transverse row, three subunits contribute to a very large minisheet of at least 30 glucan chains as depicted. The microfibril is generated by additions of these large minisheets based on succeeding transverse rows of subunits. In *Valonia* the microfibril width and thickness are almost equal, and the TC length is relatively constant.

into a microfibril, but also close association and rather extensive intermolecular H-bonding between microfibrils to form the distinctive ribbon which propels the cell through the medium. Exactly why *Acetobacter* converts up to 45% of its glucose into cellulose is unknown; however, the encasement of the cells in a cellulosic membrane may provide protection and possibly even passive dispersal via wind.

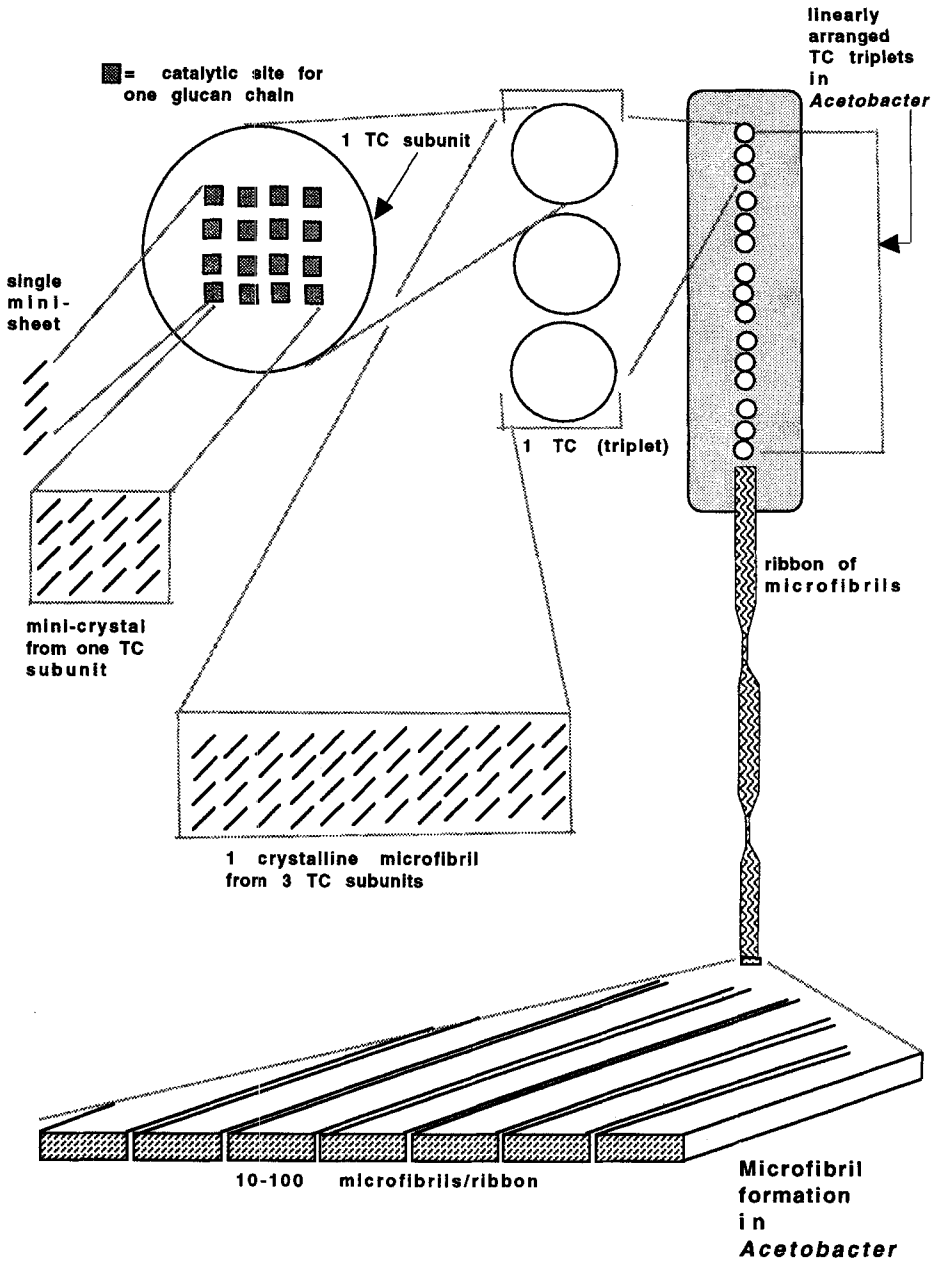

FIG. 10. Schematic representation of the geometry of the TCs in relation to the size and shapes of microfibrils and ribbon synthesized by the gram negative bacterium, *Acetobacter*. The linear TC consists of 3 subunits, and along the longitudinal surface of the cell are found numerous linearly arranged TCs. Each TC subunit contains at least 16 catalytic sites. These may be distributed among several or more protein subunits within a basket-like complex. At least 4 minisheets are made by one TC subunit. If dyes such as Tinopal are added

Williams and Canon [48] have hypothesized that the thick, gelatinous membrane may serve as a barrier to fungi, yeasts, and other organisms, giving the *Acetobacter* cells a competitive advantage to secure adequate nutrition.

Acetobacter is one of Nature's most eloquent examples of a hierarchical architecture required to assemble a massive cellulose composite ribbon. The first genes for cellulose synthase were cloned and sequenced from *Acetobacter*. Recent investigations in collaboration with Dr. Inder Saxena have given new insight into the functions of the polypeptides within the *Acetobacter* TC subunit. These will be described below.

Specific TC Examples: The Rosette TC of Higher Plants

In 1980 Suzette Mueller and Brown [49] discovered in *Zea mays, Phaseolus*, and *Pinus* a second major TC subunit geometry, the rosette. At the same time, Dr. Andrew Staehelin and his group independently discovered the same arrangement of TC subunits in the green alga *Micrasterias* [50]. In both cases the subunits are arranged with a 6-fold symmetry (Fig. 11). The rosette TC subunit as in *Valonia* has multiple catalytic sites. Each subunit synthesizes a sheet with six glucan chains. The sheets from the six subunits associate to form the microfibril which, in vascular plants, almost always undergoes twisting (Fig. 4).

The 6-fold rosette TC appears to be evolutionarily conserved among all land plants including angiosperms, gymnosperms, ferns, mosses, and liverworts. An advanced alga, *Coleochaete*, is believed to be an ancestor for land plants. This organism also has a rosette TC, but its symmetry is 8-fold [22]. Other advanced green algae, however, have rosettes with typical 6-fold symmetry that appear to be identical to those in land plants [23, 24].

Cellulose synthesized by rosette TCs in vascular plants is a mixture of cellulose Iα and Iβ suballomorphs, with a smaller proportion of Iα than found in organisms with linear TCs. It will be interesting to see if further research confirms this correlation between TC geometry (linear vs rosette) and the relative proportions of cellulose I suballomorphs synthesized. Nature's most cellulose Iβ-rich source is the tunicates [25], but so far the TCs for this organism have not been described. Perhaps something more subtle, such as the catalytic site density on the TC, may control the ultimate assembly of the suballomorph.

A COMPARISON OF SYNTHETIC AND NATIVE CELLULOSE SYNTHESIS

We shall now review synthetic cellulose assembly, concluding this section with a comparison of synthetic cellulose I formation and native cellulose I formation as well as in vitro cellulose I assembly. My involvement in research leading to the

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during synthesis, the crystallization patterns are altered at several levels, depending on the concentration of dye. For example, with low concentrations of dye, minicrystals are assembled, but they are prevented from aggregating into microfibrils. If higher concentrations of dyes are used [38], only glucan chain sheets are extruded from the subunit. These combine to form what is known as tubular cellulose which can later be converted into microfibrillar cellulose when the dye is removed either by washing [62] or by photoisomerization [46].

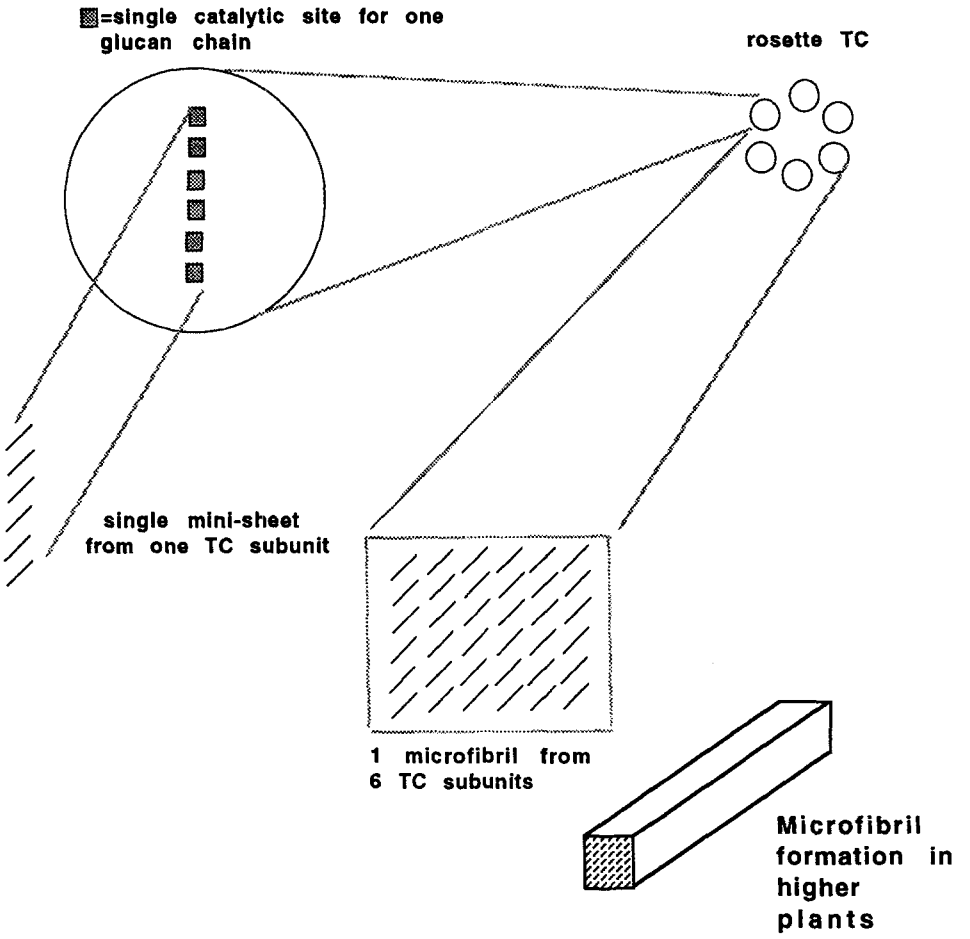

FIG. 11. Schematic representation of the geometry of the rosette TC in relation to the size and shape of microfibril synthesized in *Chara*, *Nitella*, ferns, mosses, gymnosperms, and angiosperms. The rosette TC has six subunits, and each subunit has at least six catalytic sites which produce a single minisheet. The association of minisheets assembled from adjacent subunits forms the microfibril.

assembly of synthetic cellulose I was prompted by Professor Rånby's suggestion to Professor S. Kobayashi in 1989 that he contact me (see the article by Kobayashi et al. in this issue). This led to a visit by Professor Kobayashi to my laboratory in April 1991. At that time we initiated a collaboration to continue the fruitful initial discoveries of abiotic cellulose II assembly [51]. One of my then graduate students, Jong Hwa Lee, was studying cellobiohydrolases using high resolution transmission electron microscopy, and he agreed to begin work on visualizing synthetic cellulose assembly as first reported by the Kobayashi Lab. This collaboration led to the first visualization of synthetic cellulose II assembly using cellobiosyl fluoride as the substrate in a mixed solvent of acetonitrile and acetate buffer [52]. Mr. Lee then undertook the task of determining which component of the crude *Trichoderma*

cellulase preparation was responsible for synthetic cellulose assembly. At the time, Dr. Shigenori Kuga from Tokyo University was visiting the lab, and he found, in addition to cellulose II in the products from Mr. Lee's partial purifications, a few isolated microfibrils of cellulose I. At first we were concerned that this cellulose might have been a contaminant; however, further purification and refinement of the acetonitrile/aqueous buffer ratios led to consistent and repeatable abiotic synthesis of synthetic cellulose I [53]. Recently, the cellulase responsible for synthetic cellulose I assembly has been purified to homogeneity [54]. It is a minor endoglucanase component from the *Trichoderma* cellulase mixture. Time/course TEM observations have revealed the progressive assembly of microfibrils of cellulose I from a reverse micelle (Fig. 12).

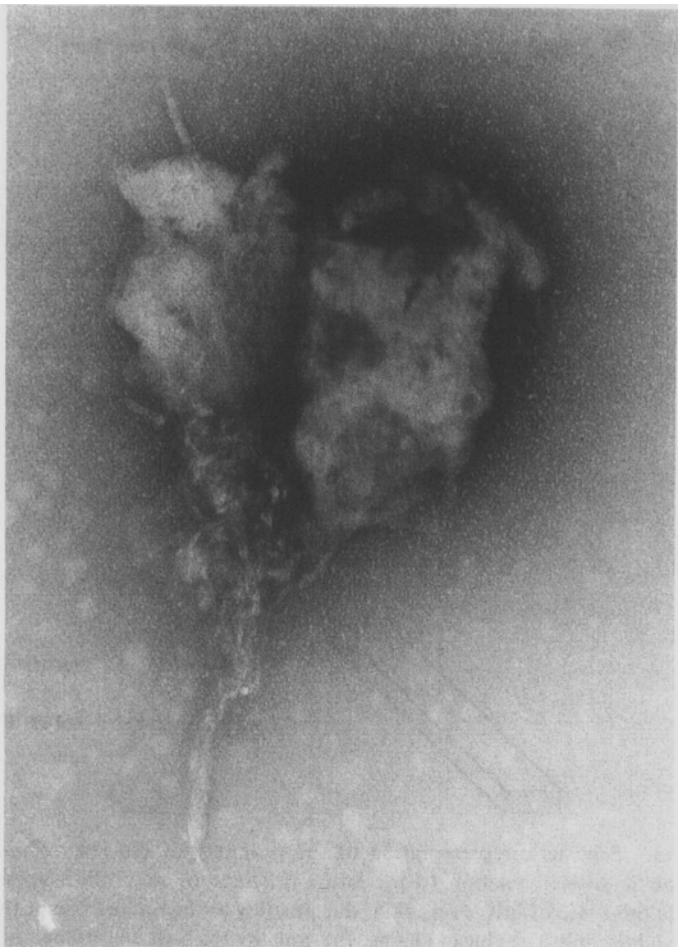

FIG. 12. Synthetic cellulose formation—A reverse micelle of partially purified endoglucanase with unidirectionally extruded cellulose I microfibrils approximately 3 minutes into the reaction after the addition of β-cellobiosyl fluoride. The microfibrils appear to originate within a depression of the micelle. (Negatively stained, TEM, 205,000×.) (Courtesy Dr. Jong Hwa Lee.)

A schematic representation of the process of synthetic cellulose assembly is presented in Fig. 13.

How is an abiotic assembly of the equivalent of Nature's metastable cellulose I allomorph possible? When the responsible enzyme has been purified to a greater degree, the catalytic site density at the surface of the micelle increases. These dense catalytic sites are preferentially ordered at the boundary of the micelle, presumably

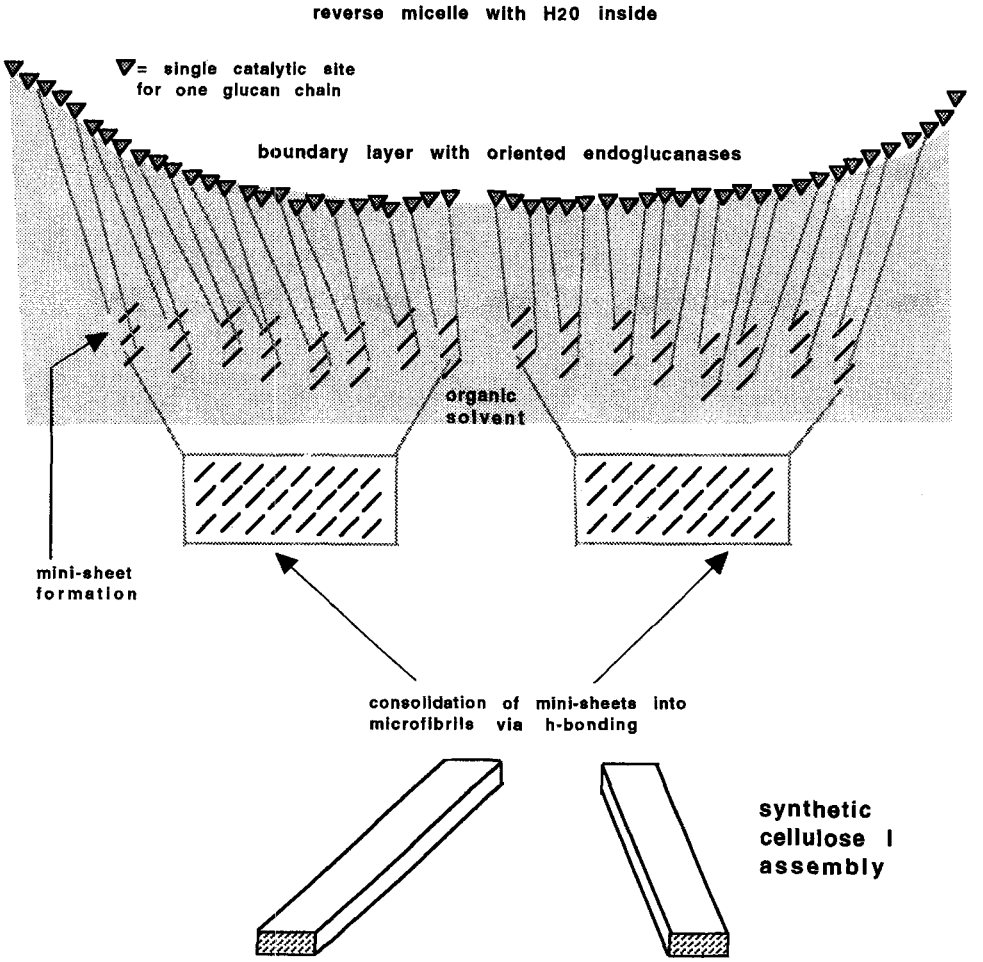

FIG. 13. Schematic representation of the geometry of the reverse micelle and its oriented catalytic sites in relation to the size and shape of microfibril synthesized. The synthetic cellulose I microfibril is very thin, due possibly to the limited size of the minisheets which may contain only 1-3 glucan chains. Presumably the tight clustering and orientation of endoglucanases at the micelle interface promote the assembly of minisheets. It is postulated that minisheet associations to form the microfibril relate to the overall distribution of endoglucanase clustering within the micelle. Perhaps cellobiose/cellobiosyl fluoride competition for the active site of the endoglucanase may also control or induce the polymerization reactions or the patterns of crystallization. It is known that very high purity β-cellobiosyl fluoride is required for synthetic cellulose assembly.

due to the amphipathic nature of the enzyme. The combination of tight packing and preferential orientation of catalytic sites increases the likelihood of a preferred direction of chain synthesis into the organic phase of the micelle. Presumably, the first stage of glucan chain association under these conditions is minisheet formation. Then the minisheets associate to form the thin microfibril. It is remarkable that metastable cellulose I has been assembled without "cell-directed self-assembly" which has been postulated as a prerequisite for cellulose synthesis in vivo (55). The reverse micelle could be thought of as a novel type of TC in that the same functions of catalytic site density and directed chain synthesis are being controlled by the artificial system. The major differences would lie in the degree of complexity of catalytic site distribution and geometry. In the TC there would be far greater complexity, thus leading to microfibrils of cellulose I with varying sizes and shapes.

Thus, we can now begin to think more specifically about cellulose I assembly, probably in the context of "self-assembly" as described by Haigler and Benziman in 1982 [55], but without the requirement for a living cell in directing the self-assembly process. Can cellulose I be assembled without the aid of an enzyme catalyst? If the endoglucanase in synthetic cellulose assembly functions primarily in bringing the monomers into close association for directed polymerization, it should then theoretically be possible to design a reactive surface to accomplish the same assembly, without the requirement of an enzyme. Obviously, a catalytic site is a prerequisite, and this site must not only direct the polymerization but also lower the energy of activation of adjacent glucan chains so that they will crystallize under strain.

Cellulose synthases of Nature are remarkably designed to accomplish these reactions as exemplified by the TC structures. The reverse micelle is a rather "feeble" TC structure but one which still contains highly ordered catalytic sites for directed polymerization. High resolution TEM has recently made possible the imaging of individual endoglucanase molecules in which a prominent open cleft for the reaction site can be visualized (Fig. 14). If these sites are not in close proximity, the more thermodynamically stable allomorph of cellulose II will be assembled.

EVOLUTIONARY IMPLICATIONS OF KNOWLEDGE GAINED FROM UNDERSTANDING SYNTHETIC CELLULOSE I ASSEMBLY

It appears that the principles underlying the synthetic assembly of a thermodynamically metastable crystalline polymer can now explain Nature's preference for a thermodynamically metastable crystalline biopolymer. Unless glucan chain order is maintained within a given threshold from the moment of polymerization until ultimate "lock-in" by crystallization, the thermodynamically more favorable form of crystalline cellulose (cellulose II) will be assembled. It will be recalled that the primitive bacterium *Sarcina* and only very few other organisms actually synthesize cellulose II naturally [56]. This suggests that during early evolutionary history of cellulose biogenesis, the cellular requirements for cellulose assembly centered on the polymerization reaction only, resulting in the preference for cellulose II assembly. Because cellulose II has a lower degree of polymerization and is composed of short rodlets of folded glucan chains or sheets, this crystalline form has certain limitations as a structural material in the cell wall. The so-called "matting" of rodlets in the cell wall would indeed confer some degree of protection; however, differential cell

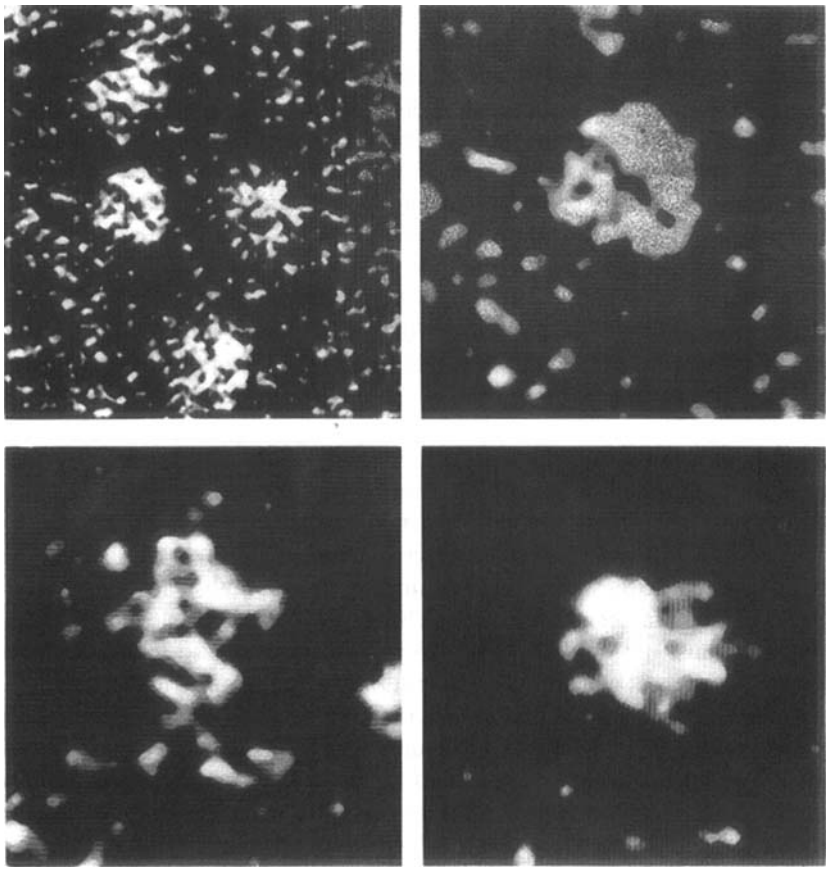

FIG. 14. Highly purified P-38 endoglucanase molecules imaged with high resolution TEM. Upper left shows a cluster of four molecules. Upper right shows an endoglucanase molecule with a single open cleft traversing the entire width of the molecule. Lower left shows the open cleft in transverse view. Lower right shows the open cleft on the opposite side of the molecule. (Courtesy Dr. Jong Hwa Lee.)

elongation and growth within the "hardened eggshell" of cellulose II would be very limited. Thus, with the advent of chain-ordering via increased catalytic site density, the stage was set for minisheet formation, leading to minicrystal assembly and then, ultimately, to microfibril assembly. As microfibrils have a much higher degree of polymerization than cellulose II, and because they are much longer and thinner with built-in flexibility, it is obvious that selection pressure favored survival of much stronger, flexible cell walls of cellulose I microfibrils. Cellular extension and directed growth leading to morphogenesis became possible with the evolution of cellulose I assembly in the eukaryotic plant cell. Cellulose I microfibrils represent only one of the numerous components of the plant cell wall. Xyloglucans, pectins, hemicelluloses, proteins, glycoproteins, and lignin all interact with cellulose microfibrils at one time during the developmental cycle of the plant. The increased functionality of the cell wall in growth and differential expansion appears to have resulted because of a fundamental switch from cellulose II to cellulose I assembly.

RECENT MOLECULAR GENETICS RESEARCH ON CELLULOSE

The first successful purification of cellulose synthase by Lin and Brown in 1989 [40] and the determination of *n*-terminal sequences of two polypeptides allowed the design of oligonucleotide probes. Meanwhile, the 83 kDa polypeptide was shown by azido-UDP-glc photoaffinity labeling to be the catalytic subunit for cellulose synthase [41]. Armed with this information and in collaboration with my coworker Inder Saxena, we first cloned and sequenced a gene for cellulose synthase [42]. This was closely followed by the investigations of Wong et al. [57] who cloned and sequenced similar genes in a cellulose synthase operon of *Acetobacter*.

DISCOVERY OF A GENE INVOLVED IN CELLULOSE CRYSTALLIZATION

We independently sequenced the genes of the operon of *Acetobacter* [43, 44] and found that by the selective mutation of one of the genes, the AcsD gene, the mutant cells were more susceptible to cellulose II production in vivo [44]. This is the first account of a component of the cellulose synthase system in which a gene product has been shown to control and modulate cellulose I assembly. A schematic diagram of the major gene products required in *Acetobacter* cellulose synthesis is shown in Fig. 15. The AcsD gene mutant also is depicted, suggesting that the function for this gene product may be to control the intermolecular ordering of the polymer chains as necessary for cellulose I assembly (see also Kobayashi et al. in this issue). It is postulated that the AcsD gene product, when present and functioning, assists in "threading" the glucan chain sheets into a narrow channel to promote sheet associations to form the minicrystal. When this gene product is not present, cellulose I microfibrils can still be synthesized by the cells but only at low rates.

When the mutant cells are agitated in any way, cellulose II is synthesized (Fig. 16). The controls (wild type cells containing the functioning AcsD gene product) continue to make cellulose I microfibrils, even upon vigorous agitation. The AcsC gene product is believed to be a structural protein which forms the major channel for export from the polymerization site through the cytoplasmic membrane, the periplasmic space, and the outer membrane (Fig. 15). When both AcsC and AcsD genes are mutated, the cells cannot export cellulose in vivo; however, cellulose II is readily assembled in vitro from cell-free preparations of this mutant [44]. These experiments reveal that the critical two genes for cellulose synthesis, the AcsA and AcsB genes, govern the polymerization reaction. Recently, we have shown that these two genes really are a single gene, the AcsAB gene [44].

UNDERSTANDING THE POLYMERIZATION REACTION FROM HYDROPHOBIC CLUSTER ANALYSIS

Using the sequence information, we undertook protein sequence comparisons using Hydrophobic Cluster Analysis (HCA) in collaboration with the laboratory of Bernard Henrissat in Grenoble. This investigation has provided new insight into understanding the polymerization mechanism for cellulose as well as other polymer

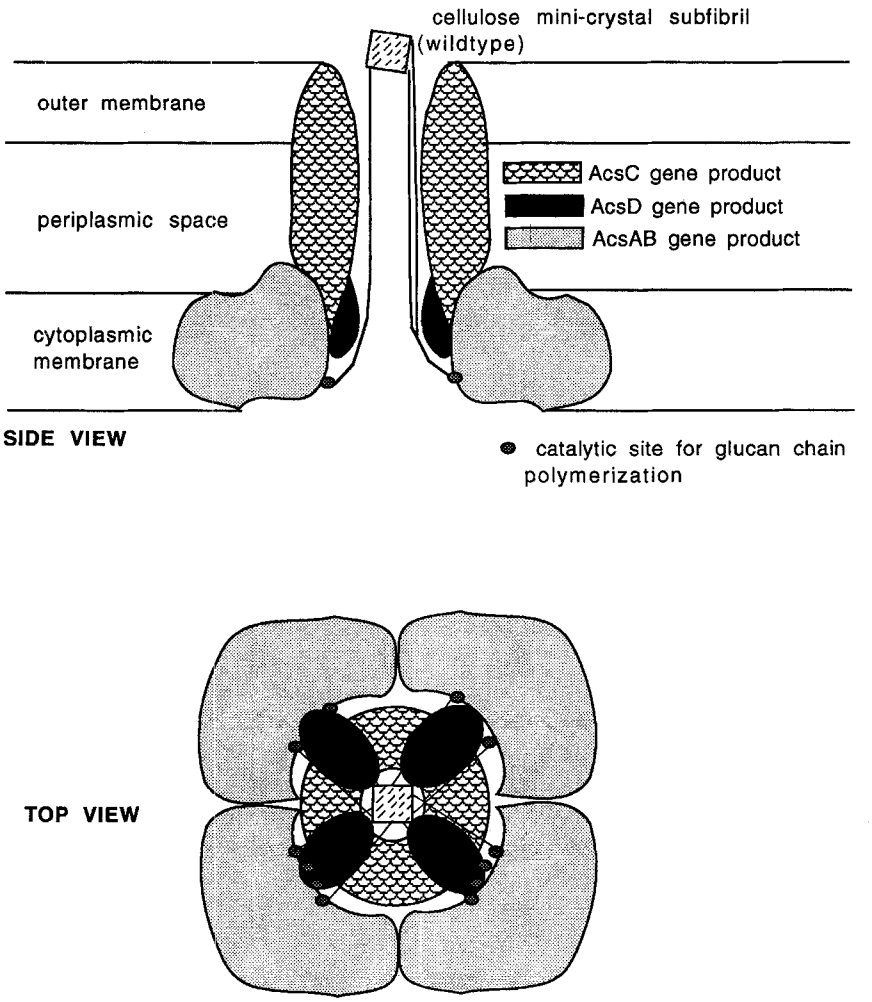

FIG. 15. Proposed organization of polypeptides in the cellulose-synthesizing complex of *Acetobacter xylinum*, based on isolation and characterization of the genes in the cellulose synthesizing operon (after Saxena et al.; relative polypeptide sizes are depicted). The catalytic domains are located in the cytoplasmic membrane along with the c-di-GMP activator binding polypeptides. The AcsC gene product is proposed to form the major channel for export of the cellulose from the cytoplasmic membrane to the cell surface. The AcsD gene product binds to the two other gene products, deeply within the channel. (Courtesy Dr. Inder Saxena.)

assemblies [45]. The hydrophobic cluster analysis has shown the presence of conserved domains in a few β-glycosyl transferases. Enzymes involved in nonprocessive reactions show the presence of only a single domain with two conserved residues whereas all enzymes show, in addition to the above, a second domain with a single conserved residue and a *QXXRW* motif. From this, we have postulated that the polymerization reaction in cellulose assembly proceeds with a simultaneous binding of two UDP-glucoses into two catalytic pockets, each oriented 180° with respect to

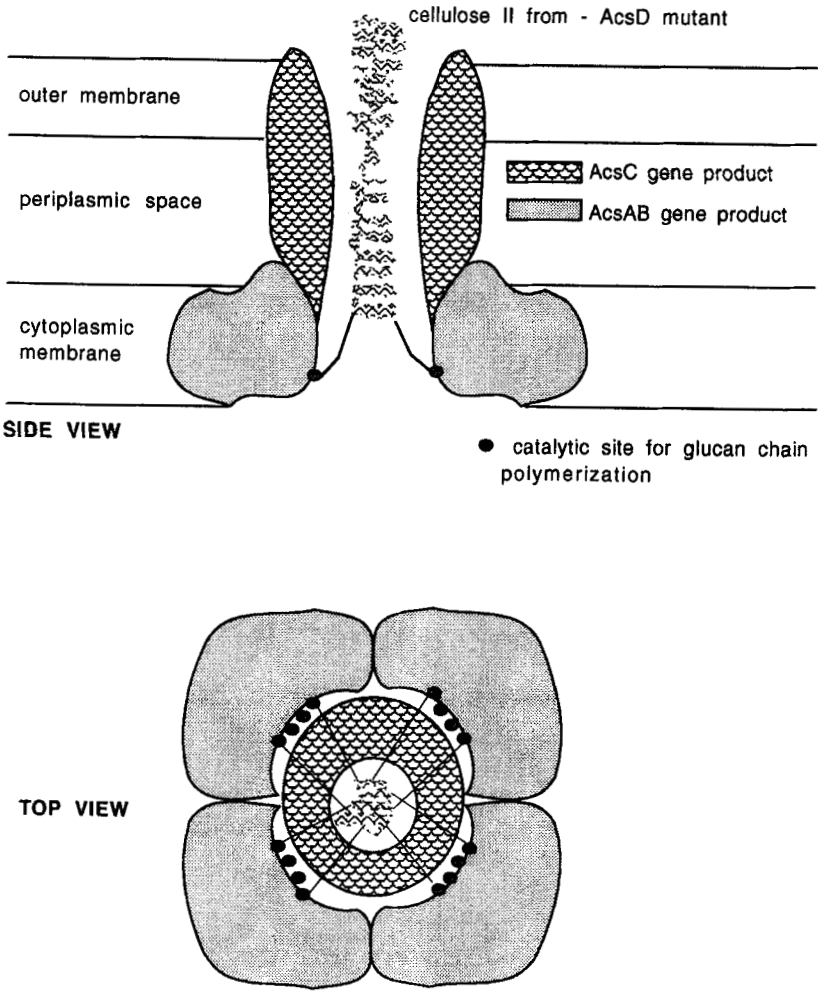

FIG. 16. Tn*phoA*/Kan^{r}GenBlock insertion mutants were used to selectively block genes in the operon. As shown, when the AcsD gene product was not produced in the mutant, cellulose I synthesis was greatly reduced, and cellulose II formation could easily be induced with mild shaking. The function of the AcsD gene product is postulated to assist in the rapid and efficient assembly of the minisheets into the minicrystal. When this product is missing, the channel is still present, but the greatly increased space allows for greater freedom of movement of the minisheets, increasing the possibility for chain or sheet folding into the cellulose II allomorph. (Courtesy Dr. Inder Saxena.)

the other such that the 2-fold chain axis is maintained (Fig. 17). A single inversion reaction is required for addition of glucose residues from a UDP-glucose into a β-linked polymer. The new insight gained by the hydrophobic cluster analysis provides a long-sought understanding into the perplexing mystery of how the glucan chain or the catalytic site in the enzyme would have to rotate 180° for each succeeding glucose addition. With this new understanding there is no such requirement for

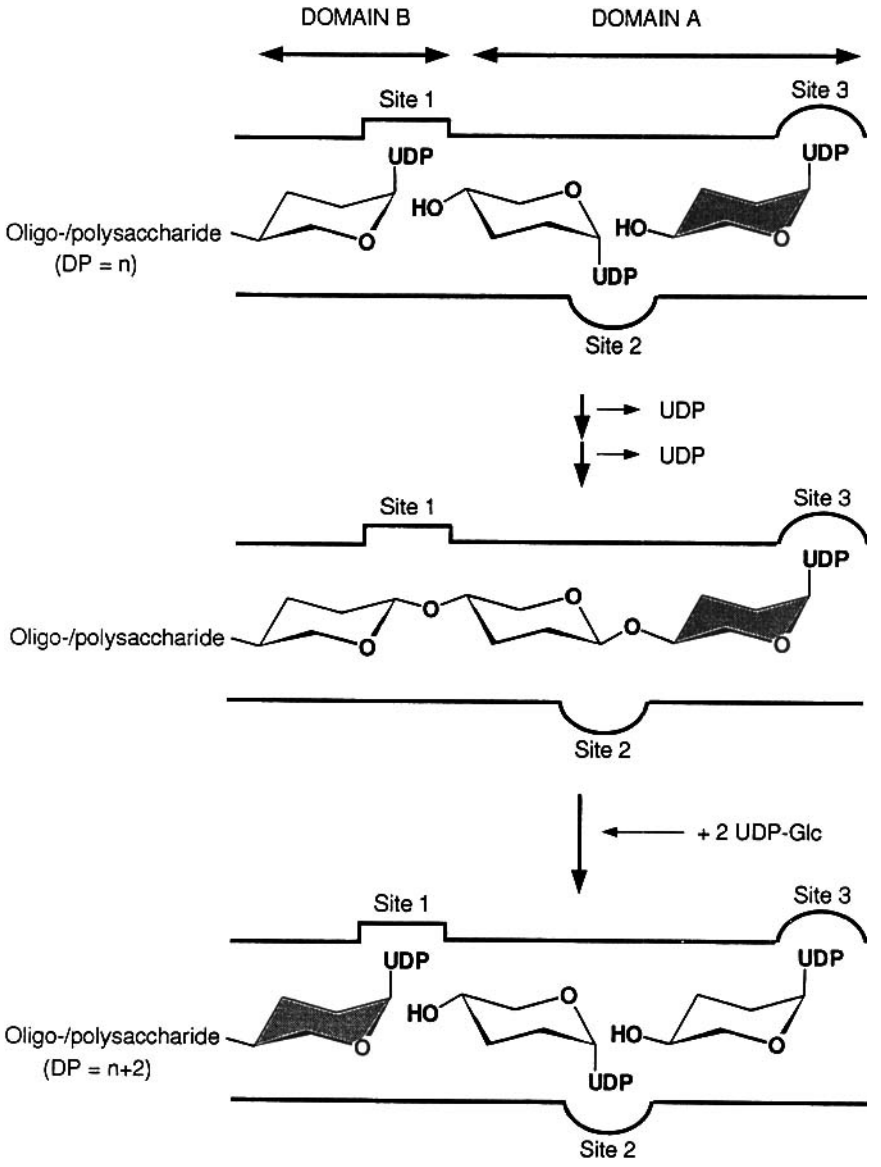

FIG. 17. Conclusions from HCA analysis of the domains of cellulose synthase. The model of simultaneous additions of two UDP-glucoses is shown. Top: Three sites are shown which are able to bind three nucleotide diphospho sugars. No primer is required since it is conceivable that one UDP-glucose would fill site 1. Middle: Two glycosidic bonds are formed either simultaneously or sequentially by a mechanism resulting in the inversion of the anomeric configuration and in the release of two UDP molecules. Bottom: The chain is then elongated by two units and bears a UDP group on its reducing end (shaded residue). Then two new UDP-glucoses can fill sites 2 and 3, ready for the next cycle of polymerization. [From Saxena et al., *J. Bacteriol., 177*, 1423 (1995).]

a rotation, and the glucan chain elongation can therefore proceed unidirectionally and be in close position with its nearest neighbor to form the minisheet. Interestingly, in synthetic cellulose production there would be no requirement for glucan chain rotation or site rotation since β-cellobiosyl fluoride is added to the growing chain by a double inversion mechanism. Thus, Nature adds two glucose units simultaneously on a diversified catalytic scaffolding while a much simpler open trough is all that is required for synthetic assembly if β-cellobiosyl fluoride is used as the substrate.

THE FUTURE—WHAT HAVE WE LEARNED AND HOW CAN WE APPLY OUR NEW-FOUND KNOWLEDGE?

It should be clear by this time that Nature is the architect from which we can learn the most. We started this journey by describing the various structural forms of cellulose, then we covered TCs, Nature's cellulose-producing engines. Next, we noted the discovery of synthetic cellulose I production, followed by a brief discussion of the evolution of cellulose biogenesis. Then we concluded with recent molecular genetics investigations in cellulose research. Where do we go from here? My major professor, Dr. Harold C. Bold, always reminded me that "Nature mocks at human categories." I shall never forget this statement, because it continuously reminds me that we can learn so much from Nature. Thus, the design for polymeric materials of tomorrow must be grounded on our respect for Nature's design.

In this presentation we have seen that synthetic cellulose I production mocks closely several critical aspects of Nature's design. Through purification of the responsible enzyme, we found that catalytic site density could be improved in the micelle, thus promoting a directed and oriented minisheet formation and microfibril assembly. We have learned indirectly through Hydrophobic Cluster Analysis of the catalytic subunit of cellulose synthase that Nature appears to have solved the 2-fold chain axis rotation problem by using *two* UDP-glucoses simultaneously binding into two catalytic sites preoriented for the ensuing β-1,4 linkage, whereas with synthetic cellulose production the dimer β-cellobiosyl fluoride accomplishes the regio- and stereoselectivity.

Can we take these examples and *artificially* construct a nanomachine for cellulose assembly? With the simple micelle TC model, it is easy to envision alterations and changes which could be helpful in the design of specific crystalline forms of cellulose. For instance, the shape of the microfibril could be controlled by the spatial positioning of the catalytic sites on an immobilized substrate. The size of the microfibril could be controlled by the number of catalytic sites relative to their specific distribution. If β-cellobiosyl fluoride is a substrate for β-1,4−glucan chain polymerization, can we find other substrates? Can we genetically engineer the cleft site in the endoglucanase to an immobilized but ordered substrate so that a totally artificial catalysis center can be fabricated? All of these concepts are rather far into the future, but who would have believed even a decade ago that synthetic cellulose I assembly was possible? Recently, we have extended cellulose I assembly in vitro using digitonin-solubilized plasma membrane fractions from cotton [58–61], mung bean, and *Arabidopsis*. Using Nature's substrate, UDP-glucose, extended chain crystalline cellulose I microfibrils can be made (Fig. 18). This provides encourage-

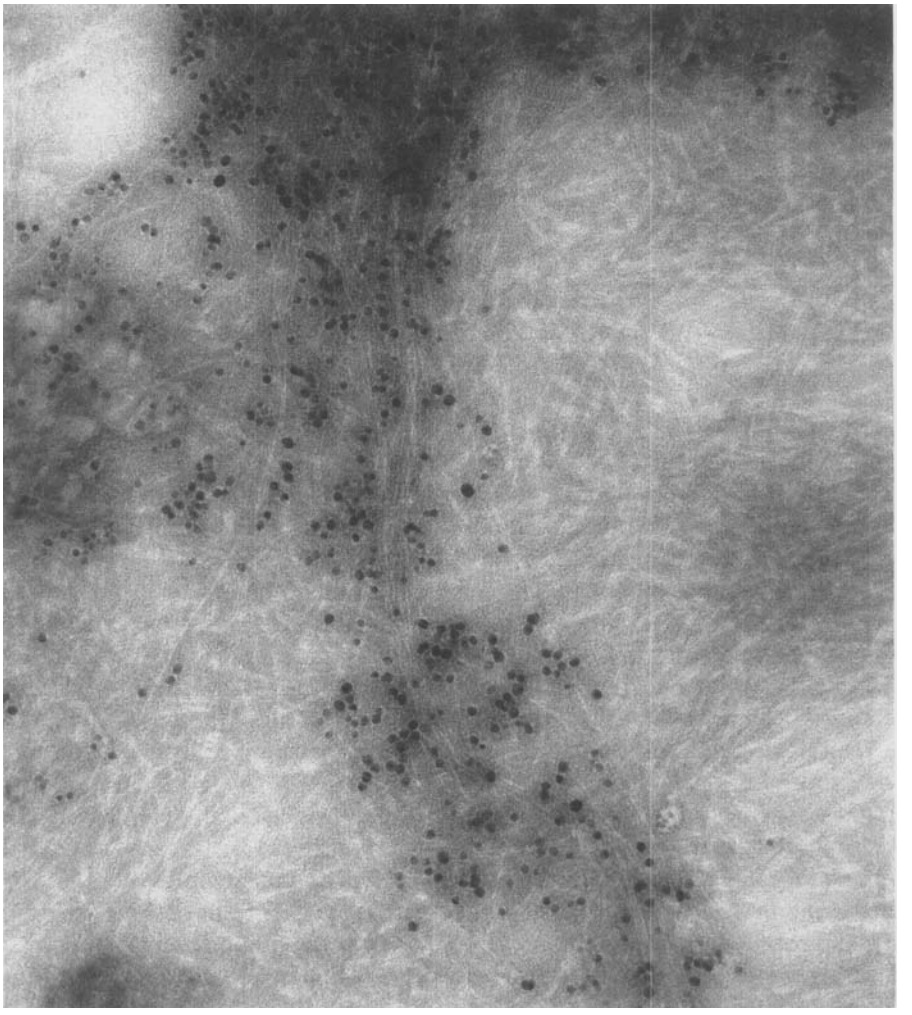

FIG. 18. Cellulose I microfibrils synthesized in vitro from a digitonin-solubilized membrane fraction of mung bean (*Vigna radiata*) seedlings. The cellulose I is specifically labeled with colloidal gold-cellobiohydrolase (dark structures attached to elongated microfibrils). The shorter structures not labeled are fibrils of callose (β-1,3-glucans) which are cosynthesized along with the cellulose. As with synthetic cellulose I, the catalytic sites of the β-1,4-glucan synthases must be sufficiently close for in vitro cellulose I to be assembled. This appears to have been achieved by a partial purification using a separate two-step digitonin solubilization procedure [61]. (Courtesy Dr. Krystyna Kudlicka.)

ment that in the relatively near future we will be well on our way toward duplicating one of Nature's most beautifully designed nanofactories for cellulose assembly, thus extending the uses of a long used biopolymer to the benefit of future humankind.

ACKNOWLEDGMENTS

I would like to take this opportunity to thank Professor Otto Vogl, Dr. Ann-Christine Albertsson, and the organizers of this marvelous symposium in honor of my good friend and colleague, Bengt Rånby. Certainly, none of this material would have been possible without my past and present students and colleagues, to whom I owe a debt of gratitude. It is impossible to thank all of them; however, I wish to convey my special appreciation to Richard Santos, Susan Cousins, Judith Sharp, Krystyna Kudlicka, Jong Lee, and Inder Saxena for their great encouragement and assistance in the preparation of this manuscript. Also, I wish to thank my colleagues Professor Shiro Kobayashi, Dr. Shigenori Kuga, and Dr. Kazuo Okuda for their unfailing devotion to cellulose and their willingness and vision to take challenges and run with them.

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