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Cellulose Biosynthesis and Function in Bacteria

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INTRODUCTION

The ever-increasing demands of industrialization have imposed extreme negative pressures on the delicate ecological balance of our planet. In particular jeopardy at present are virgin forests. Understandably, this concern motivates many efforts to comprehend the intricate mechanisms of plant life and the searches for materials to substitute for plant sources. Often, these two streams of inquiry come together, as in the study of bacteria that produce the fibrous polymer cellulose. In sheer bulk, cellulose is one of the most abundant organic macromolecules on Earth, where it occupies a great reservoir of the carbon cycle; in higher plants, its biogenesis is an integral event in cell growth and development (52, 58).

This review offers a brief introduction to some of the guiding concepts currently adopted in the investigation of cellulose biosynthesis and a synopsis of the many recent developments reported for the cellulose-producing bacteria, which include the genera *Acetobacter*, *Rhizobium*, *Agrobacterium*, and *Sarcina*. The gram-negative *Acetobacter xylinum* has been the subject of the most intensive inquiry, which permits a detailed biochemical description of cellulose biogenesis in this organism. This knowledge is rapidly appreciating in value as interest moves to other organisms as well as to novel biotechnological applications. Several excellent reviews concerning the nature of cellulose structure and biosynthesis have appeared (36, 44, 62, 95), and some of the unifying features are recounted here.

The Cellulose Fibril Is a Highly Ordered Structure

Throughout the plant kingdom, the cell wall is a tough, meshlike bulkwork in which cellulose fibrils are the primary architectural elements (47, 62). The plant cell wall is a semipermeable composite tissue of high species variability (34) and is compounded from layered deposits of cellulose fibrils in which are embedded more amorphous polymers, including neutral and acidic polysaccharides, glycoproteins, and waxy aromatic substances (40). In analogies inspired by electron-microscopic images of plant cell wall replicas (106), the structural role of cellulose has been likened to that of the nylon strands of fiber glass or to the supporting rods within reinforced concrete. Cellulose fibrils are highly insoluble and inelastic and, because of their molecular configuration, have a tensile strength comparable to that of steel (156). Consequently, cellulose imparts a unique combination of chemical resilience and mechanical support and flexibility to the tissues in which it resides. These qualitites are readily apparent in such common plant products as paper, lumber, and cotton textiles.

A typical cellulose fibril may be idealized as a cable in which the lengthwise strands are long polymeric chains composed solely of D-glucose. In each chain, the sugar monomers (present as the pyranosyl ring form) are uniformly linked in β-1,4 glucosidic bonds. The special geometry of this unbranched covalent arrangement gives rise to extended fibril structures as all of the available hydroxyl groups upon

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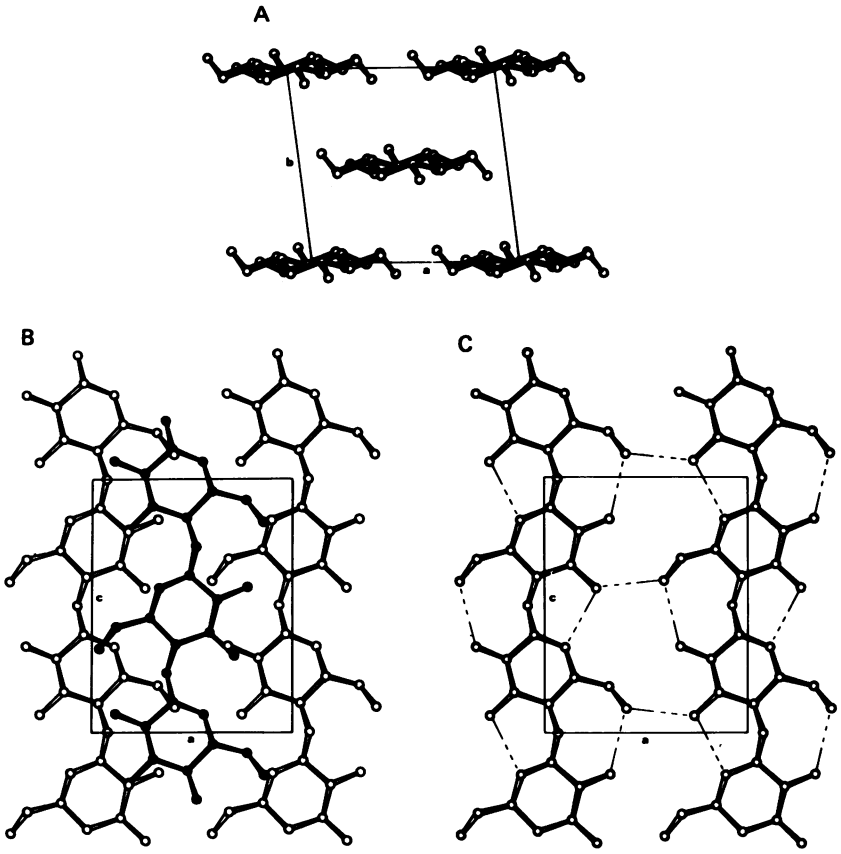

FIG. 1. Structure of cellulose I. (A) ab projection (looking along the chain axes); (B) ac projection; (C) hydrogen-bonding network in the sheet parallel to the ac plane. Reproduced from reference 23 with permission.

adjacently aligned β-1,4-glucan chains participate in inter- and intrachain hydrogen bonding. In this way, aggregates of many chains form insoluble layered sheets reinforced by the dispersion forces between their stacked heterocyclic rings. The resultant degree of order is sufficiently high for crystallographic analysis (23, 54); interpretation of X-ray and electron diffraction patterns suggests several polymorphic possibilities for the cellulose crystal, one of which is portrayed in Fig. 1.

Two prominent features of the cellulose fibril must be taken into account when considering possible modes of biosynthesis: the unidirectional polarity of cellulose fibrils and their regular, but variable, width. If individual β-1,4-glucan chains were synthesized in a disorganized, random fashion, the result would probably be a tangled mass in which any recognizable fibril structures contain chains associated in opposing directions or bent back upon themselves (119, 132). However, this entanglement does not typically occur in nature; instead, nearly all of the cellulose fibrils isolated from plants and algae bear the crystalline unit structure expected for β-1,4-glucan chains which are laterally and unidirectionally aligned (44). The term cellulose I is used for this parallel arrangement, whereas crystalline fibrils bearing antiparallel polyglucan chains—as arise during industrial mercerization when partially solubilized native cellulose reassembles into the thermodynamically stable form—is commonly referred to as cellulose II. The term microfibril has been proposed to denote the thinnest fibril structures visible in the field of the electron microscope (62). Native cellulose microfibrils occur in a spectrum of dimensions, ranging from 1 to 25 nm in width (corresponding to 10 to 250 chains) and from 1 to 9 μm in length (2,000 to 18,000 glucose residues). In general, however, although the width of microfibrils varies among different organisms, it is relatively uniform at each stage of development for a particular species (62).

Both the width of microfibrils and the parallel glucan chain arrangement within them may be accounted for by current models of cellulose biogenesis (101, 105, 112, 130) in which the sites of glucose polymerization are situated in clusters according to two basic degrees of organization. On the enzymatic level this model proposes that the catalytic sites of β-1,4 bond formation operate within sufficient proximity and with the appropriate orientation to ensure the rapid aggregation of their polyglucan chain products into a unidirectional, lateral alignment. Thus, the term cellulose synthase may actually refer to a multipolymerizing unit enzyme complex. On the cytostructural level, cellulose synthase complexes are similarly proposed to be tightly grouped with respect to the cytoplasmic membrane surface, allowing polyglucan chain aggregates to assemble rapidly and in an orderly fashion into a larger microfibril structure at their site of synthesis. Thus, although many of the self-assembly events in cellulose fibril biogenesis take place in the immediate extracellular environment, the process is considered to be tightly coupled to polymerization and directed by the morphology of the cell (63). Variations in microfibril width are probably caused by the degree of clustering on the

enzymatic and cytostructural level. The intramembranous particles observed in association with the ends of microfibrils may represent an elegant biogenetic machinery which modulates in size in accordance with the demand for microfibrils of different dimensions during the plant cell cycle (62).

It should be noted that these concepts of cellulose biogenesis, usually referred to as the ordered-granule hypopthesis (104), are grossly expressed here and many specific variations have been proposed (44, 62). Furthermore, as in the analogous case for α- and β-chitin (23), cellulose I is not an exclusive natural form and different mechanisms may be required to explain the biological occurrence of the less predominant polymorph, cellulose II (109). However, the model of cellulose synthase as an enzyme complex composed from a variable number of subunits remains an attractive one, and we are probably not far from determining whether the basic glucose polymerase of cellulose biogenesis is ubiquitous from the cellulose-producing bacteria to higher plants.

A. xylinum as a Model for Cellulose Biogenesis

The gram-negative bacterium *A. xylinum* has long been regarded as an archetype for the study of cellulose biogenesis largely by the pioneering effort of Hestrin (71) and Colvin (36) and their coworkers. The cellulose produced by this organism is of exceptionally high purity and resembles, in its crystalline unit structure and average microfibrillar width, that from many plant and algal sources (78). The advantages of using a bacterium in the laboratory are manifold, and, of course, many of the "classic" biosynthetic pathways and their regulation were first elucidated in this way. In addition to their rapid growth and ability to be maintained under controlled conditions, bacteria often offer unique possibilities in the isolation of mutants for biochemical and genetic analysis.

A single *A. xylinum* cell may polymerize up to 200,000 glucose molecules per s (73) into β-1,4-glucan chains which, extruded into the surrounding medium, typically achieve the form of a single, twisted, ribbonlike bundle of microfibrils. The ribbon elongates in direct association with the cell envelope and remains associated during cell division, as evidence by a statistical analysis of cellulose production in growing cultures (96, 108) as well as by direct observation (28). In fact, the model of cellulose biogenesis in which the stages of glucose polymerization and microfibril assembly are tightly coupled processes was first proposed for *A. xylinum*, on the basis that substances which bind to nascent polyglucan aggregates and prevent crystallization into microfibrils accelerate the rate of chain synthesis (16).

Static cultures of *A. xylinum* are characterized by a thick cellulosic surface mat, called a pellicle, in which the embedded cells of this obligative aerobe have direct contact with the liquid/air interface (123). The bacterium grows and produces cellulose from a wide range of substrates (11, 72, 149, 150) and is devoid of cellulase activity (73). Thus, in contrast to plant forms wherein cellulose fibrils make up an integral part of the complex polysaccharide cell wall matrix, a distinct advantage in studying *A. xylinum* is that its cellulose fibril product is a metabolically inert, highly pure extracellular deposit.

For many years, the study of cellulose biogenesis in *A. xylinum* was confined to physiological and morphological approaches, concerned with identifying the intracellular precursors of polyglucose chains (1) or characterizing the steps in their assembly into fibrils (27, 63). The isolation of the cellulose synthase from *A. xylinum* cells as a catalytically active species (2, 3, 13, 57), combined with the discovery of a unique regulatory system for the enzyme (113, 114, 115), has dramatically accentuated the potential of this bacterium as a model organism. Although many of the unique features of this cell-free system have been confirmed in other laboratories (29, 91, 139, 143), it is only recently that investigators have apparently succeeded in their attempts to render the cellulose synthase from other organisms accessible to biochemical analysis (5, 139). Reaffirming the general utility of bacteria to beyond the realm of laboratory models, many new commercial product lines based on bacterial cellulose have been developed (see Biotechnology Perspective, below). The advent of a highly active in vitro system has proved important because its availability now greatly expedites the molecular biologist's search for new tools with which to study cellulose biogenesis in plants and bacteria.

MACHINERY OF BACTERIAL CELLULOSE SYNTHESIS: FROM GLUCOSE TO CRYSTALLINE FIBRILS

Rise of the Sugar Nucleotide Precursor

The rate of cellulose production in *A. xylinum* is roughly proportional to the rate of cell growth and is independent of the source of carbon. This feature, together with the fact that cellulose represents a dead end with respect to glucose metabolism, led to a convenient method for the delineation of metabolic pathways in this bacterium in which the rate and pattern of incorporation of labeled carbon atoms from various substrates into the insoluble glucan polymer were determined (11, 148, 150). On the basis of such studies, the suitability of a particular substrate may be understood in terms of the two amphibolic pathways operative in this bacterium: the pentose cycle for the oxidation of carbohydrates and the citrate cycle for the oxidation of organic acids and related compounds (17, 60, 137). These pathways of carbon metabolism, some of which are unique to *A. xylinum*, are outlined in Fig. 2. The inability to metabolize glucose anaerobially in *A. xylinum* lies in the fact that it lacks phosphofructose kinase, which is required for glycolysis (60). Gluconeogenesis occurs in *A. xylinum* from oxalacetate via pyruvate, because of the unusual regulation of the enzymes oxaloacetate decarboxylase (21) and pyruvate phosphate dikinase (14). Thus, cellulose arises in this organism from a metabolic pool of hexose phosphate that is sustained directly by the phosphorylation of exogenous hexoses and indirectly via the pentose cycle and the gluconeogenic pathway. The conversion of hexose phosphate to cellulose is direct in the sense that it does not necessarily include intermediary cleavage of the carbon skeleton of the hexose moiety (121, 122). The flow of hexose phosphate carbon toward cellulose or through the pentose cycle appears to be regulated by an energy-linked control mechanism in which the crossover point could be at the ATP-sensitive NAD-linked glucose-6-phosphate dehydrogenase. Of the two distinct glucose-6-phosphate dehydrogenases operative in *A. xylinum*, only one is inhibited by ATP (17).

Cellulose production in *A. xylinum* is conditional on concurrent oxidation processes but does not depend on net protein synthesis. This is most evident in the capacity of washed cells, when deprived of a nitrogen source, to continue to produce cellulose when supplied with an adequate supply of carbon substrate. The range of utilizable substrates

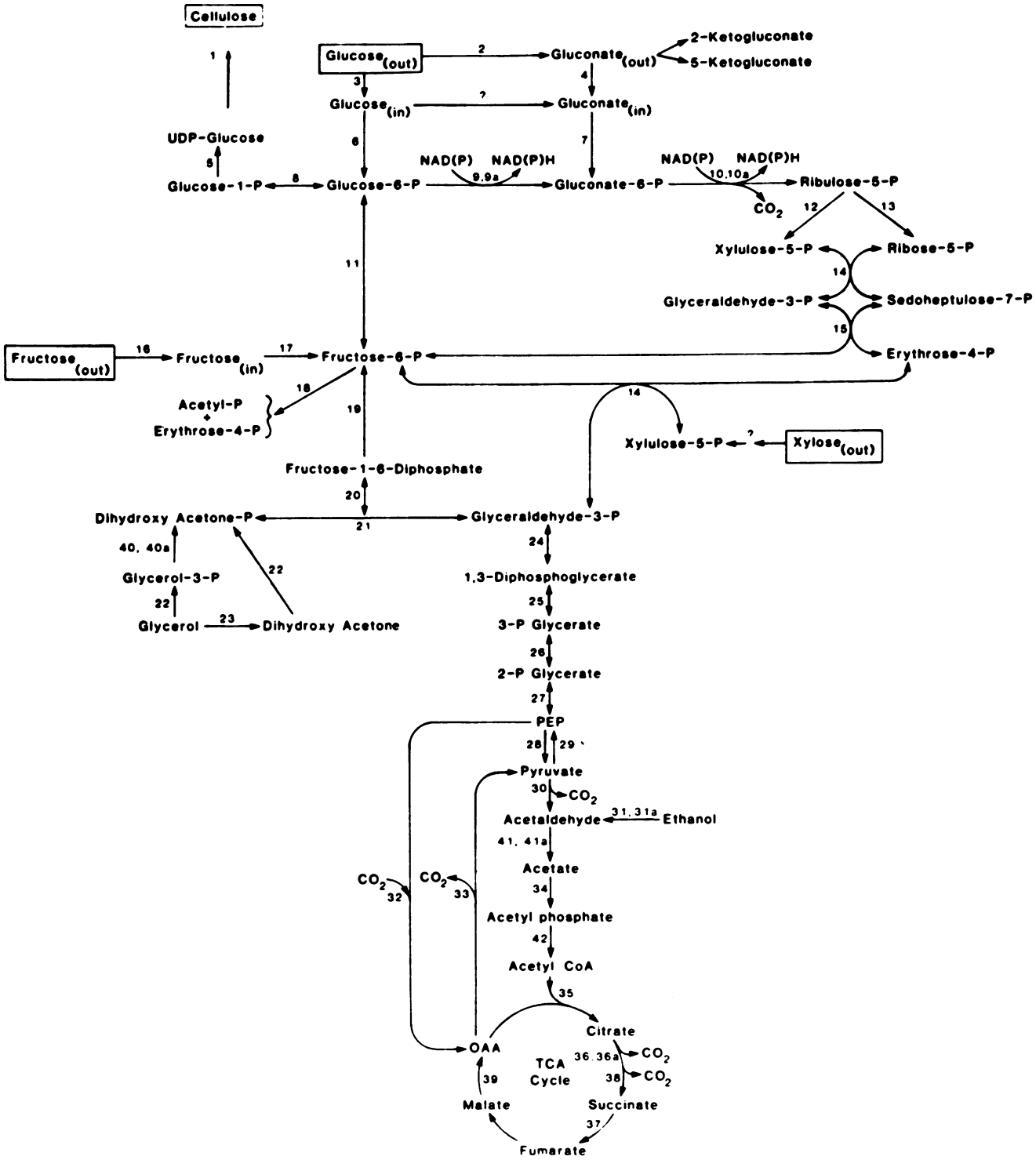

FIG. 2. Pathways of carbon metabolism in *A. xylinum*. 1, Cellulose synthase (2, 115); 2, glucose oxidase (60); 3, glucose permease; 4, gluconate permease (19); 5, UDPG-pyrophosphorylase (UTP) (143); 6, glucokinase (ATP) (19); 7, gluconokinase (ATP); 8, phosphoglucomutase (60); 9, glucose-6-P_i-dehydrogenase (NAD); 9a, glucose-6-P_i-dehydrogenase (NADP) (17); 10, 6-phosphogluconate dehydrogenase (NAD); 10a, 6-phosphogluconate dehydrogenase (NADP); 11, phosphoglucoisomerase; 12, phosphoribulose epimerase; 13, phosphoribulose isomerase; 14, transketolase; 15, transaldolase (60); 16, fructose permease; 17, fructokinase (ATP) (19); 18, fructose-6-P_i-phosphoketolase (124); 19, fructose diphosphatase (150); 20, aldolase (11); 21, triose phosphate isomerase (150); 22, glycerol-dihydroxyacetonekinase (ATP) (or glycerol kinase); 23, glycerol oxidase (150); 24, glyceraldehyde 3-phosphate dehydrogenase (NAD); 25, phosphoglycerate kinase (ATP); 26, phosphoglycerate mutase; 27, enolase; 28, pyruvate kinase (10); 29, pyruvate phosphate dikinase (ATP) (14); 30, pyruvate decarboxylase (11); 31, alcohol dehydrogenase (NAD); 31a, alcohol oxidase (138); 32, phosphoenolpyruvate carboxylase (9); 33, oxaloacetate decarboxylase; 34, acetokinase (ATP) (21); 35, citrate synthase (137); 36, isocitrate dehydrogenase (NAD); 36a, isocitrate dehydrogenase (NADP) (138); 37, succinate dehydrogenase (FAD) (15); 38, α-ketoglutarate dehydrogenase (NAD) (coenzyme A) (87); 39, malate dehydrogenase (FAD) (15); 40, glycerol-P_i dehydrogenase (NAD); 40a, glycerol-P_i dehydrogenase (FAD) (150); 41, acetaldehyde dehydrogenase (NAD); 41a, acetaldehyde dehydrogenase (FAD); 42, phosphotransacetylase (11, 60). TCA, Tricarboxylic acid.

for cellulose production demonstrated under these conditions includes hexoses; hexanoates; three-carbon compounds such as pyruvate, glycerol, and dihydroxyacetone; and four-carbon dicarboxylic acids of the citrate cycle (11, 121, 149, 150). Although the rate of cellulose synthesis in the resting-cell system is similarly unaffected by inhibitors of protein synthesis (147), inhibitors or uncouplers of the electron transport chain interrupt the synthesis (121). Such an experimental system is in many ways ideal for investigating cellulose synthesis in vivo because it allows for separation of the process from cell division and growth. The system may be adapted to compare the synthetic capacities of different strains (16, 143) and, when using a CO_2 trap for measuring total respiration, to estimate the energy efficiency of cellulose synthesis from a suitably radiolabeled sugar or organic acid (121, 148, 150). Notably, cellulose synthesis in *Agrobacterium tumefaciens* has also been demonstrated to occur in resting cells (5). A related experimental system that was recently developed (79a) involves pretreatment of *A. xylinum* cells with a nonionic detergent which renders the cells permeable to low-molecular-weight substrates and effectors while they retain their complement of cytoplasmic enzymes; upon incubation with either UDP-[^{14}C]glucose or [^{14}C]glucose and UTP in the presence of GTP or bis-(3′,5′)-cyclic diguanylic acid (c-di-GMP) (see Allosteric Effector: C-Di-GMP, below) in Mg^{2+} buffer, rates of cellulose synthesis comparable to that of the whole cell may be demonstrated. This in situ type of assay system allows for the study of cellulose synthesis under selective conditions which more closely approximate the intact cell than those afforded by a total in vitro assay with membrane fractions.

The immediate sugar nucleotide precursor of cellulose synthesis in *A. xylinum* is UDP-glucose (136). Essentially, four enzymatic steps have been characterized in cell extracts of *A. xylinum* that appear to make up the complete pathway from glucose to cellulose. These are the phosphorylation of glucose by glucokinase (19), the isomerization of glucose-6-phosphate (Glc-6-P) to glucose-1-phosphate (Glc-1-P) by phosphoglucomutase, the synthesis of UDP-glucose (UDPG) by UDPG-pyrophosphorylase (1a), and the cellulose synthase reaction. In the in vitro assay system derived from this organism, under optimal conditions the synthesis of alkali-insoluble β-1,4-glucan proceeds from UDP-Glc, when added as the sole, exogenous substrate, at rates comparable (40%) to that of the whole cell. Similarly, other forms of activated glucose, such as Glc-1-P, ADP-Glc, CDP-Glc, or GDP-Glc, do not significantly interfere with this reaction (19). The validity of the above series of reactions has also been borne out by kinetic tracer studies, in which the in vivo flow of carbon from glucose through sugar phosphates and nucleotide sugar pools into cellulose was quantitatively evaluated (136).

The critical role of the UDPG-pyrophosphorylase in cellulose production in this organism has also been verified through the analysis of a group of Cel$^-$ (cellulose-negative phenotype) mutants which were determined to be specifically deficient in this enzyme (143) (see *A. xylinum*, below). In vitro, this Mg^{2+}-dependent activity may be detected either in the direction of UDP-Glc synthesis from Glc-1-P and UTP or in the (threefold faster) reverse direction supplying UDP-Glc and PP_i as substrates. In tests of one particular *A. xylinum* strain (136) in which the latter assay method was used, UDPG-pyrophosphorylase was determined to be the only glucose-nucleotide-cleaving activity detectable, suggesting that the capacity of such cells to produce UDP-Glc is far greater than that for any other glucose donor molecule (ADP-Glc, GDP-Glc, CDP-Glc, etc.). In this regard it is noteworthy that *A. xylinum* does not engage in glycogen production (73), which in bacteria typically requires ADP-Glc (103). The kinetic properties of the *A. xylinum* UDPG-pyrophosphorylase (K_ms for UTP, Glc-1-P, UDP-Glc, and PP_i are, respectively, 0.28, 0.23, 0.44, and 0.27 mM) are consistent with its role in cellulose synthesis. The enzyme appears to be confined to the cytoplasm, where the level of activity is not influenced by the carbon source (glucose versus succinate) of the culture medium (53a). The structural gene encoding this enzyme has been cloned from an *A. xylinum* library and shown to complement the *galU* mutation in *Escherichia coli* for growth on galactose (143).

It would be of interest to examine the extent to which UDPG-pyrophosphorylase may have been conserved among bacterial species and whether the genetics and regulation of such an enzyme reflect the high demand for its product anticipated for species that produce cellulose or related polymers. These questions are particularly germane during attempts to identify specific rate-limiting steps in cellulose synthesis. Until recently, it was thought that the rate of UDP-Glc synthesis was well in excess of that required to support the observed rates of cellulose synthesis (1a). However, recent findings indicate that even among related *A. xylinum* strains of comparable cellulose-producing capacity, in vitro levels of the pyrophosphorylase vary by up to 20-fold; for example, the ratio of UDPG-pyrophosphorylase to cellulose synthase activity in vitro to cellulose synthesis in vivo is 2:1:2 for strain ATCC 23768 and 0.1:1:2 for ATCC 23769 (150a). Thus, although the capacity to form UDP-Glc is a prerequisite for cellulose synthesis, cellulose-producing bacteria may manifest dramatic variations in the patterns of UDP-Glc metabolism. Studies with *E. coli* have shown that UDPG-pyrophosphorylase-defective mutants lack detectable galactose (an epimer of glucose produced via UDP-Glc) in their cell walls (55) and lipopolysaccharide (134). A similar situation has been observed for an *A. xylinum* isolate (120), whose Cel$^-$ phenotype was thus attributed to a defect in the UDPG-pyrophosphorylase step, particularly since the cellulose synthase proper of this mutant was determined to be catalytically functional in vitro. Because UDPG-pyrophosphorylase appears to be a key—and possibly rate-determining—enzyme in cellulose biogenesis, it is hoped that more about its structure and regulatory properties will be learned in the future.

Glucose Polymerization: the Cellulose Synthase

When considering cellulose biogenesis as a universal phenomenon, much interest naturally focuses upon the cellulose synthase, since this may well be the only enzyme unique to this process. Although it was once thought that bacterial cellulose synthesis occurs extracellularly via the covalent assembly of short-chain precursors secreted from the cell (36), it is now clear that polymerization is a membrane-associated event. Much of the early work in studying the biochemistry of this process was hampered by the lack of an efficient in vitro system for demonstrating cellulose synthase activity (57), and such a system essentially became obtainable once the basic regulatory elements of the cellulose-synthesizing apparatus were known (2, 13). In catalyzing the "committed" step in cellulose biogenesis, it is not surprising that this enzyme is subject to a sophisticated system of regulation: the cellulose synthase reaction is greatly and specifically enhanced by a novel cyclic dinucle-

otide effector molecule: c-di-GMP (see Allosteric Effector: C-Di-GMP, below). Elucidating the protein structure of this enzyme and its reaction mechanism is necessary before a deeper understanding of the mechanism of cellulose biogenesis throughout the cellulose-producing species can be gained.

In vitro, cellulose synthase activity is assayed by incubation of membrane preparations in the presence of UDP-[^{14}C] glucose and Mg^{2+}, followed by quantitation of the radioactivity incorporated into the hot-alkali-insoluble β-1,4-linked glucan product. The cellulose synthase is most probably an integral membrane protein; synthase activity occurs exclusively in the membrane-associated fraction, as determined for a variety of *A. xylinum* strains and *Agrobacterium tumefaciens* (5). This activity cannot be eluted from the membrane fraction by extensive washing in either low- or high-ionic-strength buffers, by changing the pH, or by using metal-chelating agents (20). Characteristically for a membrane protein, the synthase has been effectively solubilized by treatment of membranes with digitonin (3). The catalytic and regulatory properties of the enzyme in the solubilized state are remarkably similar to those observed for the membrane-bound form. Thus, enzyme activity in both forms is Mg^{2+} dependent, is optimal at 30°C in the pH range 7.5 to 8.5, displays typical Michaelis-Menten kinetics with respect to UDP-glucose (K_m = 0.125 mM), and is competitively inhibited by the uridine 5′-phosphates UTP, UDP (K_i = 0.14 mM), and UMP (K_i = 0.71 mM) (3, 13). Similarly, the soluble enzyme, which has a molecular mass of ca. 420 kDa on gel filtration chromatography in the presence of 0.1% digitonin, retains full sensitivity to the cyclic dinucleotide regulatory system.

There are some indications that the cellulose synthase is a cytoplasmic membrane protein; these are based on the separation of crude *A. xylinum* membranes by discontinuous sucrose density ultracentrifugation and localization in relation to enzymatic markers assumed to be specific for either the outer or the cytoplasmic membrane (29). This is in contradiction to previously proposed models in which intramembranous particles that were revealed by freeze-etching of the cell envelope were taken to indicate that cellulose synthase is located in the outer membrane (28, 38). However, because cofractionation in an isopycnic density gradient is not always compelling evidence of association (107), a definitive localization study will probably require more refined techniques, such as specific immunofluorescence labeling of intact cell structures. It has also been suggested that the cellulose synthase is a glycoprotein, on the basis of its retention on a concanavalin A-Sepharose column (90).

The availability of a highly active enzyme preparation has greatly facilitated analysis of the structure and mechanism of the cellulose synthase (100b), as well as the genetic organization of the overall process (154a) (see *A. xylinum*, below). The synthase has been purified 350-fold from digitonin-solubilized membrane preparations derived from various strains of *A. xylinum*, with an overall yield of 20%; the method of product entrapment was used (82). In this purification technique, solubilized membrane preparations are incubated under conditions optimal for the synthase reaction; upon subsequent centrifugation over a glycerol cushion, the synthase may be retrieved in the pellet, associated with its insoluble glucan product. Evidence that the synthase indeed undergoes entrapment in its reaction product is that if either the UDP-Glc substrate or c-di-GMP activator is omitted from the procedure, no enzyme activity is recovered in the pellet. In addition, the purified enzyme may be partially resolubilized (up to 50%) by exposure to cellulase.

A role for three peptides, of 90, 67, and 54 kDa, as the sole constituents of the native cellulose synthase molecule has been substantiated on the basis of specific labeling techniques. In direct photoaffinity-labeling assays, which are carried out by UV irradiation of the entrapped synthase in the presence of either c-di-[^{32}P]GMP or [α-^{32}P]UDP-Glc followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography, the pattern of label incorporation indicates a differential relationship for the three peptides with respect to the structure of the active enzyme; activator- and substrate-specific binding sites are most closely aligned with the 67- and 54-kDa subunits, respectively, while the 90-kDa peptide apparently does not directly participate in formation of the binding sites because it only marginally incorporates either radiolabeled ligand. The specificity and pattern of radiolabel incorporation also closely parallel the effects of each of the ligands on the level of cellulose synthase activity, and, furthermore, the photoaffinity labeling and the cellulose synthase reaction share a similar sensitivity to *para*-hydroxymercuribenzoate (PHMB): preexposure to this -SH reagent blocks both the photolabeling and the cellulose-producing reactions, whereas the presence of ligand during the preincubation period effectively prevents PHMB inactivation.

Despite an apparent differential delegation of functions, all three of the peptides appear to represent closely related structures on the immunological level. This type of analysis has been made possible by the use of a preparation of specific anti-cellulose synthase antisera, produced against the protein translation product derived from the cellulose synthase structural gene (*bcsB*) (see *A. xylinum*, below), which itself has recently been isolated and cloned (154a). Western immunoblot analysis shows that the 90-, 67-, and 54-kDa peptides display a similar extent of labeling with respect to this highly specific antibody preparation. Furthermore, the NH_2-terminal amino acid sequence of the 90- and 67-kDa peptides shares a high degree of homology with the amino acid sequence deduced from the cellulose synthase structural gene, Thus, although the three major peptides of purified cellulose synthase appear to be functionally distinct, they are structurally related to the protein encoded by the cellulose synthase gene. One current hypothesis based on these results is that the larger (90-kDa) band represents an inactive precursor form which undergoes processing to yield the 67- and 54-kDa peptides, both of which appear to participate directly in the catalytic and regulatory functioning of the enzyme. It appears, then, that the native form of the bacterial cellulose synthase molecule (of ca. 420 kDa) is that of a hetero-oligomeric protein complex containing both catalytically active (67- and 54-kDa) and inactive (90-kDa) subunits, which share a high degree of sequence homology. It should be noted, however, that other reports have described the cellulose synthase, similarly purified by the entrapment method (although with a low yield of 2%), as made up of a single 83-kDa subunit which, on the basis of photoaffinity labeling with azido-UDP-Glc, was identified as the UDP-Glc-binding site (90, 91a). At present, these differences have not been reconciled on the basis of variations in purification methodology or strain-specific features of the enzyme.

As the basic features of the cellulose synthase reaction gradually come to light, they appear to be relatively simple. The alkaline-insoluble product formed from UDP-glucose in vitro by the synthase in both membrane-bound and solubi-

lized forms has been characterized enzymatically and chemically as a β-1,4-linked glucan (2, 3, 29). The second product of the reaction, released in amounts equimolar to those of the glucose incorporated into insoluble material, has now been determined to be UDP (20). This supports the following general equation for the cellulose synthase reaction:

$$\text{UDP-Glc} + (\beta\text{-1,4-glucose})_n \longrightarrow \text{UDP} + (\beta\text{-1,4-glucose})_{n+1}$$

This formulation excludes a direct transfer of hexose-1-P, which occurs in some of the partial reactions of bacterial mucopeptide (56) or glycoprotein (146) synthesis, in which a lipid-linked intermediate is the acceptor of the sugar phosphate. This conclusion is compatible with the failure to identify such an intermediate by in vivo labeling of *A. xylinum* cells with [^{14}C]glucose (45) and with the finding that the enzyme maintains high activity following solubilization and purification stages in which such an endogenous lipid component would most probably be removed (3, 115). Significantly, two other β-linked homopolysaccharide-polymerizing enzymes, the β-1,3-linked glucan synthase (125) and chitin synthase (82) from *Saccharomyces cerevisiae*, also do not exhibit a requirement for such a lipid component. Furthermore, none of these polymerases, including the cellulose synthase, depend upon exogenously added primer materials (95).

The apparent noninvolvement of covalent intermediates in the cellulose synthase reaction suggests that catalysis from UDP-glucose occurs via a direct substitution mechanism, in which the phosphoester-activating group at the anomeric carbon of one glucose residue is displaced by the C-4 hydroxyl group of another glucosyl residue, inverting the α configuration to form a β-glucosidic bond. Repetition of this reaction might then occur processively, meaning that the polymeric product remains tightly bound to the enzyme between elongation steps. In vitro, the high affinity of the enzyme-product complex is exemplified in the behavior of the purified synthase in the entrapped state, which is particulate but may be resolubilized by exposure to cellulase. Such a processive mechanism has also been proposed for yeast chitin synthase (82), although this enzyme, as well as the β-1,3-glucan synthase of plants (68), may be released from the product-entrapped state by relatively mild treatments. Ascertaining the validity of this mechanism, which involves a minimum of bond-cleaving and -forming steps, as well as determining the actual direction from which elongation of the polyglucan chain proceeds, will have significant implications on how later stages in the hierarchical process of cellulose biogenesis are perceived.

Assembly of the Cellulose Fibril

Although the polymerization of glucose is an important step, it is only the first of many steps in cellulose biogenesis, most of which appear to take place in the space immediately exterior to the cell surface. In no other organism have the events of microfibril assembly been observed so directly as in *A. xylinum*. The process has been divided into no fewer than four distinct phases, commencing from the cellulose synthase reaction and culminating in the construction of a single, loosely wound ribbon of fibrils containing ca. 1,000 glucan chains in total (62).

Electron micrographs of the surface of the cell envelope indicate the presence of some 50 to 80 porelike sites arranged in a regular row along the long axis of the cell and in evident juxtaposition with the extracellular cellulosic ribbon (28, 157); a complementary row of particles within the interior of the outer membrane has been described for freeze-etched preparations (26). These discrete structures of the lipopolysaccharide layer are presumed to be the sites of extrusion for precellulosic polymers in groups of ca. 10 to 15 chains known as tactoidal aggregates. Aggregates of this size, rather than individual β-1,4-glucan chains, are postulated to be the initial form of the cellulosic product because nondissociable 1.5-nm fibrils are most frequently seen when microfibril assembly is grossly disrupted, as by the high-affinity dye Calcofluor (16, 62). The existence of such tactoidal aggregates, and of analogous structures in algal preparations (16), suggests that the synthesis of many β-1,4-glucan chains simultaneously at a spatially limited site is a common feature of the assembly of cellulose microfibrils in both higher and lower organisms (44, 62). The form assumed for these premicrofibril species, judging from the results of new imaging techniques, is not a rodlike structure but rather one made up of left-handed triple helices which coassemble into twisted microfibrils consisting of nine parallel polyglucan chains (116).

The use of cellulose-binding agents which alter microfibril assembly at different phases has been the principal experimental method for characterization of the microfibril and ribbon assembly process in *A. xylinum* (62). Low-molecular-weight, cell-impermeable compounds, such as the fluorescent brighteners Calcofluor White (Tinopal) and Congo red, or the water-soluble, high-molecular-weight cellulose derivative carboxymethyl cellulose avidly binds to β-glucans in a definable, reversible manner (27, 64, 65). For example, although Calcofluor, by binding to 1.5-nm tactodial aggregates, interferes with the early-stage appearance of crystalline 3.5-nm microfibrils, a higher level of assembly in which large bundles of crystalline microfibrils fasciate to form the final, twisted-ribbon product is prevented by the presence of carboxymethyl cellulose. On the basis of such observations, microfibril assembly is postulated to occur in a stepwise, hierarchical fashion (Fig. 3). This process is usually described as cell directed because, although it occurs in the extracellular space, the mutual orientation and association of glucan chains, aggregates, microfibrils, bundles, and ribbon are apparently governed by the original pattern of extrusion sites.

Kinetically, disruption of microfibril assembly is manifested by an enhancement of the overall rate of incorporation of glucose into insoluble polyglucan chains (16). This surprising effect on the rate of polyglucan chain synthesis has led to the proposal that polymerization and crystallization are tightly coupled events in the hierarchically organized process of cellulose biogenesis. In other words, the assembly of tactoidal aggregates into 3.5-nm crystalline microfibrils must occur while their composite β-1,4-glucan chains are still undergoing elongation, because the polymerization rate is enhanced in the presence of Calcofluor. In this sense, the partial steps of crystallization and ribbon assembly (i.e., the carboxymethyl cellulose effect) are apparently limiting to the overall biogenetic process. Interestingly, high-field-strength magnetism may give rise to the disorganized form of cellulose known as band material, which resembles the disperse Calcofluor-altered product of noncrystalline tactoidal aggregates (59). Effects on rate, however, have not yet been reported for magnetic disruption of the crystallization process, and the essential nature of this effect remains obscure, although similar magnetic phenomena have been demonstrated for cellulose biogenesis in the oat *Avena sativa* as well (59).

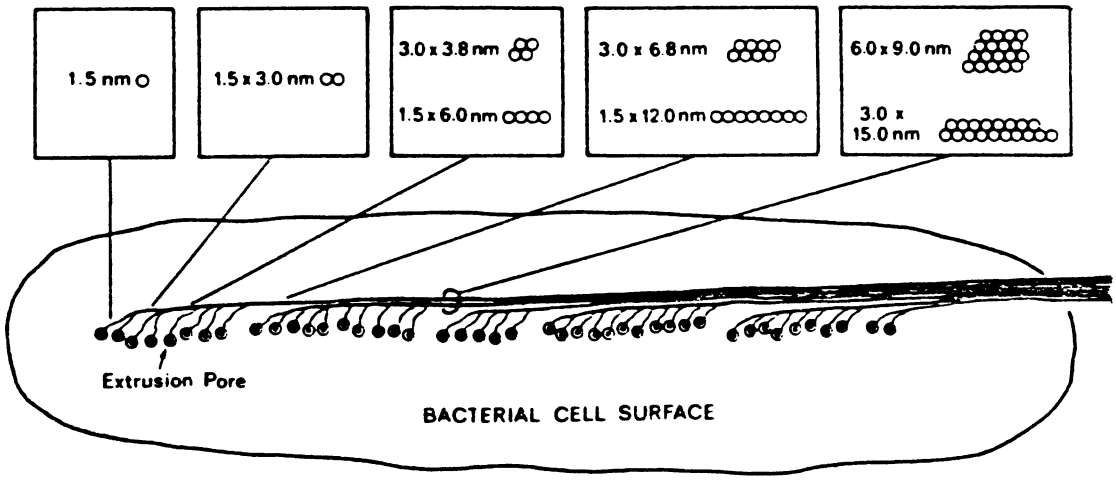

FIG. 3. Generalized model of ribbon assembly in *A. xylinum*, showing a possible mechanism of origin or microfibrils and separate fibrillar subunits within the ribbon. Shown in the boxes are possible packing arrangements of the 1.5-nm tactoidal aggregates. Reproduced from reference 62 with permission.

Although the cell-directed self-assembly concept provides an adequate framework for the formation of cellulose fibrils, many aspects surrounding the nature of the biosynthetic machinery remain an unsolved riddle. For example, although the model for the glucose-polymerizing enzyme as a cytoplasmic (29), as opposed to outer-membrane (26), component offers an explanation for the ability of the cellulose synthase to gain ready access to its hydrophilic substrate and effector molecules, it only further begs the question of how the polyglucan product traverses the periplasmic gel, peptidoglycan, and lipopolysaccharide layers of the bacterium. As proposed for the synthesis of other exopolysaccharides (152) and certain outer membrane proteins (107), cellulose synthesis may be associated with specific sites of adhesion (8) between the inner and outer membranes (27). In this case, then, because the apparent coupling of synthesis, extrusion, and assembly of β-1,4-glucan chains may be due to their common association with such an adhesion site, the cellulose synthase would resist precise definition as an exclusive component of any one membrane or compartment of the cell envelope. Our current knowledge of the synthase polymerization reaction (see Glucose Polymerization: the Cellulose Synthase, above) negates the possible involvement of a carrier species, such as a lipid-sugar intermediate. Given that catalysis is occurring through a direct substitution reaction mechanism, the process of vectorial discharge of the hydrophilic product may be envisioned as a cotranslational event, as previously proposed for the simultaneous synthesis and insertion of membrane proteins (107). Alternately, glucan chains, during the course of the elongation reaction, may be covalently anchored to a membrane component, such as a protein, lipid, or sugar residue. For example, in *Rhizobium* species, the cellulose microfibrils appear to grow out from lump- and barlike surface excrescences of the cell-shaped peptidoglycan sacculi; in this case, the microfibril may be removed by treatment with cellulase but not by gentle treatment with hot SDS followed by proteolysis (48).

If the glucan chain is covalently linked to the cell surface, this bond would probably involve the reducing end of the molecule. Regardless of the type of association involved, it is likely that the nonreducing end of the chain is situated away from the cell because it is the more stable chemically. The new techniques recently applied to demonstrating the polarity of isolated cellulose I microfibrils (35, 88) may ultimately be used in determining the direction of polyglucan chain growth. Such knowledge in turn may be applied to studying the steps of the polymerization reaction itself; some of the most outstanding issues that remain to be resolved are the nature of the cellulose synthase as either a single- or multi-β-1,4-glucan chain-synthesizing unit and whether, during the course of the enzymatic reaction, the events of chain initiation and termination are rigidly controlled.

In vitro, the enzymatic product is probably cellulose II (29), indicating that the synthase itself is not sufficient to govern proper crystallization into unidirectional microfibrils; this correlates with the observation that the immediate product exiting the cell is a tactoidal aggregate (62). But what drives the bulky cellulosic product out of the catalytic site and out from the cell? There is direct visual evidence (28), as well as indirect structural evidence based on the tunnel morphology observed for static-culture pellets (140), that *A. xylinum* cells are subject to translational motion in the direction opposite to that of polyglucan chain extrusion. Although the energy of crystallization and fibril aggregation seems a good candidate for providing the force required for the motion of newly added glucosyl residues away from the enzyme catalytic site and out through the membrane, there is some evidence which points to the contrary; namely, materials such as Calcofluor, which perturb chain association, actually increase the polymerization rate (64). Alternatively, the energy source for microfibril extrusion may be generated during β-glucoside bond formation and transduced through conformational shifts in enzyme structure. The energized state of the membrane found to be essential for in vivo cellulose synthesis may account for the force that is presumably necessary to drive and/or organize this highly complex process; specifically, dissipation of the transmembrane electrical potential by valinomycin and a K^+ gradient inhibits cellulose synthesis in *A. xylinum* (46). This force may be required to maintain a hypothetical extrusion pore in an open state, or possibly the electrical coupling reflects an exotic form of regulation in itself. Presumably, the pace of adjacent synthesis and/or extrusion sites is closely synchronized to

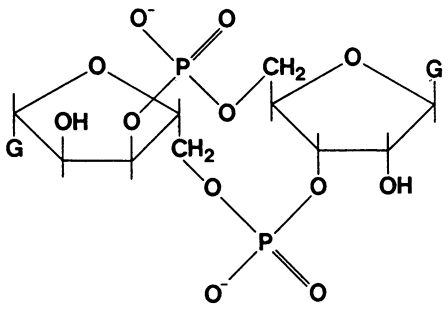

FIG. 4. Structure of c-di-GMP. G, Guanine.

ensure an orderly progression throughout the many phases of microfibril assembly upon a background of rapid fluctuations in local microscopic variables such as concentrations and energy gradients. Indeed, cellulose synthesis is so complex that the smooth flow of glucose into twisted ribbons probably involves mediation by many control systems operating simultaneously on different levels.

REGULATION BY A UNIQUE MECHANISM

From the first breakthrough in studying the biochemistry of cellulose synthesis—the isolation of a membrane preparation containing the cellulose synthase in as active a state as within the cell—much effort was concentrated toward acquiring a complete structural description of the novel nucleotide activator c-di-GMP (Fig. 4), which is essential for the high activity (114, 115). These efforts were aided by J. H. van Boom of the University of Leiden, where the first chemical synthesis of c-di-GMP was carried out, and by H. Schwarz of the Technical University of Berlin, where the novel diguanylate cyclase reaction product was subjected to fast-atom-bombardment mass-spectrometric analysis.

A conceptual scheme for the mechanism of regulation of cellulose synthesis which accounts for the results obtained from the study of cell-free preparations from *A. xylinum* is outlined in Fig. 5. The basic tenet of this model is that c-di-GMP functions as a reversible, allosteric activator of the membrane-bound cellulose synthase, which performs the committed step in cellulose biosynthesis. The concentration of c-di-GMP—the net result of its rate of synthesis and degradation—is controlled by the opposing action of the membrane-associated enzymes which together account for these two pathways. Diguanylate cyclase catalyzes the synthesis of c-di-GMP from two molecules of GTP, via the linear dinucleotide triphosphate pppGpG, in two distinct, PP_i-releasing steps. Within the cell, PP_i is rapidly cleaved to yield P_i (18). The pathway of c-di-GMP degradation is initiated by a c-di-GMP-specific, Ca^{2+}-sensitive phosphodiesterase, PDE-A, which cleaves a single phosphodiester bond in the cyclic structure, yielding the linear dimer pGpG and simultaneously inactivating the molecule. The mononucleotide residues of the inactive dimer structure are then recovered as free 5'-GMP units through the action of a second phosphodiesterase, PDE-B. The Ca^{2+} inhibition of PDE-A represents an additional locus of regulatory control; according to this feature of the system, fluctuations in Ca^{2+} levels may modulate the rate of cellulose synthesis as they influence the persistence of activator in the system.

Allosteric Effector: C-Di-GMP

The proposed regulatory scheme explains the original observations that, in vitro, in the absence of exogenously added c-di-GMP (or its precursor, GTP), the cellulose synthase exists in a basal state of inactivity or low activity, while addition of submicromolar amounts of the cyclic dinucleotide stimulates the reaction rate by factors as high as 200-fold to achieve a rate of cellulose synthesis which approaches 50% of that measured for the intact cell (2, 113). In this context, c-di-GMP may be referred to as an allosteric effector of the cellulose synthase, in the sense that it binds directly to the enzyme in a reversible manner at a regulatory site distinct from that of the catalytic, or substrate-binding, sites. This distinction is supported by several lines of evidence, which also serve to demonstrate many interesting features of the cellulose synthase.

First, the activator is readily dissociated from the synthase, as evident from the basal level of activity in which the enzyme is found in cell-free preparations, including permeabilized-cell systems (see Rise of the Sugar Nucleotide Precursor, above). Indeed, membranes exposed to c-di-

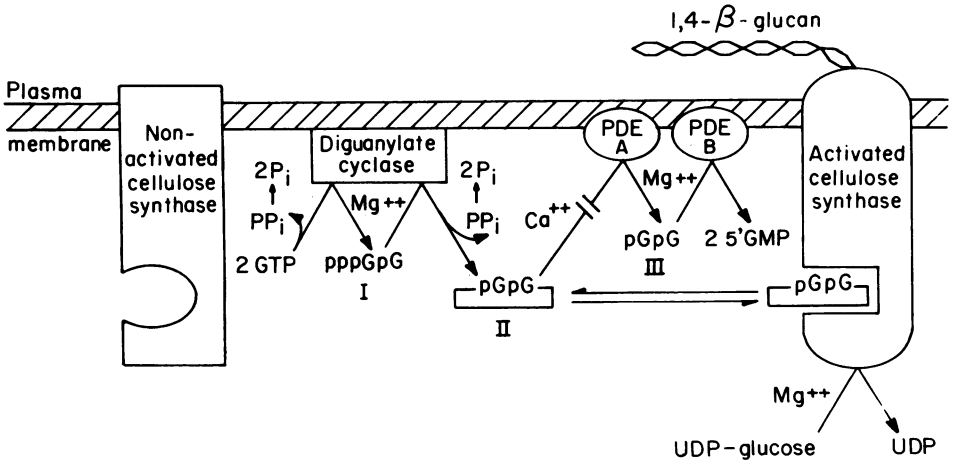

FIG. 5. Proposed model for regulation of cellulose synthesis in *A. xylinum*. For simplicity, the synthesis of a single β-1,4-glucan chain is depicted, although a more complex form of cellulose synthase, polymerizing several chains simultaneously, might be the active enzyme unit in cellulose biogenesis. Modified and reproduced from reference 115.

GMP lose all their enhanced synthase activity on being washed; this activity can subsequently be restored upon readdition of the activator (115).

Second, cellulose synthase in both a highly purified state (see Glucose Polymerization: the Cellulose Synthase, above) or immobilized within a polyacrylamide gel (139) retains sensitivity to the activator, indicating that the site through which the c-di-GMP effect is exerted is either integral to or tightly associated with the catalytically active structure of the enzyme. A different result would be expected if the cyclic dinucleotide, instead of binding directly, acted via an accessory molecule such as a kinase or a protease, which would probably be lost during purification or require a soluble phase in which to manifest its effect.

Third, the kinetics of c-di-GMP activation suggests that binding occurs in an allosteric mode. Activation of the membrane-bound cellulose synthase occurs in the first-order saturation kinetics with respect to c-di-GMP ($K_a \approx 0.35$ μM), which alters the V_{max} of the reaction but not the apparent affinity of the enzyme for the UDPG substrate.

Lastly, the identification of the subunit structure of the cellulose synthase (see Glucose Polymerization: the Cellulose Synthase, above) reveals that the c-di-GMP-binding regulatory subunit is distinct from the UDP-Glc substrate-binding site. These binding sites are mutually independent in the sense that activator does not affect substrate binding and vice versa. The interpretation of these results is that a highly specific c-di-GMP allosteric regulatory site is an integral part of the native cellulose synthase molecule.

Considering the unusual structure of c-di-GMP, the high degree of specificity displayed by its molecular targets is not unexpected. In general, modifications in the c-di-GMP structure reduce the affinity of the molecule for both cellulose synthase and PDE-A, as judged by the ability of various analogs to interact with these enzymes as activators, substrates, and inhibitors. A series of 13 analogous cyclic dimer and trimer nucleotides were synthesized in the laboratory of J. H. van Boom, University of Leiden, and tested for the ability to mimic c-di-GMP as an allosteric effector and substrate of cellulose synthase and PDE-A, respectively (114a). These compounds included cyclic homo- and heterodinucleotides, such as the mono- and dideoxy c-di-GMP nucleotides cdGpGp and cdGpdGp, respectively, as well as a diastereomeric pair of monophosphothioate derivatives in which an exocyclic oxygen of a single phosphodiester group has been substituted with sulfur, namely cGp(S)Gp:Rp and cGp(S)Gp:Sp (Table 1).

Seven of the cyclic dinucleotide analogs proved to be potent activators of the cellulose synthase; all of the other nucleotides tested were of negligible affinity for the synthase. All of the synthetic activators behave as substrates toward the Ca^{2+}-inhibited PDE-A reaction, whereas nonactivators were judged to be resistant to degradation in the presence of this membranous enzyme. In general, the same order of relative affinities for cellulose synthase estimated for the activators pertains to these compounds as inhibitors of the PDE-A reaction when using c-di-GMP as the substrate. Thus, the specificities of these enzymes for the c-di-GMP structure appear to be closely related.

The significance of the cyclic structure is evident in the lower affinity of pGpG relative to that of c-di-GMP, by a factor of 10 for PDE-A and by at least 2 orders of magnitude for the synthase. Given the twofold rotational symmetry of c-di-GMP, a similar symmetrical disposition may be reflected in its protein-binding sites, as has been suggested from studies of cyclic dinucleotide inhibition of bacterial RNA polymerase (79). Comparison of the relative affinities of cdGpGp versus cdGpdGp, cIpGp versus cIpIp, and cXpGp versus cXpXp reveals that a given substitution is always more deleterious to the active structure when carried out in both GMP residues; this suggests that both residues participate in the recognition process. This might be taken as evidence that binding of a cyclic dinucleotide occurs in either one of the two alternate 180° rotational orientations that the molecule may adopt and that both binding modes contribute, in proportion to their frequency of occurrence and relative efficacy, to the observed potency of the analog. In such an analysis, however, the effect of substitutions on the overall conformation of the molecule is not taken into account; cyclic dinucleotides can adopt a variety of asymmetrical conformations in solution (24) and in the crystalline state (53); this necessarily limits many conclusions regarding the structure-function relationship.

TABLE 1. Analogs of c-di-GMP

Nucleotide	Relative affinity for[a]:	
	Cellulose synthase	Phosphodiesterase A
cGpCp (c-di-GMP)	1.00	1.00
cdGpGp	1.00	3.33*
cdGpdGp	0.17	1.00*
cIpIp	0.08	0.22*
cIpGp	0.33	0.63*
cApAp	0	0.01
cCpCp	0	0.01
cCpGp	0	0.01
cUpUp	0	0.01
cXpXp	0	0.18*
cXpGp	0.07	0.91*
cGp(S)Gp:Rp	0.04	0.31*
cGp(S)Gp:Sp	1.00	3.33*
cGpGpGp	0	0.16
pGpG	0	0.11
pppGpG	0	0.11
pppG (GTP)	0	0.01
ppG (GDP)	0	0
pG (5'GMP)	0	0
ATP	0	0

[a] Affinities of cyclic dimers, trimers, and related nucleotides are expressed relative to that of c-di-GMP for in vitro cellulose synthase activation or for inhibition of PDE-A activity, with ^{32}P-labeled c-di-GMP as the test substrate. In addition to inhibiting the enzyme, compounds with values marked with an asterisk undergo the PDE-A degradation reaction at rates comparable to that of c-di-GMP.

Positive Control: Diguanylate Cyclase

As with its cyclic mononucleotide-forming counterparts, adenylate cyclase and guanylate cyclase, the diguanylate cyclase of *A. xylinum* appears to occur in both a cytoplasmic and a membrane-associated form. In cell extracts the former predominates and has been the most extensively studied here. To a certain extent, the 20% polyethylene glycol-containing buffer routinely present during cell rupture can explain the enrichment of the enzyme in the membranous fraction. However, it has been shown that the resultant membrane preparation from cells broken in the absence of polyethylene glycol bear diguanylate cyclase and GTP-stimulated cellulose synthase activity, although this activity is markedly reduced (20).

The pathway of c-di-GMP synthesis attributed to diguanylate cyclase proceeds from GTP via the diguanosine tetraphosphate intermediate pppGpG (112a, 115). Each of

the two 3′,5′-phosphodiester bond-forming, PP_i-releasing steps in the pathway appears to be catalyzed by a single enzyme on the basis that a separate pppGpG-forming activity has not been isolated during the course of purification. In contrast, accumulation of the intermediate is more evident in incubations of purified diguanylate cyclase preparations than in crude cell extracts. This might indicate that within the cell, pppGpG is efficiently channeled into c-di-GMP without accumulating as a free, intracellular pool. Reflecting this aspect are the kinetic properties of the enzyme, of which both the affinity and catalytic rate parameters have been roughly estimated to favor the second reaction over the first by up to an order of magnitude. The apparent K_m value of 90 μM for GTP conforms with the presumed physiological levels of this nucleotide (25).

Presumably, the purpose of the diguanylate cyclase reaction is to communicate information about the metabolic state of the cell inherent to the intracellular concentration of GTP, such as overall energy charge and biosynthetic capacity, to the cellulose synthase via a high-affinity, rapidly diffusible, metabolically stable messenger molecule, the c-di-GMP activator. In the classic cyclic nucleotide systems, however, the concentration of the nucleoside triphosphate substrate is usually considered to be a secondary factor to the more prominent signals of G-protein and receptor-coupled cascades; in analogy, then, the activity of diguanylate cyclase would be subject to additional modulation. Although at this stage the nature of such an effector is still speculative, a low-molecular-weight, heat-stable material derived from mung bean extracts was shown to specifically inhibit the GTP-stimulated cellulose synthase reaction (12). Further analysis has shown that the inhibitor affects the cyclase reaction in a manner which is both reversible and noncompetitive with GTP (147a). Characterization of such an inhibitor is the object of avid pursuit because, whether it be a common metabolite or a novel compound, knowledge of its structure should lead to the uncovering of additional loci of regulation in the c-di-GMP system.

Although native diguanylate cyclase appears to be a multisubunit enzyme, the precise nature of its subunit composition has not been completely ascertained. The enzyme has been purified ca. 2,000-fold by affinity chromatography based on an immobilized form of the GTP substrate (113, 114). Polyclonal antibodies raised against two different peptides (59 and 61 kDa), both highly enriched during the course of the purification, have been shown to bind to the native enzyme but not to be cross-reactive with respect to the original antigens (112a). Thus, the native, soluble form of diguanylate cyclase, which has a molecular mass of 190 kDa, appears to be a hetero-oligomeric protein.

Because little is known about the cyclic dinucleotide content of the cell, much interest attaches to the function of nucleotides other than GTP in the reactions catalyzed by diguanylate cyclase. On the basis of inhibition data the enzyme appears to be highly selective toward GTP relative to other common nucleoside di- and triphosphates (13, 114). Furthermore, the Mg^{2+}-dependent binding of diguanylate cyclase to an immobilized ligand is not subject to displacement with free ATP, CTP, GDP, PP_i, or c-di-GMP. Although the synthetic analog guanosine-5′-(γ-thio)triphosphate (GTP-γ-S) is a substrate for the enzyme, the "nonhydrolyzable" GppNHp is not (13, 114). However, so far only dGTP and ITP have been tested directly as substrates (by high-pressure liquid chromatography [HPLC] analysis); the results indicate that the enzyme will utilize dGTP, albeit at relatively low efficiency, to yield the corresponding cdGpGp and cdGpdGp as products. On the basis of PDE-A and cellulose synthase reaction data (Table 1), these molecules would not be expected to be functionally distinct from c-di-GMP, and it is not clear whether they would be formed within the cell, considering the much higher concentration of GTP than dGTP (25). The fact that cellulose synthase and PDE-A appear to be compatible with a number of potentially naturally occurring cyclic dinucleotides suggests either that selective pressures within the cell to discriminate between structures was low, owing to the high efficiency of the cyclase in producing solely c-di-GMP, or that the relatively wide specificity is an integral feature of the system, possibly indicating a diversity in the cyclic nucleotide population of the cell. Analysis of nucleoside triphosphates as potential diguanylate cyclase substrates, as well as analysis of the cyclic dinucleotide content of the cell, should help to answer these questions.

Negative Control: C-Di-GMP Phosphodiesterase

Although two phosphodiesterases are associated with the degradation pathway, the role of PDE-A is more critical in that it inactivates c-di-GMP by cleaving the molecule to form pGpG. This form of negative control is irreversible in the sense that, at least within the in vitro system, the pGpG degradation intermediate is not subject to rephosphorylation (in the presence of ATP) but rather is rapidly degraded to produce two molecules of 5′-GMP. This latter reaction, catalyzed by the activity of PDE-B, evidently serves a salvage role, regenerating monophosphate units from one round of c-di-GMP formation and breakdown for de novo GTP synthesis.

The Mg^{2+}-dependent PDE-A and PDE-B reactions have been clarified as being due to the action of distinct enzymes mainly by the criteria of sensitivity to divalent cations, differential degree of intracellular distribution, and immunochemical analysis, in addition to substrate specificity (100a). The ratio of PDE-A to PDE-B activity in membrane preparations is approximately 10:1; in soluble extracts this ratio is reversed. Antiserum which effectively inhibits PDE-A activity has no inhibitory or binding effect on the PDE-B enzyme. Furthermore, in contrast to the PDE-B reaction, the Mg^{2+}-requiring ($K_a \approx 4$ mM) PDE-A activity is inhibited at low ($K_{1/2} \approx 50$ μM) concentrations of Ca^{2+} ions. Kinetically, the PDE-A reaction obeys a first-order rate pattern ($K_m \approx 0.25$ μM), typical of an enzyme bearing a single substrate-binding site.

The c-di-GMP-specific, Ca^{2+}-sensitive PDE-A has a crucial regulatory role and has been studied in further detail, with particular emphasis on regulatory properties and substrate specificity. A subunit molecular mass of 85 kDa has been assigned to the membrane-associated PDE-A, which in soluble form displays a native mass of ca. 190 kDa. The inhibitory effect of Ca^{2+} on the rate of hydrolysis of c-di-GMP appears to result directly from a reduction in the catalytic constant (V_{max}) of PDE-A activity, rather than from a K_m effect (114a). The mechanism of inhibition does not appear to be allosteric, as via a specific Ca^{2+}-binding site which paralyzes the enzyme when occupied, but rather may involve specific interaction with the cyclic dinucleotide substrate. PDE-A activity is also sensitive to the presence of polyethylene glycol; inhibition of c-di-GMP degradation offers an adequate explanation for the previously reported potentiating effect of this hydrophilic, protein-aggregating polymer on the synthase reaction under cell-free assay conditions (115).

PDE-A is highly specific for the c-di-GMP structure (Table 1). Although the cyclic dinucleotide-binding sites of both PDE-A and the cellulose synthase would appear to be closely related on the basis of both absolute and relative affinities for c-di-GMP and related analogs, PDE-A appears to have wider specificity than the cellulose synthase. Their overlapping specificity is evident when considering that all of the cyclic dinucleotide activators of cellulose synthase serve as substrates in the PDE-A reaction and that a number of other cyclic dinucleotides (i.e., cApAp, cCpCp, and cUpUp) show low or no affinity for either enzyme. However, the existence of at least one exception in this comparison, namely that cXpXp is a substrate for PDE-A activity but not an activator of the synthase, demonstrates that the specificities are not completely overlapping. In addition, PDE-A activity is inhibited by a variety of guanyl nucleotides which do not affect the synthase; these include cCpGp, cGpGpGp, pGpG, GpG, pppGpG, and GTP. The affinity of the phosphodiesterase for these last two compounds, whose structures digress significantly from that of the c-di-GMP substrate, may represent an additional locus of regulation based on inhibition of PDE-A activity. These results are compatible with the observations that PDE-A activity (in soluble form) binds to GTP-affinity columns and may be eluted with free ligand. The physiological basis for this inhibition may lie in the fact that both GTP and pppGpG are substrates in the activator-forming diguanylate cyclase reaction.

Therefore, although the two enzymes PDE-A and cellulose synthase share a similar high degree of specificity for the c-di-GMP structure, their cyclic dinucleotide-binding sites are not identical. In addition, the cellulose synthase reaction is insensitive to Ca^{2+} ions, whereas PDE-A is unaffected by PHMB. Indeed, highly purified preparations of cellulose synthase are devoid of detectable PDE-A activity or protein subunits, as judged by Western blot analysis with anti-PDE-A antiserum (100b). These observations have led us to conclude that the cellulose synthase and PDE-A are structurally and functionally distinct enzymes.

The use of the phosphothioate linkage in c-di-GMP analogs raises the prospect of obtaining a highly potent activator of cellulose synthase which is stable to enzymatic degradation. Both of the monophosphothioate analogs tested so far (Table 1) resist degradation at the modified bond, with the *S*-diastereomer revealing far greater potential as an activator and as a PDE-A-resistant molecule. These compounds may be considered as prototypes for the design of a nonhydrolyzable c-di-GMP analog, such as a diphosphothioate structure in which both linkages contain a substituted exocyclic oxygen in the *S* configuration. Examples of nonhydrolyzable but biologically effective nucleotide phosphothioate analogs abound, and derivatives of this type could be particularly informative when implemented in whole-cell studies (49).

Why C-Di-GMP?

The components and properties of the in vitro cellulose-synthesizing system derived from *A. xylinum* indicate that a highly specialized and complex system exists in the cell for regulation of this process. However, the raison d'être for such a novel mode of regulation requires some clarification because neither the precise stage in cellulose synthesis nor other cellular processes (if any) have been identified for c-di-GMP. As a unifying theme in this discussion, the c-di-GMP regulatory system will be posed against the many unsolved aspects of cellulose biogenesis in the hope that the unanticipated appearance of this novel molecule in a cellulose-producing bacterium will stimulate deeper probings into the nature of this process and into cellular regulatory mechanisms in general.

The type of regulation in which an allosteric effector such as c-di-GMP exerts its effect on the V_{max} of its enzyme target is usually associated with the physiological situation in which the intracellular concentration of substrate is well above the saturating level; indeed, in *A. xylinum* cells the intracellular level of UDP-Glc (>1 mM) is in great excess relative to the synthase K_m value for this substrate (147b). The functioning of this regulatory system in relation to the overall physiology of the organism is still difficult to ascertain, mainly because *A. xylinum* is an obligate aerobe and appears to maintain a uniform ratio of cellulose production to carbon oxidation (CO_2 release) and growth rates under a variety of conditions (149, 150). This phenomenon could be attributed to the optimized state of the regulatory system, designed to keep the polymerization process in step with the pace of cellular metabolism by a mechanism whereby synthase activity is linked to other GTP-dependent polymerization processes, such as nucleic acid and protein synthesis.

On the basis of the stoichiometry of the cellulose synthase reaction, the metabolic cost of inserting glucose into a β-1,4-glucan chain is two high-energy phosphate bonds. Because the ratio of glucose polymerized to glucose oxidized is approximately 3:1 under optimal conditions (147b), the cell appears to invest ca. 10% of its total energy budget in this process. Thus the cellulose synthase, which performs the committed step in cellulose biogenesis, is found under strict regulatory control. Other factors may also control the overall capacity of the cell for producing cellulose, because high-cellulose producers of potential industrial value have been isolated (see Biotechnology Perspective, below). Such strains may be enriched in the copy number of the cellulose synthase itself or may vary in other components associated with the novel regulatory system or essential aspects of membrane structure.

Paradoxically, the activity levels of the c-di-GMP-forming and -degrading enzymes, as measured in vitro, both fall within the same range as that of the cellulose synthase. On this basis it would appear that as much energy is invested in the regulation of cellulose synthesis as in the overall process of glucose polymerization. If indeed the rapid turnover of c-di-GMP is an integral feature of the system, this would allow for equally rapid variations in intracellular concentrations of the activator in response to additional regulatory signals such as Ca^{2+} ions. However, in recognition of the possibility that in vitro rates do not faithfully represent conditions within the intact cell, the estimated turnover rate of c-di-GMP might actually be much lower.

These aspects should be considered in regard to the intracellular concentration determined in ^{32}P-labeled *A. xylinum* cells for c-di-GMP (5 to 15 μM [79a]), which is an order of magnitude higher than that predicted from the affinity of its known binding sites in vitro. This is often the case for other cyclic nucleotides, namely cyclic AMP (cAMP) and cGMP. Thus, during the course of studies on the repression of the pyruvate phosphate dikinase level (see Rise of the Sugar Nucleotide Precursor, above) in *A. xylinum*, it was found that cells grown on succinate contain a 20-fold-higher level of cAMP (10 μM) than glucose-grown cells do (1). Presumably the role of the adenine nucleotide messenger here is in the regulation of the so-called catabolite repression responses characterized for other gram-negative bacteria. It would be interesting to characterize further the

adenylate cyclase system from *A. xylinum* and compare its mode of function with that of the c-di-GMP system.

From an alternative perspective, the c-di-GMP regulatory system may serve in an organizational capacity. For example, the structural complexity of the cellulose microfibril and the overall cell-directed nature of ribbon formation imply that the polymerizing sites must be maintained in rigid coordination; the c-di-GMP regulatory system might conceivably function in such a capacity. The membrane-associated state of the cyclase, as well as of the regulatory phosphodiesterases, raises the possibility that within the membrane structure of the intact cell these enzymes are situated in close proximity to their regulatory target, the cellulose synthase. Together, these may make up the discrete proteinaceous complexes ascribed by electron-microscopic studies as the presumed cellulose-synthesizing sites of the cell membrane structure (26, 157).

The cyclic dinucleotide may fulfill such a functional role in the organization of the polymerization process. Presumably, the overall polymerization process occurs in the three separate phases, namely that of polyglucan chain initiation, elongation, and termination, followed by a new initiation event; however, a clear distinction between these would not be applicable if, as may be the case, synthase units produce a number of chains simultaneously or the capacity of an individual enzyme unit is limited to the production of a single glucan chain. The present assay method does not distinguish among these various possible stages, although recently a technique for the determination of average chain length has been used to analyze the product of the in vitro reaction (29), with the startling result that each newly synthesized polyglucan molecule contains approximately 5,000 glucose residues. Unfortunately, the latter result has been reported for only one set of experimental conditions (at saturating c-di-GMP concentrations). A detailed analysis of the catalytic mechanism of cellulose synthase based on degree of polymerization values on even the relatively superficial level of kinetic parameters would impose a formidable technical challenge, considering that cellulose synthase units might be engaged in different stages of chain elongation when isolated (108). As a consequence of this quagmire, it is difficult to ascertain which, if any, particular rate-limiting step in the polymerization process is accelerated in the course of c-di-GMP activation.

Another type of organizational requirement proposed for cellulose synthesis in *A. xylinum* was originally formulated from the assembly-disrupting effect of Calcofluor White (see Assembly of the Cellulose Fibril, above). This effect has been interpretated to indicate that the rate of polymerization is rigidly synchronized between adjacent synthesizing sites or possibly throughout the cell membrane to ensure the orderly assembly of the fibril structure on all levels (16). If the c-di-GMP regulatory system functions in this pacemaker capacity, then within the system is probably incorporated some type of feedback response which is not yet apparent experimentally.

Regardless of the overall mechanism of catalysis, c-di-GMP binding to the cellulose synthase appears to invoke a critical conformational change, as is most dramatically evident in the 50- to 200-fold activation effects observed in vitro. Such a wide variation in the level of activity bestows an on-off quality to the regulation process and raises interesting questions regarding the autonomy of the enzyme; this result may also point to a signaling role for c-di-GMP in chain initiation and/or termination. This issue warrants further exploration because the feature of absolute dependence on the cyclic dinucleotide activator may come to bear on a deeper understanding of the mechanism of cellulose synthesis in *A. xylinum* as well as in other organisms.

COMPARATIVE BIOCHEMISTRY OF CELLULOSE SYNTHESIS

Other Bacteria

Whereas the biosynthetic apparatus of all strains of *A. xylinum* tested to date are essentially identical, among the other cellulose-producing bacteria, only *Agrobacterium tumefaciens* has been examined further. There is now good evidence that cellulose synthesis occurs in this gram-negative bacterium by a mechanism similar to that of *A. xylinum*. This was first indicated when UDPG:β-glucan synthase activity in solubilized membranes derived from this organism and separated by native PAGE was shown to be stimulated in the presence of c-di-GMP (139). Membrane preparations of this organism have recently been shown to possess a Mg^{2+}-dependent cellulose synthase activity, which is stimulated up to 20-fold in the presence of c-di-GMP (5). The synthase displayed normal Michaelis-Menten kinetics with respect to the substrate UDP-glucose, with an apparent K_m of 0.15 mM. c-di-GMP has been detected in acid extracts of ^{32}P-labeled cells grown in various media. Furthermore, enzyme systems responsible for c-di-GMP formation and degradation, namely diguanylate cyclase and PDE-A, respectively, have been demonstrated in in vitro assays of *A. tumefaciens*. These findings suggest that the c-di-GMP regulatory system of cellulose synthesis is operative in this organism. This has been substantiated by immunochemical analysis with anti-*A. xylinum* cellulose synthase antibodies, which revealed the presence of immunologically related peptides in extracts derived from *A. tumefaciens* cells (100b). Taken together, these studies are gradually revealing a common enzymatic mechanism for bacterial cellulose biogenesis and its regulation.

Plants and Algae

Because the basic molecular structure of cellulose has been preserved throughout evolution, nearly all of the questions asked about bacterial cellulose biogenesis are germane to the investigation of higher organisms. Plant and some algal cells construct multilayered walls based on cellulose microfibrils, as do some tunicates (44, 62).

Perhaps the most unifying feature, recognized now for many years, is at the cytostructural level (26, 44, 62). In plants and algae, cellulose microfibril synthesis is apparently associated with stereotypically organized arrays of intramembrane particles, known as terminal complexes and rosettes, which appear to be analogous to the extrusion pore arrangement described for the *A. xylinum* cell envelope. The clustering of such particles, as revealed by freeze-fracture studies of plant and algal membranes, closely mirrors the pattern of microfibril orientation and spacing within the innermost cellulosic layers. Cell-directed mechanisms of microfibril assembly have been proposed for plant cellulose biogenesis, and, indeed, cellulose-binding materials interfere with microfibril deposition at the level of the cell wall (27).

The biological occurrence of terminal complexes and rosettes has been classified according to phylogenetic history, wherein the distinction is made between fixed and mobile bigenetic apparatus (27, 77, 89). In many eukaryotes, the intramembrane particles of cellulose synthesis are pre-

sumed to engage in lateral movement within the cytoplasmic membrane, which is channeled in appropriate directions as a result of the underlying microtubular cytoskeleton (61, 69, 93, 111, 130). Such a mobile mechanism of deposition, which is presumably driven by the force of microfibril crystallization, accounts for the encapsulated state of the plant cell within a layer of continuous fibrils. The pattern of cellulose deposition is a key determinant in plant growth because the axial orientation of cell expansion is limited to the area of weakest opposition to turgor pressure. In contrast, the sites of cellulose synthesis described for *A. xylinum* and other prokaryotes consist of fixed intramembrane particles (27, 89).

Regrettably, an efficient demonstration of cellulose synthase activity in a cell-free system from plants has not yet been realized. Any definitive comparison of bacterial and plant cellulose biogenesis on the enzymatic level is therefore premature. On the basis of biochemical evidence (43), UDP-Glc appears to be the most likely candidate for the sugar nucleotide precursor in plant systems. Most workers attempting to demonstrate this enzyme activity by using plant cell membranes in vitro use UDP-[^{14}C]glucose as the test substrate. Often, efforts are stymied by competing UDP-Glc-utilizing transferase reactions, such as sucrose synthase and, notably, the β-glucan synthase responsible for callose formation. The synthesis of callose, an insoluble, unbranched β-1,3-linked glucose polymer, is stimulated as part of the cellular wound response which is evidently evoked upon preparation of cell extracts (43, 44). The requirement for a transmembrane electrical potential for cellulose synthesis in cotton (7) has been taken to signify the essentiality of an intact membrane to the polymerization process itself; however, in *A. xylinum*, for which a similar requirement has been demonstrated, the catalytic apparatus may be obtained in vitro in a highly active form. One, perhaps significant, distinction between eukaryotes and prokaryotes (e.g., *A. xylinum*) is the insensitivity of the latter group to the herbicide 2,6-dichlorobenzonitrile, which is an apparently specific inhibitor of cellulose synthesis in algae and plants (47). Judicious application of the bacterial activator c-di-GMP in attempts to demonstrate plant cellulose synthase activity in vitro has proved fruitless in our laboratory and others (44). Similarly, activators of the plant β-1,3-glucan synthase, including an endogenous β-glucolipid compound and the alkyl β-glucosides which mimic it (30), do not affect the *A. xylinum* system (29b).

One stimulating model that has been proposed includes a common step in the mechanism for plant cellulose and callose biosynthesis (44). Conceived on the basis of the model of lactalbumin modulation of galactosyltransferase substrate specificity, the model presumes that there is a single plasma membrane-localized glucosyltransferase with the capacity to produce either β-1,4-glucan or β-1,3-glucan product in accordance with the state of the membrane: at normal transmembrane electrical potential and low intracellular Ca^{2+} levels, the enzyme acts as a cellulose synthase; upon membrane damage which dissipates the potential and elevates the Ca^{2+} level, the callose synthase activity is activated and the cellulose synthase activity is suppressed. This regulation is presumed to be mediated by a dissociable, acidic, 18-kDa peptide which binds to 2,6-dichlorobenzonitrile.

In contrast, it has been possible to study β-1,4-glucan synthesis in membranes from the fungus *Saprolegnia monica*, which also synthesizes β-1,3-glucans. The two synthases have been solubilized and extensively purified. In their native forms, both are of high molecular weight and apparently contain subunits of different sizes (28a, 50). This system shares a common regulatory feature with bacterial cellulose synthesis in being sensitive to GTP. In this system, the nucleoside triphosphate depresses the synthesis of β-1,3-glucan and stimulates β-1,4-glucan formation. Recently it has been reported that c-di-GMP stimulates the β-1,4-glucan synthase activity but not the β-1,3-glucan synthase activity of this organism. The activator was efficient on solubilized enzymes, increasing, as in the bacterial system, the V_{max} without modifying the K_m for UDP-Glc (56a).

Of great potential importance is recent immunochemical evidence which indicates a more direct link between the bacterial and plant systems (100b). The preparation of anti-cellulose synthase antibodies from the *A. xylinum* protein, demonstrated to cross-react with a similar set of peptides from *Agrobacterium tumefaciens*, also labels, upon Western blot analysis, a number of peptides from wheat, cotton, mung bean, and pea extracts. Recent affinity-labeling studies have revealed the presence in cotton extracts of a peptide which specifically binds both c-di-GMP and UDP-Glc and which has the same molecular weight as that of the peptide reacting with the above antibodies (5a). These recent findings suggest a high degree of homology between the cellulose synthase of diverse organisms and ultimately may lead to a more unified theory of cellulose biogenesis.

Related Bacterial Exopolysaccharides

The evidence from which to ponder upon a unified mechanism for the polymerization reactions and noncovalent discharge and assembly steps in the biosynthesis of bacterial exopolysaccharides in general still appears to be too disperse and meager. However, excellent reviews of this topic are available (135, 152). It should be noted that many of the same types of queries have not yet been answered for the problem of outer membrane and secreted proteins (107). Bacterial cellulose synthesis may offer many interesting experimental possibilities for studying membrane-mediated biosynthesis and secretion, because this energy-dependent process occurs in a discretely organized pattern and may be directly visualized. In most of the cases mentioned below, the biological function of the exopolysaccharide is not patently evident. On the surface at least, it would seem likely that the membrane-associated β-glucan synthases found in bacteria, if not throughout nature, share more in common than just UDP-Glc.

In addition to crystalline cellulose, *A. xylinum* strains have been reported to produce a variety of water-soluble β-linked glucose polymers such as β-1,2-glucan chains (117) and substituted β-1,4-glucan chains in which every second or third residue bears a side chain composed from glucose, mannose, and glucuronic acid in various proportions (41, 144). In some cases these exopolysaccharides have been determined to arise from a diverse range of carbon sources and to occur concomitantly with cellulose synthesis. In its structure and mode of biosynthesis, the high-molecular-weight pentasaccharide-substituted β-1,4 backbone polymer of *A. xylinum* known as acetan (41) closely resembles the xanthan gum product of the plant pathogen *Xanthomonas campestris* (80). In vitro, the membrane-associated synthesis of these polymers proceeds from UDP-Glc via the initial formation of a diphosphoprenyl cellobiosyl, which is subsequently modified in reactions from other nucleotide sugars (GDP-mannose, dTDP-glucuronic acid) to give rise to the heptasaccharide monomeric unit of the in vivo product.

Such a lipid-linked cellobiose intermediate is observed only in strains which produce acetan; an interesting observation, however, is that when acetan production in cellulose-producing colonies is lost upon aging, the altered colony morphology is accompanied by an enhanced rate of cellulose synthesis. The polymerization in vitro of the corresponding lipid-linked pentasaccharide monomer of *X. campestris* has been demonstrated (80). Membrane preparations from Cel^+ *A. xylinum* strains have also been observed to utilize UDP-Glc in forming a high-molecular-weight β-1,2-homoglucan (117); however, such a material has been detected in only small quantities in vivo. Numerous other water-soluble acetanlike and xanthanlike exopolysaccharides have been reported for *A. xylinum* (135). No relationship has been established to suggest that any of the above structures participate as intermediates in cellulose synthesis, which may be the sole exopolysaccharide-producing process in many strains, in which it is insensitive to antibiotics such as bacitracin and tunicamycin (1a), which inhibit glycosylation reactions at the level of the lipid intermediate.

Because acetan can be considered a substituted cellulose, it is remarkable that the same organism has different machinery for producing two structurally related exopolysaccharides (41). It could be argued that homopolysaccharides such as cellulose do not need to preassemble repeating units on prenyl derivatives and can be produced by the classic stepwise addition of the corresponding monosaccharide, but this is not the case for the well-studied systems that make another exohomopolysaccharide, namely the polysialic capsular polysaccharide from *Escherichia coli* and *Neisseria meningitidis* (97, 142). In this system, polymerization appears to occur via a mechanism in which NeuNAc is transferred from CMP to the nonreducing end of an endogenous polysialic acceptor via the undecaprenol monophosphate saccharide as the intermediate; this novel mechanism may have evolved to maintain the α configuration at C-2.

Despite the dissimilarity to the proposed direct transfer mechanism for cellulose synthesis, the sialyl transferase system is interesting from the point of view of membrane dynamics (141). (Actually, two different isomeric forms of polysialic acid bearing α-2,8 and α-2,9 linkages have been described [152].) Polysialic acid synthesis has been shown to proceed in vitro from a low-density vesicle fraction containing components of both the inner and outer membrane, suggesting that it might represent a Bayer-type of adhesion site (153). The mature-length polymeric product appears first to pass through the periplasm and may be found in exocellular form connected to the cell via a 1,2-diacylglycerol (152).

Like *A. xylinum*, both *Agrobacterium* and *Rhizobium* species produce a heteropolysaccharide, but one that is composed from an octasaccharide repeating unit containing glucose, galactose, and pyruvate in the ratio 7:1:1 (22, 74, 75, 158). Synthesis of this polymer appears to occur via the corresponding polyprenol diphosphate saccharide, which grows from the addition of galactose followed by glucosylation in a sequence of β-1,4, β-1,6, and β-1,3 bonding, as determined in vitro by using radiolabeled UDP-Glc or UDP-Gal as a substrate (131). The succinoglycan *Agrobacterium* sp. is synthesized simultaneously with other exopolysaccharides, including curdlan, a β-1,3-glucan which appears to contain some degree of organized tertiary structure and yields insoluble gels (86). An apparently unrelated gel-forming neutral exopolysaccharide has been characterized for certain *Rhizobium* spp. (159).

Low-molecular-weight classes of periplasmic β-1,2-glucans are reported to occur in a wide range of gram-negative bacteria (84). Studies have been performed on in vitro synthesis of linear β-1,2 chains from UDP-Glc in membrane preparations from *E. coli*, in which the native products, known as membrane-derived oligosaccharides (MDOs), typically occur as β-1,6-branched derivatives in linkage to phosphoglyceride and/or succinate, as well as in membranous preparations from *Agrobacterium radiobacter* and *Rhizobium phaseoli*, in which the predominant in vivo species is an unbranched cyclic molecule (4) containing 17 or more residues. The precursor role of UDP-Glc in MDO biosynthesis was founded upon biochemical and genetic evidence (84). In in vitro MDO biosynthesis, the requirement for Mg^{2+}, exogenously added primers such as octyl β-glucoside, and acyl carrier protein has been characterized, although the stimulatory mechanism of acyl carrier protein is unknown. A direct-substitution, processive mechanism has been proposed for the β-1,2 transglucosylation reaction. MDO biosynthesis appears to play an important role in diglyceride metabolism, and, like the MDO-like materials of other gram-negative genera, its occurrence in the periplasm is extremely sensitive to the osmotic balance and possibly the K^+ ion flux.

Cellulose synthesis resembles that of other bacterial exopolysaccharides only insofar as UDP-Glc appears to be a common substrate for the transglucosylation step and the biosynthetic machinery is associated with the cell membrane. Considering the current level of knowledge, it might be speculated that UDP-linked sugars are a general form of the substrate in the membrane-associated production of β-linked homoglucans, such as β-1,4- and β-1,3-glucan in eukaryotes (30, 44, 50, 125) and β-1,4- and β-1,2-glucan in bacteria. The chitin synthase of *S. cerevisiae* also uses UDP-*N*-acetylglucosamine (82). Although sensitivity to GTP and the apparently processive nature of the reaction (i.e., lack of lipid intermediate requirement) indicated in some of the more well-studied cases (50, 114, 125) may be other unifying features, no additional common denominators have yet been identified for β-glucan synthases.

BIOLOGY OF BACTERIAL CELLULOSE: FUNCTION AND GENETICS

Although in many ways *A. xylinum* may be the most thoroughly characterized organism with respect to cellulose synthesis, a wide range of bacteria, including *Pseudomonas*, *Achromobacter*, *Alcaligenes*, *Aerobacter*, *Azotobacter*, *Agrobacterium*, *Rhizobium*, and *Sarcina* species, produce cellulose (42). These may also be gram-negative, obligative aerobes or, alternatively, anaerobes bearing a gram-positive cell wall structure, perhaps reflecting a diverse evolutionary history for cellulose biogenesis (Fig. 6). All of the cellulose-forming bacteria known to date are distinguished by the extracellular nature of the glucan product and the formation of cell aggregates (known as flocs), which is attributable to the high self-affinity of cellulosic material. In contrast, plant cellulose is an intrinsic component of the cell wall. However, bacterial cellulose also appears to fulfill a structural role in the sense that it may confer mechanical, chemical, or biological protection within the natural habitat, as in *A. xylinum* and *Sarcina ventriculi*, or facilitate cell adhesion processes necessary for symbiotic or infectious interactions, as in *Rhizobium* and *Agrobacterium* species. These last two genera are closely related in other respects, including on the DNA level (70). In some cases the biological advantages have not been clearly established. Many of the cellulose-

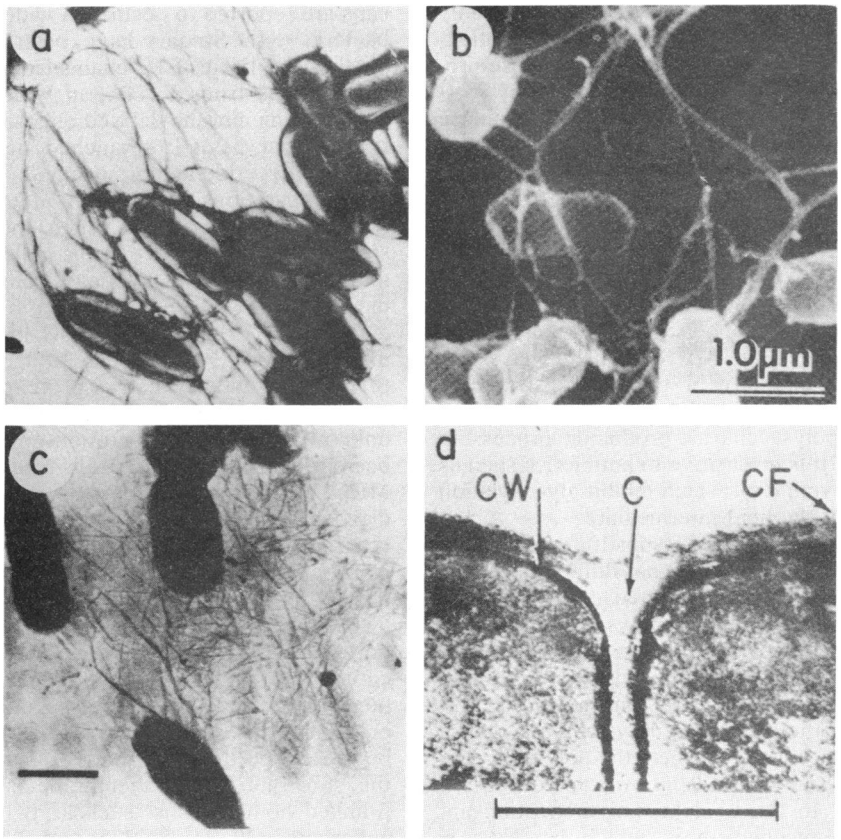

FIG. 6. Electron micrographs of cellulose-producing aerobic gram-negative bacteria. (a) Intertwining cellulose ribbons formed by *A. xylinum*. Magnification ×7,000. Reproduced from reference 16. (b) Cellulose fibrils elaborated by *Agrobacterium tumefaciens*. Reproduced from reference 100 with permission. (c) Cellulose microfibrils produced by growing *Rhizobium trifolii*. Bar, 1 μm. Reproduced from reference 102 with permission. (d) Electron micrograph of cellulose-producing anaerobic gram-positive bacterium. Thin section through *Sarcina ventriculi* cells. CF, Cellulose fibrils; CW, cell wall; C, intercellular cellulose with fibrous structure. Bar, 1 μm. Reproduced from reference 32 with permission.

producing bacteria also form other extracellular polysaccharides, and the relationship between these and cellulose is not clear either. Of notorious difficulty is the specific determination of cellulose in biological systems, especially in the presence of other β-glucans; these aspects have been discussed previously (44).

A. xylinum

Colonies of *A. xylinum* in static culture produce a readily visible film of cellulose which covers the surface of the growth medium. Under suitable conditions (14), as much as 50% of the supplied carbon substrate may be assimilated into cellulose to produce a remarkably thick pellicle (Fig. 7). When a broth culture is shaken or stirred, *A. xylinum* grows more rapidly as a result of the increased oxygen tension in the medium. Under these conditions, a well-organized pellicle is not formed but, rather, round balls of cellulose are typically observed (123). The inclusion of cellulolytic enzymes in the nutrient medium was reported to further augment the growth of cells in shaken cultures without affecting their cellulose-synthesizing capacity once the cellulase in removed (26a, 47a). In general, cellulose synthesis occurs at a rate that is linear with respect to the cell concentration (73, 81) and, as mentioned above, is constitutive with respect to the carbon source supplied for cell growth. Rates, however, are strain specific, and, indeed,

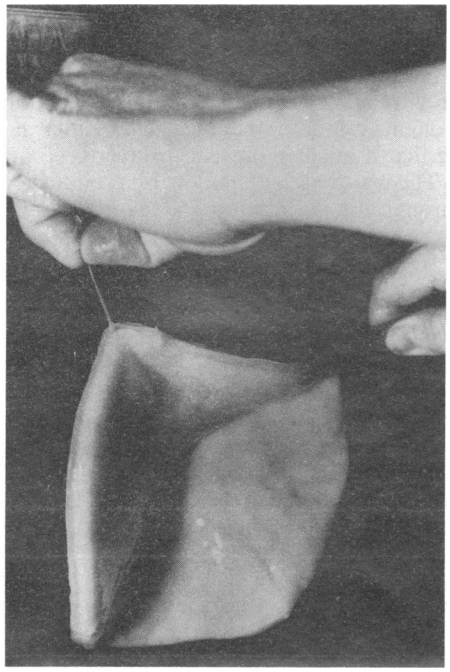

FIG. 7. Cellulose pellicle formed by a static culture of *A. xylinum* after 24 h at 30°C.

cellulose overproducers, which form thick pellicles under static conditions (154) and/or thicker aggregates during growth in rotary shakers, have been reported (8a).

Presumably, the purpose of this extracellular material is to provide a firm surface matrix in which the embedded cells of *A. xylinum*, an obligate aerobe, may benefit from close contact with the atmosphere (37, 123). In addition to the notion that the cellulose mat serves as a flotation device, alternative environmental roles for cellulose produced by *A. xylinum* have been adduced (154). In experiments measuring colonization by *A. xylinum*, molds, and other bacteria on pieces of apple, the cellulose pellicles promoted colonization of *A. xylinum* on the substrate and provided protection from competitors which used the same substrate. Cellulose pellicles were also observed to protect *A. xylinum* cells from the killing effects of UV light. Thus, cellulose pellicles produced by *A. xylinum* may have multiple functions in the growth and survival of the organisms in nature. Curiously, electron-microscopic analysis of *A. xylinum* pellicles has revealed the presence of tunnellike lacunae arranged at regular intervening intervals between microfibril bundles (140). This morphology may indicate a previously unsuspected high degree of organization in pellicle formation.

Aeration of cultures of most *A. xylinum* strains by stirring or shaking gives rise to spontaneous non-cellulose-producing mutants, a phenotype which appears to enjoy the selective advantage when oxygen is in abundance (123). Such Cel^- mutants may be isolated and may prove to be stable and nonreverting over the course of years of cultivation (123). Alternately, they may revert to the Cel^+ phenotype when subjected to static culture conditions (145). The precise frequency of such mutational events is best ascertained by plating and identification of Cel^+ and Cel^- colonies by either direct visualization or specific staining reactions, because performing the initial isolation by sampling directly from liquid cultures may be influenced by the greater accessibility of Cel^- cells relative to cellulose producers, which are embedded in their pellicular or floccular product. Significantly, high-cellulose-producing strains have been isolated from which the spontaneous appearance of the Cel^- phenotype appears with practically undetectable frequency (8a).

Chemical mutagenesis of *A. xylinum* cells induces a high frequency of Cel^- mutations. In one strain tested, the majority of such mutations apparently do not reside in the structural genes of cellulose synthesis, because production of this polymer could be activated phenotypically by antibiotics that block RNA or protein synthesis (145). Transient cellulose negativity has been reported for a wild-type *A. xylinum* strain upon cultivation on a defined minimal medium (154). These observations may indicate that cellulose synthesis in *A. xylinum* may be subject to some type of on-off regulation, which deserves further characterization. Indeed, it has never been established whether all of the cells, or only a select proportion, within a pellicle-forming culture are actively producing cellulose.

On the molecular level, the genetics of cellulose synthesis in *A. xylinum* are rapidly coming of age. Although the genes governing cellulose synthesis were once thought to be confined to the often complex plasmid arrangement occurring in this species, some Cel^+ strains have been reported to be cured of plasmids. Recently, an operon encoding four proteins required for bacterial cellulose synthesis (*bcs*) in *A. xylinum* has been isolated by genetic complementation with strains lacking cellulose synthase activity (154a). Nucleotide sequence analysis indicated that the operon is 9,217 bp long and consists of four genes. The catalytic molecular masses of the proteins encoded by *bcsA*, *bcsB*, *bcsC*, and *bcsD* are 84.4, 85.3, 141.0, and 17.3 kDa, respectively. The genes appear to be translationally coupled and transcribed as a polycistronic mRNA with an initiation site 97 bases upstream of the coding region of the first gene in the operon. Results from genetic complementation tests and gene disruption analyses demonstrate that all four genes are essential for maximal cellulose synthesis. The second gene in the operon (*bcsB*) encodes the catalytic subunit of cellulose synthase. The structure of the first 24 amino acids of the sequence deduced from this gene is remarkably similar to that of signal peptides of secreted proteins in other bacteria. This suggests that cellulose synthase is synthesized as a precursor and is processed and deployed in the membrane (see Glucose Polymerization: the Cellulose Synthase, above). The exact functions of the other three gene products in cellulose synthesis are unclear. However, it appears that they probably play a role either in the complex processes of transport and crystallization of the cellulose product (see Assembly of the Cellulose Fibril, above) or in the regulation of cellulose synthase activity. In this connection it has also recently been reported for another strain of *A. xylinum* (120a) that the gene coding for an 83-kDa peptide presumed to represent the UDP-Glc-binding site of cellulose synthase has been cloned and sequenced. The relationship between the gene and in vivo cellulose synthesis has not yet been established by genetic analysis. It will be of interest to compare the DNA sequence of this gene with that of the *bcs* operon.

The use of gene libraries constructed from the *A. xylinum* genome has also led to the identification of critical enzymatic steps in the pathway of cellulose synthesis, namely that catalyzed by UDP-Glc pyrophosphorylase (143) (see Rise of the Sugar Nucleotide Precursor, above). The structural gene coding for this enzyme was first evident upon shotgun transconjugation from *E. coli* cells harboring the library of three Cel^- strains of *A. xylinum* obtained by chemical mutagenesis. In each case, the same 2.3-kb fragment was detected as the complementing agent. Biochemical analysis by in vitro assay for all the enzymes currently known to be involved in either the pathway of glucose polymerization (hexokinase, phosphoglucomutase, UDP-Glc pyrophosphorylase, and cellulose synthase) or its regulation (diguanylate cyclase, PDE-A, and PDE-B) identified a 100-fold decline in the level of UDPG pyrophosphorylase as the common deficiency. This conclusion was confirmed upon successful complementation of a non-galactose-utilizing *E. coli* strain with the same gene fragment, a result which also demonstrates the apparent ability of the *E. coli* system to recognize *A. xylinum* promoter regions.

The ability of some strains of *A. xylinum* to produce acetan (144) (see Related Bacterial Exopolysaccharides, above) warrants further attention. An apparent connection between this polymer and cellulose has been proposed on the basis of the observation that synthesis of the heteropolysaccharide is inhibited concurrently with induction of cellulose synthesis by tetracycline in certain strains (145). These phenomena may point to the existence of additional regulatory features of cellulose biogenesis.

Agrobacterium tumefaciens

The plant pathogen *Agrobacterium tumefaciens* is a free-living soil organism that is readily isolated and grown in the laboratory. Glucose metabolism by this gram-negative, obligative aerobe involves the Entner-Doudoroff pathway (55%) and the pentose cycle (44%), whereas terminal respi-

ration includes both the tricarboxylic acid and the glyoxylic acid cycles (6). Cultures of this organism produce cellulose from a wide variety of substrates. Cellulose synthesis is associated with flocculation, and such flocs may be dispersed by the addition of cellulase (42). As in *A. xylinum*, cellulose fibrils also appear to arise from the side of *Agrobacterium* cells; however, the ultrastructure of the final product differs significantly in each case. Although an apparently advanced mechanism of microfibril assembly is operative in *A. xylinum*, leading to distinct ribbons and a pellicle of great strength and hydrophilicity, the analogous process in *A. tumefaciens* results in the formation of simple bundles and flocs (27, 100). *A. tumefaciens* also produces other exocellular polysaccharides, as described in an earlier section (see Related Bacterial Exopolysaccharides).

The cellulosic product of this organism has been verified on the basis of enzymatic (5, 100) and infrared (42, 100) analyses; X-ray diffraction analysis revealed a pattern typical of cellulose I (42). Cellulose synthesis in vivo, quantitatively assessed with [^{14}C]glucose as the tracer, occurs at a rate of about 1/10 that of *A. xylinum*. This difference is similarly reflected in the lower level of cellulose synthase activity found in cell-free membrane preparations from *A. tumefaciens* (5). As related in a preceding section, the two bacteria appear to share the same enzymatic and regulatory mechanism for cellulose synthesis (see Other Bacteria).

Cellulose synthesis in *A. tumefaciens* is apparently a constitutive feature of the genus, because cells synthesize cellulose during growth in the absence of plant cells (5, 100) and resting cells maintain a high capacity for cellulose synthesis (5). The addition of plant extract, such as Soytone, to synthetic media enhances cellulose synthesis, and it was suggested that a substance released from wounded tissue may act similarly (99). In this connection it is interesting that floc formation was more pronounced in cultures grown in media with either a high C/N ratio or low substrate concentration (42). These effects suggest that extracellular signals may influence the rate of cellulose synthesis.

A. tumefaciens is best known for its parasitic invasiveness in plants as the causative agent of the formation of tumors (crown galls), which afflict most dicots, some gymnosperms, and a few monocots (99). Following attachment to the plant cells, the bacteria begin to multiply in the host tissues. The induction of tumors is associated with the transfer of the large Ti DNA plasmid, a small part of which is subsequently integrated into the nuclear genome of the plant cell. The ability of *Agrobacterium* DNA to integrate stably into the plant host genome has led to its widespread exploitation as a vector for the genetic transformation of higher-plant cells with recombinant DNA molecules.

The function of cellulose in this bacterium is related, at least in part, to the pathogenicity of the bacterium. An early step in the infection process is the site-specific attachment of the bacteria to the plant host cells (92). During this phase, the bacteria synthesize cellulose fibrils that cover the surface of the plant cells, thus facilitating attachment (99). Cellulose is not sufficient for the attachment process, because nonattaching, cellulose-forming mutants of *A. tumefaciens* have been isolated (98). Cellulose is also not completely necessary for bacterial virulence, because transposon mutants deficient in cellulose synthesis retain their pathogenecity (98). However, the binding of these mutants to plant cells is weaker than that of the parent strain; cellulose-producing bacteria cannot be removed from carrot suspension culture cells after they have bound without removal of some of the carrot surface with enzymes such as cellulase. The Cel$^-$ mutants, on the other hand, may be easily removed by vigorous vortexing (98). Similarly, the ability of the wild-type strain to produce tumors is unaffected by rinsing the site of infection with water, whereas the tumorigenicity of Cel$^-$ mutants is greatly reduced by such treatment. Thus, the major role proposed for cellulose fibrils in *A. tumefaciens* is in the firm, irreversible anchorage of the bacterium in large numbers to the host cells, thereby promoting the production of tumors (98).

That cellulose synthesis is an intrinsic property of *A. tumefaciens* is demonstrable not only in the ability of resting cells to produce cellulose, but also on a genetic basis. Indeed, cellulose fibrils have been observed in strains that are devoid of the Ti plasmid or any other plasmid (100), so that the genes for cellulose synthesis are probably located on the bacterial chromosome, as has been established as well for *A. xylinum* (154a). Furthermore, chromosomal transposon mutants range from Cel$^-$ to overproducing phenotypes (98). The genes involved in cellulose synthesis and the attachment interaction have been mapped to within the same area, suggesting that there is a region of the *Agrobacterium* chromosome which is concerned with the plant host cell surface interaction in general (110).

Rhizobium spp.

The roots of leguminous plants are associated with bacterial nodules in perhaps one of the most important symbiotic relationships of the biosphere (39). The atmospheric nitrogen-fixing rhizobia occur as both free-living and colonizing bacteria, which in general display a high degree of specificity for their plant host. The tremendous amounts of energy required for nitrogen fixation are manifested in the catabolic sequences of this aerobic, gram-negative bacterium; whereas the fast-growing strains are capable of growth on a wide variety of carbon substrates, the more limited, slow-growing strains exhibit great nutritional diversity with respect to aromatic substrates (133). Whereas all strains use the Entner-Doudoroff pathway and Krebs cycle, the pentose phosphate pathway is restricted to fast growers (133).

Cellulose synthesis is an intrinsic property of *Rhizobium* strains; cells produce cellulose fibrils as an extracellular product during growth in the absence of plant cells. Fibril formation is strongly dependent on the growth phase (126, 127); during cultivation in standing cultures, a surface pellicle is formed (126). The cellulosic product of this organism has been characterized on the basis of enzymatic analysis (127) and infrared spectroscopy (102, 127); X-ray diffraction analysis has revealed a pattern characteristic of cellulose I (102). The cellulose fibrils produced by *Rhizobium leguminosarum* (1 to 10 per cell) range from 5 to 6 nm in diameter and up to 10 μm in length (126).

As with *A. tumefaciens*, the major proposed role for cellulose fibrils elaborated by *Rhizobium* species is in the plant-microorganism attachment process (102, 126, 127). This process has been studied in considerable detail because it is critical to the root nodulation symbiosis. Among the several factors defined for the two-step attachment process, the first phase involves a Ca^{2+}-dependent soluble cell surface protein (rhicadhesin), which is also present in *Agrobacterium* species (128, 129). Bacterial cellulose fibrils are associated with a subsequent phase in the attachment process; their involvement could be demonstrated by inhibition experiments with isolated *Rhizobium* fibrils as well as commercially available cellulose or carboxymethyl cellulose or, alternatively, by pretreatment with cellulase (127). In addi-

tion, a correlation has been established between cellulose-synthesizing capacity and attachment ability for *Rhizobium* cells; whereas fibril-negative strains almost completely lost their attachment ability, fibril overproducers showed increased attachment (102, 126, 127). The production of cellulose as a second step in the attachment process is not affected during growth under low-Ca^{2+} conditions (127). A growth-medium-dependent, species-specific attachment, which is probably mediated by host plant lectins (66), has also been proposed to participate in the second phase of attachment (85).

In fast-growing rhizobia, many essential nodulation genes, including the genes coding for host specificity, are located on a large plasmid, the so-called Sym plasmid (83). However, genetic examination of transposon fibril-negative and fibril-overproducing mutants showed that the corresponding insertions were not located on this plasmid (127). Thus, as is the case for the other gram-negative, cellulose-producing bacteria described here, the cellulose genes of rhizobia probably reside on the bacterial chromosome.

Sarcina ventriculi

Sarcina ventriculi is a gram-positive, obligate anaerobic soil bacterium that is capable of growing on sugars within an extraordinarily wide pH range (from 2 to 10) (31). The major end products of glucose metabolism are ethanol, acetate, CO_2 and H_2. Glucose is catabolized to pyruvate by the Embden-Meyerhof pathway, and the organism apparently possesses two alternate mechanisms for the anaerobic cleavage of pyruvate (31). Enzymes that may be involved in cellulose synthesis, namely hexokinase, phosphoglucomutase, and UDP-Glc pyrophosphorylase, have been detected in extracts of *S. ventriculi* (33). Cultures of this organism produce cellulose (or a closely related compound) which remains tightly associated with the cell wall and has been assigned the structural function of maintaining the cells within the large many-celled packets characteristic of this organism (31). This interpretation is in line with reports that strains of *S. ventriculi*, which form little or no cellulose, tend to form packets consisting of relatively few cells which are loosely attached to one another (31). The cellulose is not deposited in the cell wall, and removal of the cellulose by cellulase or by treatment with cellulose solvents does not affect the cell wall, which remains intact. The cellulosic nature of this intercellular cementing material, which may account for up to 18% of the total dry weight of the cells (33), is based mainly on solubility properties and digestion with a crude cellulase preparation. Electron microscopy of *S. ventriculi* cells indicates that this material has a distinct fibrillar appearance (32). Its morphology in relation to the cell is markedly different from that observed in other cellulose-forming bacteria (Fig. 6). Whether this morphology can be attributed to the gram-positive cell wall structure of *S. ventriculi* is an intriguing possibility, especially in view of reports that the cellulose produced by this organism is of the cellulose II type (54). In addition to the role assigned to cellulose as a cell-cementing substance, it has been suggested that it may have also a nutritional role in facilitating the diffusion of nutrients from the growth medium to cells located in the interior region of packets (31). The possibility that the cellulose layer contributes to the remarkable acid tolerance of this organism appears unlikely considering the physiology of *S. maxima*, which exhibits a similar tolerance but is devoid of an intercellular cellulose layer (31).

BIOTECHNOLOGY PERSPECTIVE

Bacterial cellulose is of commercial interest for many of the same reasons that cotton fields and forests attract the industrialist's attention: cellulose is a lightweight but extremely sturdy material which may be cut and woven into any shape required. Importantly, new materials with desirable properties may be obtained by chemical treatments of natural cellulose. In addition to paper and textiles, many common products of plant cellulose include those based on derivatization reactions (e.g., nitrocellulose, celluloid, and rayon).

Cellulose of bacterial origin is distinguished by its high degree of crystallinity, its mechanical strength and absorptive capacity, and its overall chemical purity with regard to contaminating sugar and protein polymers (51, 73, 80b, 107a, 151, 156). To a certain extent, this purity obviates the extensive processing, such as mercerization and pulping, necessary in the conventional production of paper products from lumber. In the present era, though, it is not expected that cellulose obtained from bacteria will replace plant cellulose in traditional, bulk-product applications in which agriculture and forestry have proven to be adequate sources. However, the deepening understanding of cellulose biogenesis on the molecular level, combined with the advent of new fermentation technologies, is rapidly broadening the accessibility of bacterial cellulose to the marketplace.

Although *A. xylinum* is known chiefly for its cellulose-producing capacity in static culture, such growth and production methods are extremely inefficient on an economic basis. Ideally, industrial strains should be highly efficient in their utilization of nutritional sources for growth, maintenance, and polymer production; should utilize cheap and readily available carbon sources and vitamins; and should be genetically stable under the environmental stresses imposed by growth in the fermentor. Some of the major obstacles encountered in the industrial adaptation of *A. xylinum* were the wasteful and noxious accumulation of metabolic by-products from otherwise desirable carbon sources and the marked tendency of wild-type strains to revert to non-cellulose-producing mutants under the oxygen-enriched conditions of stirred-batch fermentations. The isolation of a genetically stable strain with a substantially reduced ability to form gluconic acid was achieved by relatively standard techniques of mutagenesis and selection; this strain is reported to allow rapid and reliable culture on a glucose substrate, from which the major product is fibrillar cellulose. For example, the sustained production of reticulated, highly crystalline cellulose over 70 h at a rate of 0.26 g/liter/h was achieved by using such an *A. xylinum* strain under shaken-culture conditions (8a, 80b). Lower, but equally impressive, values have been reported by other firms (29a, 51a, 89a, 155), which use at least slightly different fermentor designs as well as presumably distinct strains.

The choice of fermentor design is particularly critical because it must reconcile the opposing requirements for, on the one hand, vigorous mechanical agitation of the rapidly growing *A. xylinum* (obligate aerobe) culture and, on the other hand, the sensitivity of the cellulose fibril and fibrillar matrix to mechanical disruption, especially when the force is imposed in situ, i.e., as during the actual period of fibril assembly. Thus, upon agitated culture a highly branched, three-dimensional, reticulated structure may result, which is suitable for the production of high-quality paper (80b, 80c), whereas in static culture a normal cellulose pellicle with a lamellar structure and less significant branching tends to be

produced, which is ultimately usable as a wound dressing (151). Indeed, it has been suggested that the route to desirable modifications in the fibrillar and/or macroscopic nature of the cellulosic product may be achieved by varying fermentor design factors such as the shape of vessels and agitating impellers (151).

Alternately, *A. xylinum* strains may actually bear distinct and inherent patterns with regard to the overall fibrillar structure of their cellulose product, possibly reflecting differences at the molecular level, such as polyglucan chain length or perhaps even altered polarity (109); thus, bacterial cellulose may offer the possibility of custom-designed cellulose matrices, selectively woven at the level of individual bacterial cell surfaces. Additional possibilities are conceivable when considering the potential of bacterial cellulose production by anaerobes such as *S. ventriculi* (85a).

A very promising line of advance toward obtaining industrially valuable strains is in the direct genetic manipulation of the genes coding for the catalysts of cellulose synthesis, their adjunctive regulatory enzymes, and the relevant associated membrane structures such as the postulated extrusion pores. To date, eight different proteins have been established to participate directly in the biosynthetic pathway and its regulation; these are UDP-Glc pyrophosphorylase, the cellulose synthase, diguanylate cyclase, PDE-A, PDE-B, and the recently discovered *bcsA*, *bcsC*, and *bcsD* gene products (154a) (see *A. xylinum*, above). Each of these proteins is a candidate target for obtaining enhanced or reduced expression by genetic engineering; as mentioned above, the genes for cellulose synthesis reside on the bacterial chromosome. On the basis of in vitro activity levels measured under optimal conditions, the step catalyzed by the cellulose synthase appears to be the rate-limiting one. However, as mentioned above in the section on *A. xylinum*, the level of enzymes less specific to the process, such as UDP-Glc pyrophosphorylase, may also be demonstrably limiting in certain strains. Obviously, whether any particular factor is rate limiting is also a function of the given set of physiological conditions; for example, in poor media the permeability of glucose may be the slowest step, whereas when glycerol is used as the substrate, the rate of gluconeogenesis may be the bottleneck. It is likely that the maximal capacity of bacteria such as *A. xylinum* to produce cellulose has not reached full expression, although reports on this process, as well as on industrial production of other bacterial exopolysaccharides (67, 118), indicate that the ultimate potential of bacterial cells to produce such polymers is both greater and more obtainable than expected.

So far the products developed from bacterial cellulose include wound dressings (151), high-quality additives to paper (80b, 80c), fiber glass filter sheets (85a, 156), chewing gum (151), food stabilizers (102a), and acoustic diaphragms for audio instruments (80a). Eventually, the wide availability of bacterial cellulose will undoubtedly spawn new applications and inventions, based on this polymer and on other exopolysaccharides as well.

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