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Chapter 1

General Introduction:

Cyanobacterial cellulose synthesis in the light of the photanol concept

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Introduction

The energetics of life on Earth has evolved as a cycle of redox reactions of the elements carbon, hydrogen, and oxygen: Sunlight drives the conversion of carbon dioxide and water into the biomass of oxyphototrophs and chemo-heterotrophic organisms re-oxidize the products of oxygenic photosynthesis back to carbon dioxide and water, in a process in which they transiently store reduced forms of carbon in chemo-heterotrophic biomass. However, during the billions of years of evolution, a considerable amount of biomass/carbon has been removed from the biochemical carbon cycle and deposited as reduced, diagenetically converted organic matter (mainly gas and oil).

Since the beginning of the industrial revolution mankind has massively exploited the free energy contained within these fossil sources of energy, which has led to a notable increase in atmospheric carbon dioxide levels. This development has generated serious concerns about global warming. Hence, and also for political reasons [1], there is a considerable incentive to develop sustainable alternatives to the exploitation of fossil fuel. By far the majority of the free energy available on Earth is ultimately derived from sunlight, and it is available in large excess of the energy demands of mankind [2]. It is therefore rational to exploit sunlight directly.

Many ways to achieve the direct use of sunlight have been exploited since the first oil crisis in the early 1970s. Hydroelectricity, photovoltaics, and wind energy are the most notable examples. All of these produce electricity, which is – together with liquid fuel (and heat) – the main free-energy carrier on Earth. There still is a need for the development of new methods for the sustainable production of a liquid fuel in the form of solar generated biofuel.

One may think that in the future liquid fuel will be produced by chemical devices (often referred to as “artificial leaves”), which would convert carbon dioxide into more reduced forms of carbon. Despite significant effort, only limited progress has been made along these lines [3], particularly with respect to the formation of covalent carbon–carbon bonds. Since the turn of the century the idea has gained momentum that natural oxygenic photosynthesis would be more suitable for sustainable biofuel production. The main driver

for this idea is the autocatalytic nature of formation of the devices that produce the fuel in the first place, namely the living cells of plants, algae, or cyanobacteria.

In two forms this approach of exploiting natural biosynthesis already finds large-scale application: the conversion of crop plants (e.g., maize, or one of its sub fractions such as (ligno)cellulose) into methane via the use of an anaerobic digester and of its polysaccharide fraction into solvents such as ethanol or butanol, via industrial acetone–butanol–ethanol (ABE) fermentation. Yet, in a third mode the lipid fraction of crops can be converted into biodiesel. However, all these methods suffer from the disadvantage that arable land and valuable crops – which can also be used for food or feed applications – are used to produce energy, in a world in which the future food supply is a serious concern.

Shifting to the use of algae or cyanobacteria would alleviate this latter problem, because these aquatic organisms can be grown in various forms of photobioreactors. Hence, activity aimed at the production of biodiesel through large-scale growth of various algae has gained significant momentum. But even in this approach there is an important element of inefficiency: The carbon fixed in the Calvin–Benson–Bassham cycle is first converted into all the complicated building blocks for biomass formation (amino acids, lipids, sugars, nucleotides, vitamins, etc.), after which cellular anabolism converts these “monomers” into new cells. These cells then have to be processed, in other words they are fractionated and/or fermented, after which another processing step (such as ethanol fermentation) is required to make the final product.

The “photanol” concept

To further increase the overall efficiency of the process of solar biofuel formation it has been proposed to combine phototrophic and fermentative metabolisms in a single organism: a kind of “photofermentative” chimera. If the activity of the added fermentative pathway can be tuned properly, one could achieve a situation in which such a photofermentative organism functions as a photo-catalyst for the reaction: $\text{CO}_2 + \text{H}_2\text{O} \rightarrow$ “biofuel product” + O_2 . This proposed concept has been suggested as being applicable to the synthesis of various products, ranging from lactic acid and ethanol, for which simple fermentative metabolic pathways suffices [4], via sugars [5], to longer chain alkanes and alcohols [6]. Elsewhere, we have referred to this concept of solar biofuel production as the “photanol concept” and several initiatives have been undertaken to test its economic

viability. The concept can be applied to all oxyphototrophs, including plants (for which the synthesis of poly- β -hydroxy butyrate can be taken as an example), but is most easily applied in cyanobacteria because of their much better genetic accessibility and more simple structure.

Generally, fermentative pathways function best when oxygen is absent. The photanol concept was conceived from the observation that organisms such as yeasts also use fermentative ethanol production extensively under aerobic conditions, when glucose is in excess. Nevertheless, the compatibility of a heterologously expressed metabolic pathway with the condition of high oxygen concentration in the cytoplasm is an important element in the design of a photofermentative chimera: the chance of functionally expressing the well-known clostridial butanol fermentation pathway in an organism that carries out oxygenic photosynthesis will be small. Even less likely is the possibility of combining methanogenesis with oxygenic photosynthesis, because in this case, not only the oxygen-sensitivity of the methane-forming enzymes must be addressed, but also the incompatibility of the metabolic intermediates and the cofactors.

Maximizing photosynthetic efficiency

Light, as a source of energy in biology, is rather delicate to handle because it comes in packages (photons), many of which carry much more free energy than what is contained in, for example, a single covalent C–C bond. Hence various forms of non-productive chemistry are readily initiated by (electronically excited) molecules, the most notable of which is the formation of reactive oxygen species, such as singlet oxygen and superoxide. In agreement with this notion, many regulatory processes have been discovered in phototrophic organisms that allow for the processing of this radiation energy directly into heat if – temporarily – the forward reaction towards the productive biological process is blocked. The best-known example of such processes is the non-photochemical quenching of excitons in the antenna system of many oxyphototrophs, when – due to transient over-excitation of particularly photosystem II – the primary electron acceptor of this system is already in the reduced form at the time of arrival of the next electron. But such processes have also been identified much later in the chain of events that converts the solar radiation into the chemical free energy of NADPH and ATP (i.e. the two free-energy intermediates that drive the reactions in the Calvin–Benson–Bassham cycle), for example the Mehler reaction. The

Mehler reaction transfers excess electrons arriving from photosystem I directly to oxygen, conserving ATP but also producing superoxide radicals, which are disproportionated to hydrogen peroxide and water. Cyanobacteria, in contrast, possess a Mehler-like reaction, mediated by flavodiiron proteins, in which oxygen is reduced directly to water without formation of oxygen radicals [7,8]. Nevertheless, less energy-dissipating forms of regulation have also emerged, such as the state transitions, in which the cross section of the two photosystems is adjusted via reversible migration of the antenna pigments to these photosystems, so that the rate of excitation of the two photosystems is in balance under widely varying conditions of the spectral composition and intensity of the available ambient radiation. It is thought that the redox state of the plastoquinone (PQ) pool, which receives electrons directly from PSII, regulates these state transitions [9,10]. State transitions and their mechanism is further discussed in Chapter 7.

Successful application of the photanol concept requires that the photosynthesis process in future cyanobacterial cell factories proceeds with the utmost efficiency, preferably so that all photons present in the spectral window of photo-synthetically active radiation will be used productively and not be dissipated into heat prematurely. Intrinsically, for thermodynamic reasons, oxygenic photosynthesis is inefficient and never exceeds ~30 %. Actual photosynthetic yields in agriculture rarely exceed 1% and the theoretical maxima for crops are 4.5 % and 6 % for C(3) and C(4) plants, respectively [11]. For cyanobacterial photosynthesis, yields of up to 10 % of the total radiation impinging on the Earth's surface have been reported from small lab-scale photo-bioreactors, and it is a challenge to design photo-bioreactor systems in which such high yields will be attainable at large scale (and at an affordable price). A significant hurdle in meeting this challenge is the dynamical fluctuations in light intensity to which cells in such large-scale reactors will be exposed. The two main factors that govern these fluctuations are:

- the limited penetration depth of the light in dense cell cultures
- the circadian and seasonal alterations in light color and intensity.

It will take intelligent reactor design to meet this challenge of optimization.

To complicate matters even further, biological engineering is also among the tools that are available to make this optimization process a success. A clear example of the use of this technique has already been carried beyond the stage of proof of principle: by varying

the total complement of antenna pigments, the penetration depth of sunlight into a photobioreactor, and thereby the relative fraction of photosynthetically productive cells in it, can be increased [12]. However, with this approach the possibilities are by no means exhausted. Obvious candidate approaches are alteration of the mutual stoichiometry and content of the two photosystems and alteration of the color of the photosynthetic pigments [13]. Optimization of the “dark reactions of photosynthesis” (such as the *in vivo* activity of RuBisCO) is also among the possibilities, or even introduction of far-red-absorbing proton-pumping rhodopsin into the cyanobacterial membrane(s).

Downstream processing of solar biofuel products

Low-molecular-weight biofuel products

The “distributed” nature of sunlight has important consequences for the geometry of solar bioreactors in which cyanobacteria can be grown under ambient solar irradiation and the optimal cell densities in these bioreactors. High cell densities cause a limited penetration depth of light into the photobioreactor, whereas large volumes of cells in a photobioreactor that would not receive light will only contribute to (maintenance) energy dissipation, rather than product formation. Therefore, most large-scale facilities for growth of unicellular oxyphototrophs have an average aerial depth of between 0.1 and 0.3 m, which can be obtained by many different ways of arranging tubes, plates, or even plastic bags.

Solar biofuel production differs considerably from traditional ABE fermentation. In ABE processes, fermentative bacteria convert high concentrations of polysaccharide or sugar into solvents such as ethanol and butanol, such that these products – often facilitated by high solvent tolerance of the fermentative bacteria or yeasts – accumulate to high concentrations. Furthermore, the high biomass density causes substantial heating of the compact reactors. Recovery of the biofuel products can then be achieved through distillation, filtration and/or pervaporation techniques. The economic feasibility of this downstream processing is inversely correlated with the solvent tolerance of the fermentative organism selected. This tolerance can be increased by random screening or through rational engineering. In this respect, it is relevant to note that a yeast strain was recently described that tolerates up to 17% (v/v) ethanol [14].

As of yet, the product levels achieved in applications of the photanol concept using a cyanobacterium have led to moderate product accumulation levels [15-18]. Nevertheless, investigations aimed at increasing the product (ethanol) tolerance in cyanobacteria may become relevant in the future. In contrast to its conventional biofuel counterpart produced from, for example, cellulose with a chemotrophic organism, the solar biofuel product is not produced in a complex broth, but rather in a solution in which the solar biofuel product – after cell-separation – is the dominant component. Ethanol recovery after photofermentation is probably best achieved by multistep separation, or by evaporation (using solar heating) and subsequent condensation [19].

One way to circumvent downstream processing problems is to synthesize a volatile or insoluble product, allowing phase separation. Various products can be used for phase separation, for instance ethylene or butylene. Proof of principle for the production of some of these products has already been given [20]. However, the requirements for the maintenance of a proper gas atmosphere in mass cultures of cyanobacteria for solar biofuel production – in other words a high level of carbon dioxide in order to avoid photorespiration and preventing super-saturation of oxygen – makes it difficult to recover volatile products. Nevertheless, there is hope that newly developed methods will circumvent these problems and optimize product recovery and cultivation conditions [21].

Another way out of the high costs of product recovery from dilute solutions may be found in the coupling of processes, so that the dilute solar biofuel product is converted by a high-affinity biological process into a secondary product in a (photo)chemical conversion in which product separation can be based on phase separation [Bekker, M. and Hellingwerf, K.J., manuscript in preparation].

Macromolecular solar biofuels

A radically different approach would be to synthesize a high molecular weight compound such as polysaccharide, polyester, or lipid. These macromolecules are easier to separate and minimize the energy expenditure for downstream processing. Examples include the conversion of the Calvin–Benson–Bassham cycle intermediate(s) into poly- β -hydroxy butyrate, poly-(D,L)-lactic acid, glycogen, or long-chain triglycerides. Nevertheless, such an approach would suffer from the disadvantage that it relies on the storage capacity of the cell. Moreover, a high intracellular content of the polymer would be required in order to

make its extraction economically feasible. In order to circumvent the restriction of cellular capacity, a polymer that is deposited extracellular therefore has the preference.

It has long been known that certain chemoorganotrophic bacteria, in particular members of the genera *Acetobacter*/*Gluconobacter*, are able to produce cellulose, which is deposited just outside the cell envelope. When grown with non-limiting amounts of glucose, *Gluconacetobacter xylinus* produces and deposits massive quantities of the insoluble “crystalline” cellulose [22]. The genes involved in cellulose synthesis in these bacteria as well as their regulation, have been characterized (see below).

In contrast, little is known about cellulose production in cyanobacteria. Although it has been known for a long time that cyanobacteria can produce cellulose [23,24], the mechanism and regulation of the process have never been investigated. The literature on cellulose production in cyanobacteria reports production of non- or semi-crystalline cellulose [23,24]. A reason for the semi-crystalline nature of the cellulose may be the complex mixed-polysaccharide cell-wall layers produced by cyanobacteria. This might also complicate its purification should it be used for biofuel production. Nevertheless, a genetic approach has already been implemented to produce or increase production of cellulose from cyanobacteria [25-28]. This was achieved by introducing the *bcsAB* genes of *G. xylinus* together with other genes to help boost cellulose production into the cell via conjugative transfer, with *E. coli* cells harbouring the cargo plasmid. Cellulose was produced in these transgenic strains but only in non-crystalline form.

Although the mechanism of crystallization of cellulose remains unknown, the observations described above now allow us to formulate an alternative biosolar cell for the production of macromolecular biofuel (see Fig. 1.1). By bypassing the endogenous regulation of cellulose synthesis or through regulated heterologous expression of cellulose synthesis genes, it should be possible to construct a cyanobacterial cell that converts a large portion of its fixed carbon into exocellulose. Whether the latter macromolecule can be used directly or whether it first must be converted into a more convenient-to-handle liquid fuel remains to be seen. One approach that may help to increase production of cellulose, albeit non-crystalline, would be to knockout the pathway of photosynthetically fixed carbon to glycogen, the main carbon and energy storage compound of cyanobacteria [29]. The substrates for glycogen and cellulose synthesis are similar: ADP-glucose and UDP-glucose,

respectively. In this respect introduction or over-expression of UDPG-pyrophosphorylase might also be a successful approach.

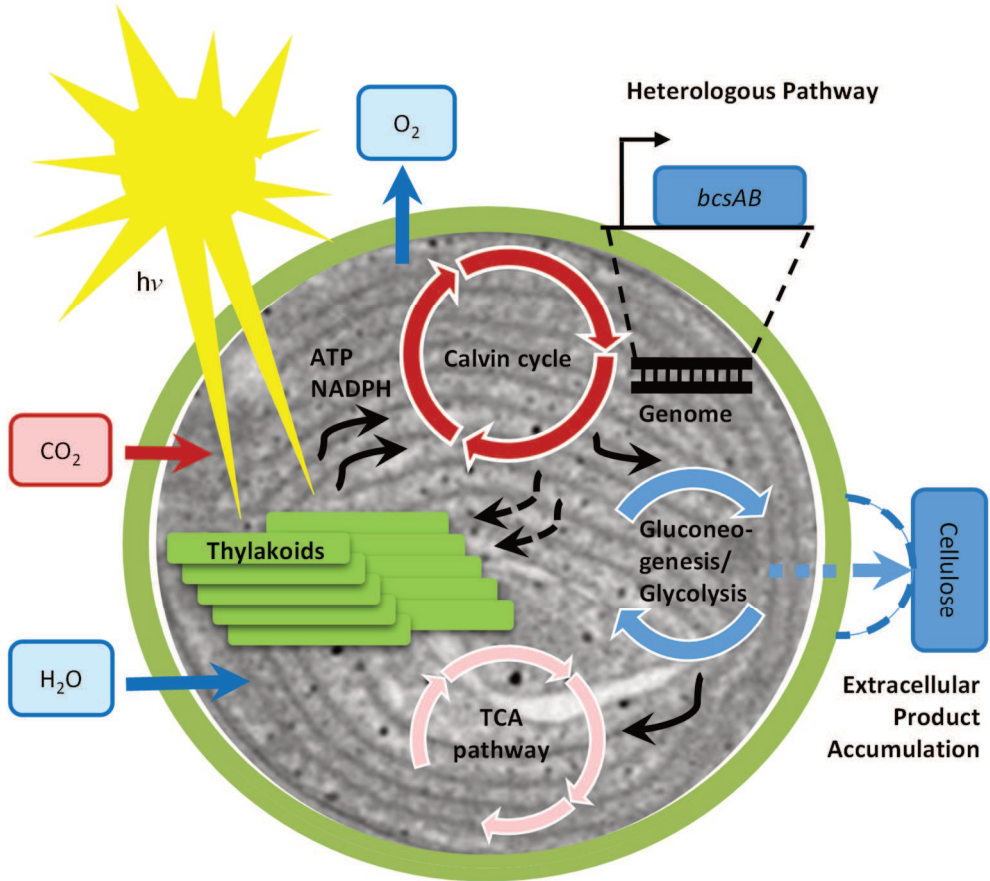


Figure 1.1 The cellulose synthesizing biosolar cell

Cellulose synthesis

Cellulose structure and physiological function

Cellulose is one of the most abundant polymers on earth. It is an important component of cell walls of plants and algae, but it is also produced by a variety of bacteria and by some fungi and animals [30]. Cellulose is a complex compound with a wide variety of structures, such as wood, paper, and cotton. In contrast to this complexity the basic structure of a cellulose fibril is simple: it consists of only one ingredient, namely D-glucose. The D-glucose monomers are linked at a 180° angle in β -1,4 glucosidic bonds to form long un-branched polymeric chains. The special geometry of this unbranched covalent arrangement gives rise to extended fibril structures, because all of the available hydroxyl groups upon adjacently aligned β -1,4-glucan chains participate in inter- and intra-chain hydrogen bonding. In this way, aggregates of many chains form insoluble layered sheets reinforced by the dispersion forces between their stacked heterocyclic rings [22]. Nearly all cellulose isolated from nature has a crystalline structure in which the poly- β -1,4-glucan chains are aligned in a parallel manner; this is known as cellulose type I. This crystalline cellulose is metastable and can be transformed to cellulose type II, for instance by acid treatment. Cellulose II is rare in nature and has an antiparallel arrangement. For cellulose microfibrils to be assembled in such a metastable way the individual glucan chains must be held in close proximity by intermolecular hydrogen bonds and van der Waals forces [22]. Therefore, these chains must be synthesized in close proximity to one another. In 1964 the use of the freeze-fracture technique made it possible to investigate the surface of cellulose synthesizing cells. As a result, highly ordered membrane-bound structures were observed and the “ordered granule hypothesis” for the assembly and orientation of cellulose microfibrils was proposed [31,32]. This vision was later confirmed with freeze-fracture experiments on the alga *Oocystis*, in which the so-called linear terminal complexes (TC) – multi-subunit arrays at the end of microfibrils – were observed [33,34]. Differences in the arrangements of terminal complexes lead to different shapes and sizes of the final crystalline product.

To obtain more knowledge about cellulose production and fibril assembly, attempts were made to produce cellulose *in vitro* using membrane preparations. This approach was frustrated by the fact that only low amounts of cellulose are produced from

bacterial preparations. In cellulose preparations derived from higher plants little or no cellulose synthase activity was observed against the high background of callose (β -1,3-glucan) synthase activity [34]. Callose synthesis is induced as a result of plant wounding. However, *in vitro* production of cellulose is possible: in bacterial preparations by the addition of the regulatory compound c-di-GMP or its pre-cursor GTP [35] and in plants by using developing cotton fibres [36] instead of plant-cell preparations.

While the function of cellulose in plants is obvious being the component responsible for the rigidity of the cell wall, its role in bacteria, including cyanobacteria, is not clear. Bacterial extracellular cellulose may play a role as a protective shield against environmental stresses and external (mechanical) forcing, or it may facilitate the adhesion of the bacterial cell in case of infection or for symbiotic interactions, as is the case with *Agrobacterium* sp. and *Rhizobium* sp. [22]. In the case of *Gluconacetobacter xylinus* it has been suggested that the massive amounts of cellulose that this organism produces when growing in a static culture, and which forms a floating pellicle, helps this obligate aerobic bacterium to move to the surface in order to obtain access to oxygen [22]. One interesting hypothesis is that the cellulose sheath might protect the cell against UV irradiation [22]. This, in particular, is interesting for cyanobacteria because these phototrophic organisms are often exposed to high levels of UV light.

The presence of cellulose in cyanobacteria has gone unnoticed for a long time. One of the first detailed reports was the discovery of cellulose in a new cyanobacterial isolate, *Crinalium epipsammum*, which was shown to accumulate cellulose to 21.6% of its dry weight [23], but before that, the presence of extracellular cellulose had been suggested in heterocystous cyanobacteria, based on light and electron microscopy [37]. One report suggested that cellulose is an integral component of the heterocyst cell wall and essential for protecting nitrogen fixation from oxygen inactivation in these specialized cells [38], but subsequent studies of the heterocyst envelope did not confirm the presence of cellulose. However, genes encoding cellulose synthase appear to be widespread among cyanobacteria and representatives of four out of the five sections of cyanobacteria produced cellulose [24]. *C. epipsammum* is a filamentous, non-heterocystous, cyanobacterium from section III. It was isolated from algal crusts that grow on the sand surface of blow-outs in the dunes near The Hague, The Netherlands (Fig. 1.2). These algal crusts are characterized by harsh

conditions, especially drought, large temperature fluctuations, and low nutrient availability. One of the noticeable characteristics of *C. epipsammum* is its desiccation resistance. This organism is immotile and incapable of fixing nitrogen. *C. epipsammum* does not contain phycoerythrin and its phycobiliprotein content is low. Also, the DNA of this organism has an exceptionally low GC content and these characteristics may reflect its low nitrogen habitat and its incapability of fixing nitrogen.

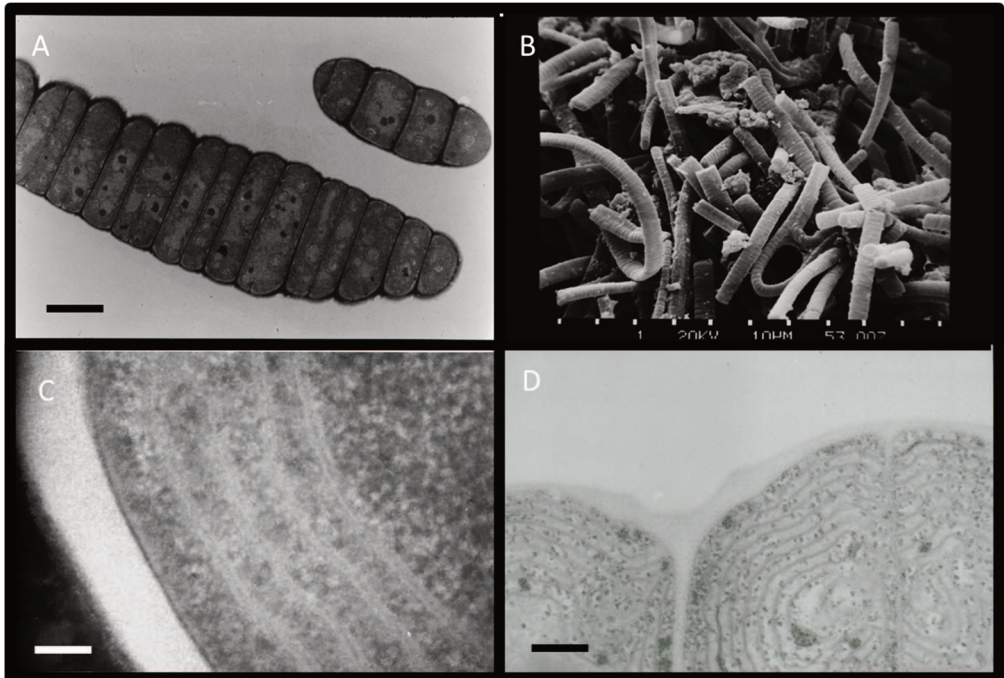


Figure 1.2. (A) Transmission electron microscopy image of *Crinalium epipsammum*. The thick cell wall with some sheath material is notable. The thylakoids and carboxysomes (polyhedral bodies) are clearly visible. (B) Scanning electron microscopy image showing the flat morphology of the trichomes of *Crinalium epipsammum*. (C) Detail of the extraordinarily thick cell wall of *Crinalium epipsammum*, which is more similar to those of Gram-positive microorganisms (cyanobacteria have a Gram-negative cell envelope). Some sheath material is visible on the outer layer of the cell wall. (D) Transmission electron microscopic image stained for polyglucose. The glycogen granules are visible between the thylakoids and the cell wall is also lightly stained, which might hint at the presence of cellulose. The scale bars in A, C, and D are 5, 0.5, and 0.5 μm , respectively.

The cellulose detected in *C. epipsammum* might be present in non-crystalline form [23]; the presence of cellulose with low crystallization has also been reported for other

cyanobacterial strains [24]. In native form neither cellulose I nor II was detected in *C. epipsammum* but in its extracted form cellulose II was found, probably as the result of the alkali treatment. The location of the cellulose in *C. epipsammum* is uncertain. While in other bacteria the cellulose is exuded outside the cell, this was apparently not the case in *C. epipsammum*. It was hypothesized that the cellulose might be an integral part of the cell envelope. The cell envelope and the genes involved in synthesizing it in cyanobacteria reflect the environmental conditions under which the organism thrives [39]. *C. epipsammum* has an unusual morphology; instead of cells that are round in cross section, *C. epipsammum* is characterized by a flat, oval form. Considering the turgor, this would require special adaptation of the cell wall, which is unusually thick in *C. epipsammum*. Hence the function of cellulose in *C. epipsammum* could be the formation of the rigid cell wall, although it is unknown how this could be achieved. Moreover, it is also unknown what the function of this flat, band-like trichome could be. Another possible function of cellulose related to the habitat of *C. epipsammum* could be its water-retaining capacity; this might help the organism to withstand periods of drought [40].

In the thermophilic unicellular cyanobacterium *Thermosynechococcus vulcanus*, cellulose accumulates particularly under illuminated, low-temperature conditions and results in cell aggregation [41]. It is suggested that this is an acclimation to light stress by self-shading of the cells in the aggregates [42]. The disruption of a putative cellulose synthase gene in *T. vulcanus* prevents the accumulation of cellulose as well as the aggregation of cells [41]. Cellulose has also been suggested as being important as a biofilm matrix [43].

In the Precambrian, the oxygen-free atmosphere was devoid of ozone and UV could therefore reach the Earth's surface. Cellulose might have evolved in Precambrian cyanobacteria in order to protect them from UV light. No compelling evidence for any of these possibilities is available.

The cellulose synthase gene of *Acetobacter xylinum* has been genetically transformed into the filamentous heterocystous cyanobacterium *Anabaena* PCC7120, leading to expression and production of extracellular cellulose [26]. The transgenic *Anabaena* showed increased photosynthetic efficiency and growth rate, but it is unclear whether this was caused by the production or presence of cellulose.

The cellulose synthesis genes

The protein responsible for cellulose synthesis was first discovered in bacteria in 1989 [44] and was shown to bind UDP-glucose [45]. The operon, including the gene encoding cellulose synthase, was identified through a complementation study of a cellulose-deficient mutant of *Gluconacetobacter xylinus* [46]. Subsequently, cellulose synthase operons were identified in other bacterial species such as *Agrobacterium tumefaciens* [47] and *Rhizobium leguminosarum* bv *trifolii* [48]. All cellulose producing organisms require a cellulose synthase, which forms the β -1,4-glucan chain from UDP-glucose (*bcsA* in bacteria and *cesA* in eukarya). Extracellular cellulases are thought to break the glucan chains to avoid excessive mechanical stress during the crystallization process [34]. In bacteria, the gene for cellulase, often named *bcsZ*, is located within, or adjacent to, the cellulose synthase operon [47-49]. In plants, mutations in cellulase genes are known as korrigan, and can cause dwarfism, radial swelling of root tips, and collapse of xylem vessels [50]. Besides *bcsA* and *bcsZ*, several other genes are present within the cellulose synthase operon in bacteria, namely *bcsB*, *bcsC*, *bcsD* in *G. xylinus* [51] and *celD* and *celE* in *A. tumefaciens* and *R. leguminosarum* [47,48].

BcsB used to be considered the regulatory subunit of cellulose synthase because it had been reported that it binds the regulatory molecule c-di-GMP [34,51]. However, this observation was later questioned since the 90 kD polypeptide that co-purifies with cellulose synthase does not bind c-di-GMP [52]. Amikam and Galperin [53] identified a novel c-di-GMP binding domain in bacteria: PilZ. This domain was also identified near the C-terminus of the BcsA protein of *G. xylinus*. This strengthens the idea that BcsB is not the regulatory subunit of cellulose synthase. More recent studies show that BcsB is a periplasmic protein that may guide the glucan chain across the periplasmic space towards the outer membrane [54]. Additionally, BcsB anchors onto BcsA with its C-terminal domain, likely stabilizing the transmembrane domain of BcsA and allowing the glucan chain to pass through [55].

BcsC, which is required for *in vivo* but not *in vitro* cellulose synthesis, is present in enterobacteria, pseudomonads and in *G. xylinus* [56]. BcsC is related to bacterial proteins involved in pore formation such as VirB from *A. tumefaciens*, Tra2 from *Escherichia coli*, and Ptl from *Bordetella pertussis* [51]. BcsC is predicted to consist of a large periplasmic domain

involved in complex assembly and a β -barrel domain in the outer membrane for translocation of the cellulose fibrils [57,58].

BcsD, which is required *in vivo*, but not *in vitro* for cellulose biosynthesis in *G. xylinus* [51], forms what appears to be an interior cylinder of the terminal complex pore (see below for description) providing a spiral passageway through which four glucan chains can pass simultaneously [59].

Molecular biology, biochemistry, and phylogenetic analysis

The most extensively researched and best characterized protein involved in cellulose biosynthesis is cellulose synthase (Ces), which is a membrane-bound protein located in the plasma membrane. The N-terminus of the plant- and some of the green-algal Ces proteins contain a RING-type zinc finger that is not present in Ces proteins from other organisms. This domain is followed by two trans-membrane domains and the central cytosolic domain, then another six trans-membrane domains and the C-terminus, at the position where in bacterial Ces proteins a PilZ domain is located (Fig. 1.3). Among different organisms the cellulose synthases show low homology, with exception of the central cytosolic “catalytic” domain [50]. This domain contains the catalytic site present in all processive glycosyltransferases known as the D,D,D,QXXRW motif [60]. This domain can be split into four subdomains, U1–U4 (Fig. 1.3), in which the first three subdomains each contain one aspartic acid and the last domain contains the QXXRW motif in which X can be any amino acid [56]. These subdomains can be interspaced with different organism-specific regions (Fig. 1.3). The *ces* genes from some cyanobacteria, green algae, plants, and the amoeba *Dictyostelium discoideum* all contain the CR-P (plant conserved region) between the U1 and U2 domains [24,61,62]. Between the U2 and U3 domains in plants and in some green algal *ces* genes, a class-specific region (CSR, previously known as hypervariable region) is present. These eukaryotic genes also contain several introns [62,63]. The presence of different types of cellulose synthases in cyanobacteria and algae and the appearance of the interspacing regions within the catalytic domain provide an insight into the evolution of the cellulose synthases.

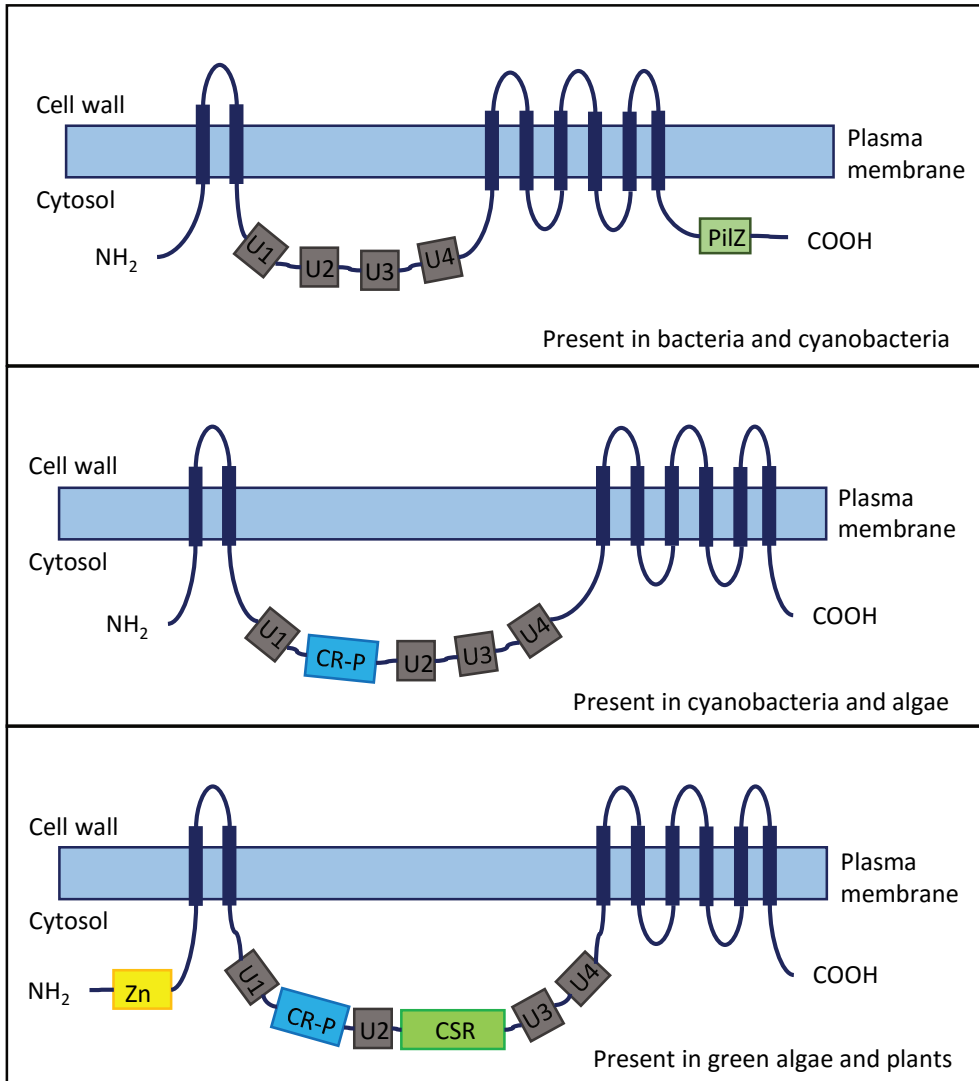


Figure 1.3. Membrane topology of cellulose synthase proteins. Blue bars indicate trans-membrane segments. PilZ, PilZ domain; Zn, RING-type zinc finger; U1–U4, catalytic domain; CR-P, plant specific region; CSR, class specific region. Reproduced and adapted from [50] with permission.

In 2001 Nobles, Romanovicz, and Brown [24] demonstrated that cellulose biosynthesis is a common feature in cyanobacteria and, most importantly, phylogenetic analyses suggest that the cellulose synthase genes from plants are derived from cyanobacteria [24,64,65]. In cyanobacteria two types of cellulose synthases have been identified [65]. For example, the cellulose synthase of *Thermosynechococcus elongatus*

(accession# NP_682585.1) has the same domain topology as other bacterial BcsA proteins, while the *ces* gene of the diazotrophic cyanobacterium *Nostoc* sp. PCC 7120 (accession# NP_487797.1) has lost its PilZ domain and gained an insert between the U1 and U2 domains, giving it the same domain topology as many algal Ces proteins. In phylogenetic studies comparing the cellulose synthases of all known cellulose-synthesizing classes of organisms it became evident that the eukaryotic cellulose synthases, with the exception of the Ces proteins from *C. intestinales* and *A. fumigatus*, have a unique common ancestor in the cyanobacterial *ces* gene that contains the CR-P region [65].

The appearance of the CSR domain within the catalytic domain and the N-terminal RING-type zinc finger within the *ces* gene seem to be related to the configuration of the active cellulose synthase complexes on the plasma membrane, known as terminal complexes (TCs). By freeze-fracturing of the plasma membrane of cellulose producing cells the TCs can be seen as multi-subunit arrays at the ends of the microfibrils [33]. In bacteria the TCs are arranged in a straight line along the plasma membrane [22,51] but in higher plants they form hexagonal arrays known as rosettes [66]. The largest variety in TCs is found in green algae, which exhibit different sizes of linear terminal complexes, hexagonal rosettes, and even octagonal rosettes in *Coleochaete scutata* (Fig. 1.4) [32,67]. In eukaryotes, the cellulose synthase enzyme complexes are formed in the endoplasmatic reticulum and then transferred to the Golgi apparatus, where they form large globules or tetrads. These globules and tetrads are transported to the plasma membrane where they unfold and aggregate to form the active TC [32]. This controlled formation of TC in eukaryotes may have enabled this high variety in TC formation in green algae, which also led to the development of the rosette. Rosettes are thought to be fully assembled before they reach the plasma membrane [50]. In order to form a rosette TC, two units of three different types of Ces proteins are required. These different Ces proteins most likely bind to each other through their zinc-finger domains [68]. If one of these Ces proteins is missing, no rosette is formed [69].

The proteins that comprise the rosette in plants are referred to by CesA followed by a number. In *Arabidopsis*, CesA1, CesA3, and CesA6-like (CesA2,5,6, or 9) proteins form the rosette responsible for primary cell-wall formation, while CesA4, CesA7, and CesA8 form rosettes for secondary cell-wall deposition. Because of the different order of discovery

of these genes in different organisms, the numbering has become confusing. The CSR in the catalytic domain appears to be specific for the different types of CesA proteins but is highly variable between different CesAs [70]. It would be useful to renumber the rosette-forming CesAs based on their CSR.


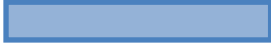








Organism	TC type	Microfibril cross section (nm)
Bacteria	Linear	
<i>Gluconacetobacter xylinus</i>		~100~  1.5
Eukaryotic algae		
<i>Pelvetia Sphacclaria</i>		14  2.6
<i>Vaucheria</i>		21  1.5
<i>Oocystis</i>		25  10
Land plants and algae	Rosette	
<i>Spyrogyra</i>		3.5  3.5

Figure 1.4. Terminal complex organization on the cell membrane in different organisms and cross sections of the resulting cellulose microfibrils. Reproduced and adapted from [32] with permission.

Physiological regulation

In bacteria, cellulose synthesis is regulated by cyclic diguanylic acid (c-di-GMP). C-di-GMP was first discovered as an enhancer of cellulose synthesis in *G. xylinus* in 1987 by the group of Benziman [35]. Turnover of this second messenger was thought to be mediated by diguanylate cyclase (DGC), which forms c-di-GMP from two molecules of GTP, and phosphodiesterase (PDE), which linearizes c-di-GMP to 5'-pGpG. pGpG is then further

degraded to 5'-GMP by non-specific cellular PDEs [22,35]. In 1998, the genes that encode these enzymes in *G. xylinus* were identified [71]. In total three operons that mediate c-di-GMP turnover were identified and termed *cdg1–3*. Each operon encodes one PDE protein followed by one DGC protein and all turnover proteins contain both a GGDEF and an EAL domain [71]. These domains are named after their conserved sequence motifs and they form the catalytic site of the DGC and PDE enzymes, respectively.

The nature and the location of the c-di-GMP receptor of bacterial cellulose synthase, however, remained a point of discussion. There was some evidence that the BcsB subunit of the cellulose synthase in *G. xylinus* could bind c-di-GMP [52], but this has never been confirmed. In 2006, the first c-di-GMP binding domain was identified through a bioinformatics approach [53]. This domain was named PilZ and found to be encoded within the *bcsA* subunit of the cellulose synthase in *G. xylinus*. In recent years, interest in c-di-GMP has increased greatly after it became apparent that c-di-GMP does not just regulate cellulose synthesis but that it is part of a far more ubiquitous second messenger system in bacteria (reviewed by [72-74]).

The GGDEF and EAL domains are located C-terminally from, often multiple, sensory and signal transduction domains, including PAS, BLUF, haemerythrin, GAF, CHASE, and MASE. These different N-terminal domains are capable of responding to a wide range of signals: phosphorylation, protein binding, binding of gaseous molecules, or even light (Fig. 1.5). PilZ domains are often associated with regulatory, catalytic, or transport domains [53] and PilZ-containing proteins function in a variety of cellular processes including the virulence of animal and plant pathogens, motility, and the synthesis of exopolysaccharides, such as cellulose or alginate (Fig. 1.5; [74] and references therein). Finally, the FleQ regulator from *P. aeruginosa* (involved in transcription control) and RNA aptamers specifically bind c-di-GMP [75,76].

GGDEF and EAL domains are abundant in bacterial genomes, but absent from the genomes of Archaea and Eukarya [77], and the same is true for the PilZ domain [53,78]. This suggests that c-di-GMP signalling is a trait that is exclusive for Bacteria. Therefore, cellulose synthesis in eukaryotes must be regulated in a different way. In secondary cell wall synthesis in plants, NAC and MYB family transcription factors are key players. Deletion or overexpression of these transcription factors generally leads to loss or low levels of

secondary cell wall formation and ectopic secondary cell wall deposition, respectively [50] and references therein.

Phosphorylation sites have been identified for a number of *Arabidopsis* Cesa proteins. Phosphorylation may target the proteins for degradation [79]. This is further supported by the finding that the half-life of a Cesa protein in cellulose producing cotton cells is less than 30 min, which is much shorter than for the average membrane protein [80]. This phosphorylation signal could be part of a feedback communication with the cell wall, where the cellulose is deposited. This could be achieved through the action of membrane-spanning receptor-like kinases, which trigger a kinase cascade [50].

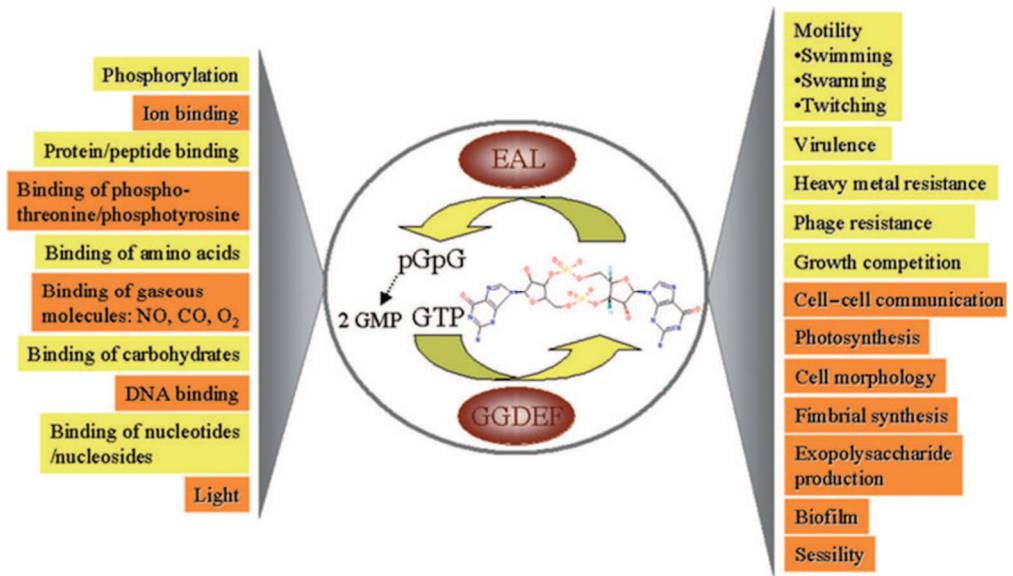


Figure 1.5. Known input signals and output of c-di-GMP metabolism. GGDEF and EAL domains conduct the turnover of c-di-GMP. pGpG is degraded to two GMP by an unknown phosphodiesterase. Various domains N-terminal of GGDEF or EAL receive and transmit the input signals on the left. The output behaviour by variation of c-di-GMP concentration is shown on the right. Reproduced with permission from [72].

Concluding remarks

The increasing global demand for efficiently and sustainably produced forms of transport fuel and feedstock chemicals provides ample opportunity to further develop the photanol concept to products beyond the short-chain alcohols. The detailed knowledge already available about cellulose synthases and their regulation, plus emerging insights into the process of cellulose secretion in cyanobacteria make cellulose to an attractive polymer for the application of the photanol concept in an economically viable production process. The toolbox of classical physiology, combined with synthetic biology, provides ample opportunities to take up this challenge.