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Bacterial cellulose biosynthesis: diversity of operons, subunits, products and functions

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Summary

Recent studies of bacterial cellulose biosynthesis, including structural characterization of a functional cellulose synthase complex, provided the first mechanistic insight into this fascinating process. In most studied bacteria, just two subunits, BcsA and BcsB, are necessary and sufficient for the formation of the polysaccharide chain *in vitro*. Other subunits – which differ among various taxa – affect the enzymatic activity and product yield *in vivo* by modulating expression of biosynthesis apparatus, export of the nascent β -D-glucan polymer to the cell surface, and the organization of cellulose fibers into a higher-order structure. These auxiliary subunits play key roles in determining the quantity and structure of the resulting biofilm, which is particularly important for interactions of bacteria with higher organisms that lead to rhizosphere colonization and modulate virulence of cellulose-producing bacterial pathogens inside and outside of host cells. Here we review the organization of four principal types of cellulose synthase operons found in various bacterial genomes, identify additional *bcs* genes that encode likely components of the cellulose biosynthesis and secretion machinery, and propose a unified nomenclature for these genes and subunits. We also discuss the role of cellulose as a key component of biofilms formed by a variety of free-living and pathogenic bacteria and, for the latter, in the choice between acute infection and persistence in the host.

Keywords

bacterial genomes; bacterial host interaction; biofilm structure; environmental bacteria; polysaccharide export; nanocellulose

Cellulose production

Cellulose, poly- β -(1 $\rightarrow$ 4)-D-glucose, is the key component of plant cell walls and the most abundant biopolymer on this planet. In fact, it was the view of the cellulose ‘cells’ in cork that prompted Robert Hooke to coin the term cell in the first place. Cellulose is incredibly stable and can withstand washing in strong hot acid or alkali, heating, stretching, and other challenges. You are probably reading this text printed on paper, wearing a cotton T-shirt,

and sitting in a wooden chair by a wooden table, which all consist largely of cellulose. While most cellulose on this planet is now being produced by plant cellulose synthase complexes, this enzyme clearly has bacterial origin: there is no doubt that its genes have been acquired by plants from cyanobacterial ancestors of their chloroplasts [1, 2].

Being a major component of the global carbon cycle, cellulose is a favorite substrate for many diverse bacteria and fungi; for higher organisms, including cows that carry cellulolytic bacteria in their rumen; and also for biotechnologists that investigate ways of converting cellulose into biofuel and various bioactive compounds. Despite the recent success in elucidation of the molecular mechanisms of cellulose biosynthesis in plants, many aspects of this process still remain obscure, particularly the mechanisms of cellular export of the nascent polysaccharide chain and arrangement of the cellulose fibrils into a quasi-crystalline structure [3–5]. Bacterial cellulose biosynthesis has long been used as a simpler and genetically tractable model to study its biosynthesis in plants. Even after this model system became dispensable, studies of bacterial cellulose biosynthesis proved extremely important in their own right, providing valuable insights into the mechanisms of polysaccharide export, as well as regulation of bacterial cell responses to oxygen and nitric oxide (NO), cell motility, cell-cell interactions, biofilm formation and dispersal, and a variety of other environmental challenges. Cellulose biosynthesis has been documented in a wide variety of bacteria, including the nitrogen-fixing plant symbiont *Rhizobium leguminosarum*, soil bacteria *Burkholderia spp.* and *Pseudomonas putida*, plant pathogens *Dickeya dadantii*, *Erwinia chrysanthemi* and tumor-producing *Agrobacterium tumefaciens*, and the well-known model organisms *Escherichia coli* and *Salmonella enterica* (Figure 1; [3, 6, 7]). Cellulose and its derivatives have been identified as significant extracellular matrix components of biofilms and play key roles in modulation of virulence of important plant and human pathogens [8, 9].

From a practical standpoint, bacterial synthesis of cellulose (so-called nanocellulose) is seen as a convenient and effective way to produce stable recyclable fibers for use in wound-dressing and in a variety of emerging nanotechnologies [10, 11]. Genomic data revealed unexpected diversity of cellulose synthase operons even in closely related bacteria, indicating substantial differences in cellulose secretion mechanisms. We review here the recent progress and future challenges in understanding the processes of cellulose biosynthesis in various bacterial lineages.

Diversity of the *bcs* operons

Substrate synthesis for cellulose production starts from the glycolytic intermediate glucose-6-phosphate. The first committed reaction, isomerization of glucose-6-phosphate to glucose-1-phosphate, is catalyzed by phosphoglucomutase (EC 5.4.2.2). Glucose-1-phosphate then reacts with UTP, forming uridine-5'-diphosphate- α -D-glucose (UDP-glucose) in a rate limiting reaction catalyzed by UTP-glucose-1-phosphate uridylyltransferase (EC 2.7.7.9). Finally, cellulose synthase (BCS, EC 2.4.1.12) transfers glucosyl residues from UDP-glucose to the nascent β -D-1,4-glucan chain. Channeling copious amounts of UDP-glucose to cellulose biosynthesis leads to reprogramming of the cellular metabolism favoring gluconeogenesis [12].

A four-gene *bcsABCD* operon involved in cellulose biosynthesis (Figure 2) was initially identified in *Komagataeibacter (Acetobacter) xylinus* (Box 1). Products of the first two genes, BcsA and BcsB (Table 1), were essential for the BCS activity *in vitro* [13–15]. However, all four proteins were required for maximal cellulose production *in vivo*, indicating that BcsC and BcsD were involved in exporting the glucan molecules and packing them at the cell surface. *BcsC* mutants were unable to produce cellulose fibrils, whereas *bcsD* mutants produced ~40% less cellulose than the wild-type [13]. The *K. xylinus bcs* locus included three more genes: *eng* (later renamed *bcsZ*) and *ccpA* upstream of *bcsABCD* genes, and *bglX* downstream of them (Figure 2, Ia). The products of *bcsZ* and *bglX* are an endoglucanase and a β -glucosidase, respectively (Table 2). Such enzymes could be expected to participate in hydrolysis, rather than synthesis, of β -D-glucans, and their roles in cellulose biosynthesis have long remained obscure. The product of the *ccpA* gene was required for cellulose production, earning it the name of ‘cellulose-complementing protein A’ [16]. It affects the expression levels of BcsB and BcsC, interacts with BcsD, and appears to assist the arrangement of glucan chains into crystalline ribbons [17–19]. Accordingly, we propose renaming this gene *bcsH* (Table 2).

Box 1

A brief history of cellulose synthase

Bacterial cellulose biosynthesis has been observed many years ago by ancient Chinese growing the so-called Kombucha tea mushroom (Figure I), a syntrophic colony of acetic acid bacteria and yeast, which metabolizes sugar to produce a slightly acidic tea-colored drink and forms a thick cellulosic mat at its surface [86]. Cellulose was first described in plants in 1838 by French scientist Anselme Payen, in whose memory American Chemical Society has established an annual award (see <http://cell.sites.acs.org/anselmepayenaward.htm>). Thirty years later, British chemist Adrian J. Brown identified cellulose as a key component of the gelatinous pellicle formed upon vinegar fermentation by “an acetic ferment, *Bacterium xylinum*” [87]. The cellulose-producing acetic acid bacterium, discovered by Brown, proved to be a very convenient model organism for studying cellulose biosynthesis. Over the years, this organism has been known under a variety of names, including *Acetobacter xylinum* and *Gluconacetobacter xylinus*. Two years ago it was renamed once again, to *Komagataeibacter xylinus* [88], and is referred to here as *K. xylinus*. However, many publications still refer to this organism as *G. xylinus*; this is also true for its recently sequenced genome (GenBank accession CP004360). Genomes of several closely related bacteria are also available (Table I).

The culture of *K. xylinus* growing in liquid media is extremely efficient in producing a surface pellicle that consists of pure cellulose fibers [76]. Studies of the cellulose biosynthesis by *K. xylinus* led to the purification of the respective enzyme complex and the identification of its activator, cyclic dimeric (3'→5') guanosine monophosphate (c-di-GMP), which proved to be a universal bacterial second messenger that also regulates biofilm formation, motility, virulence, the cell cycle, bacterial cell differentiation, and other fundamental physiological processes in a wide variety of bacteria [9]. Cellulose and its derivatives have been identified as significant extracellular matrix components of

biofilms and play key roles in modulation of virulence of important plant and human pathogens [8, 9].

When did cellulose first appear on this planet? It is being produced by many diverse bacteria including members of the cyanobacterial lineage, which apparently branched from other bacteria as early as 3.0–3.5 billion year ago [1, 30]. As cellulose protects against environmental stresses such as UV radiation and desiccation and is found in modern bacteria-containing hot spring sulfur-turf mats [81, 89], it was even proposed as a potential biosignature for detecting signs of life in extraterrestrial rock materials [90].

Figure I. Kombucha tea mushroom, a syntrophic community of yeast and acetic acid bacteria of the *Gluconacetobacter*/*Komagataeibacter* group [96, 99].

Table I
Genome sequences of the cellulose producing *Gluconacetobacter* (*Komagataeibacter*) species

Organism name (latest)	TaxID	Genome					Reference
		Status	GenBank entry	Contigs	Total size, bp	Encoded proteins	
<i>Komagataeibacter xylinus</i> E25	1296990	Comp- lete	CP004360 - CP004365	6 ^a	3,447,425	3,674	[91]

Organism name (latest)	TaxID	Genome					Reference
		Status	GenBank entry	Contigs	Total size, bp	Encoded proteins	
<i>Komagataeibacter medellinensis</i> NBRC 3288 ^b	634177	Comp-lete	AP012159 - AP012166	8 ^a	3,513,191	3,195	[92]
<i>Gluconobacter oxydans</i> DSM 3504	1288313	Comp-lete	CP004373, AJ428837	2 ^a	2,886,777	2,463	–
<i>Gluconacetobacter</i> sp. SXCC-1	1004836	Draft	AFCH00000000	64	4,233,336	4,895	[93]
<i>Komagataeibacter hansenii</i> ATCC 23769 ^c	714995	Draft	ADTV01000000	71	3,636,659	3,303	[94]
<i>Komagataeibacter europaeus</i> LMG 18890	940283	Draft	CADP00000000	321	4,227,398	3,485	[95]
<i>Komagataeibacter europaeus</i> LMG 18494	940284	Draft	CADR00000000	216	3,991,281	3,312	[95]
<i>Komagataeibacter europaeus</i> 5P3	940285	Draft	CADS00000000	256	3,989,313	3,364	[95]
<i>Komagataeibacter oboediens</i> 174Bp2	940286	Draft	CADT00000000	200	4,181,473	3,355	[95]
<i>Komagataeibacter rhaeticus</i> AF1	1432055	Draft	JDTI00000000	225	3,939,137	3,358	[96]
<i>Komagataeibacter kakiaceti</i> 25156	1234672	Draft	BAIO00000000	947	3,133,102	2,314	[97]
^a Genomes of <i>K. xylinus</i> , <i>K. medellinensis</i> and <i>G. oxydans</i> consist of a chromosome and, respectively, 5, 7 and 1 plasmids. ^b <i>Komagataeibacter medellinensis</i> NBRC 3288 does not produce cellulose [92]. ^c <i>Komagataeibacter hansenii</i> has been also referred to as <i>Gluconacetobacter kombuchae</i> [98].							

Some strains of *K. xylinus* also have a second *bcs* operon which encodes a single long BcsAB fusion protein, and two additional genes, *bcsX* and *bcsY*. Their products have not been characterized, although sequence comparisons predicted that BcsY could function as a transacylase, probably participating in the production of acetylcellulose or a similarly modified polysaccharide [20].

An early analysis of the cellulose synthase operons in *E. coli* and *S. enterica* [6, 21] indicated the presence of the same *bcsA*, *bcsB*, and *bcsC* genes and two additional genes, *yhjQ* and *bcsZ* (Figure 2, IIa). This locus also contains a divergent operon, *bcsEFG*, which is also required for cellulose production [22, 23]. Subsequent analysis revealed the existence of an additional short gene, *yhjR*, preceding the *yhjQ-bcsABZC* operon, and showed that *yhjQ* is required for cellulose biosynthesis [24]. Accordingly, *yhjQ* gene has been renamed *bcsQ*. More recently, deletion of *yhjR* has been shown to affect biofilm formation, indicating a role in cellulose production [23].

In *A. tumefaciens*, *R. leguminosarum*, and many other bacteria, *bcs* operons include *bcsA* and *bcsB* genes but lack *bcsC* (Figure 2, IIIa) [25–27]. The *bcs* locus of *A. tumefaciens* includes two convergent operons, *celABCG* and *celIDE*. The first three genes are orthologs of *bcsA*, *bcsB*, and *bcsZ*, respectively, whereas the other three appear to be specific for this kind of operon. Of these, *celE* was essential for cellulose formation [27]. Similar operons are also seen in members of the *Actinobacteria* and *Firmicutes* phyla, which lack the outer membrane and apparently employ cellulose secretion machineries that are quite distinct from those seen in proteobacteria.

Classification of the *bcs* operons

The diversity of *bcs* operons prompted several attempts to classify them [6, 7, 20]. Here, we propose dividing all characterized *bcs* operons into three major types, found, respectively, in *K. xylinus*, *E. coli*, and *A. tumefaciens* (Figure 2). Each of these major types of *bcs* operons can be divided into several subtypes, based on the gene order and gene content, as shown in Figure 2.

The distinguishing feature of the first type of *bcs* operons is the presence of the *bcsD* gene, whose product is believed to be localized in the periplasm and appears to direct the glucan chain towards the pores in the outer membrane [28, 29]. Type I *bcs* operons are found in certain representatives of alpha, beta, and gamma subdivisions of Proteobacteria (Figure 3). In some type Ib operons, *bcsA* and *bcsB* genes are fused, resulting in a single catalytic subunit of ~1,500 amino acid residues, which might be post-translationally cleaved.

The second, *E. coli*-like type of *bcs* operons is widespread among the members of beta and gamma subdivisions of Proteobacteria. Its distinguishing feature is the presence of the *bcsE* and *bcsG* genes (and the absence of *bcsD*). Two short genes, *bcsF* and *yhjR*, are often present but may be either missing or just not annotated in the genomic sequence.

The third type of *bcs* operons includes *bcsA* and *bcsB* genes but not *bcsD*, *bcsE*, or *bcsG* (Figure 2). Such operons usually include *bcsZ* and an additional gene, referred to here as *bcsK*, that encodes a BcsC-like TRP domain-containing protein but without the BcsC_C domain (see below).

Finally, there is a class of *bcs*-like operons that contain *bcsA* but lack *bcsB* and most other *bcs* genes. Such *bcsB*-missing operons have been seen in cellulose-producing cyanobacteria *Anabaena* sp. PCC7120 and *Thermosynechococcus vulcanus* [1, 30]. In the absence of BcsB, binding of the BcsA-synthesized nascent glucan polymer on the outer face of the membrane and its translocation outside the cell must be carried out by different, yet uncharacterized proteins. Potential candidates include the membrane-fusion family protein HlyD (Figure 2, IVa) and an uncharacterized protein BcsW, which are encoded in the same operons as *bcsA*. Thus, type III and type IV *bcs* operons encode potential new components of the cellulose export machinery, whose functions remain to be elucidated. Obviously, type IV operons represent a mixed bag and additional operon types could be identified once their BCS products are experimentally characterized.

Acquisition and loss of *bcs* genes

Some organisms, including opportunistic human pathogens *Klebsiella pneumoniae* and *Raoultella ornithinolytica*, carry both type I and type II *bcs* operons, whereas their close relatives carry either a single *bcs* operon or none at all (Figure 3). The presence of two *bcs* operons likely results from lateral gene transfer with extra *bcs* genes providing the organism with greater flexibility with respect to cellulose production and biofilm formation. A more common picture is acquisition and loss of individual *bcs* genes, which may result in substantial differences between species from the same genus (Figure 3). The genus *Burkholderia* exhibits the greatest diversity with at least four patterns of *bcs* genes. In

Rhizobium etli and *Pseudomonas syringae*, *bcs* gene patterns differ even within species. The significance of such unusual gene arrangements of *bcs* genes remains to be studied.

Another interesting development is inactivation of certain *bcs* genes that is seen in the genus *Shigella* (Figure 3). While *Shigella boydii* carries the same *bcs* operon as *E. coli*, three other species have frameshift mutations in *bcsA* (*Shigella flexneri* and *Shigella sonnei*) or other *bcs* genes (*Shigella dysenteriae*) that should prevent them from producing cellulose. The loss of cellulose biosynthesis is probably an adaptation of these organisms to their parasitic lifestyle.

Structure and functions of individual subunits

Bacterial synthesis of cellulose occurs at the cytoplasmic side of the (inner) membrane and elongation of the nascent molecule must be tightly linked to its secretion. Accordingly, in addition to the catalytic glucosyltransferase subunit BcsA, BCS complexes include a variety of subunits that ensure orderly export of the growing polysaccharide chain. We present here a brief description of the BCS subunits.

BcsA and BcsB

BcsA and BcsB are two catalytic subunits that are present in all BCS enzymes experimentally characterized so far. Crystal structure of the BcsA-BcsB complex from *Rhodobacter sphaeroides* has been solved recently [31]. BcsA consists of eight transmembrane (TM) segments and two large cytoplasmic domains, a glycosyltransferase domain in the middle and a C-terminal fragment that contains the c-di-GMP-binding PilZ domain (Figure 4). BcsB is located in the periplasm and is anchored in the membrane by a single transmembrane helix. An intriguing feature of the BcsAB structure is a channel leading from the glycosyltransferase domain across the membrane and into the periplasm [31]. The size of this channel allows it to accommodate several glucoside units, indicating that BCS likely couples addition of new glucoside residues to the nascent glucan molecule with its translocation across the membrane and into the periplasm [31]. In *K. xylinus*, the BCS complex is localized along the longitudinal axis of the cell, which might aid arrangement of glucan chains into crystalline cellulose ribbons [29].

BcsC and BcsK

BcsC is a periplasmic protein that consists of an N-terminal α -helical part formed by several tetratricopeptide repeat (TPR) domains and a C-terminal part that is structurally similar to the β -barrels of outer membrane proteins. This organization is similar to that of AlgK-AlgE pair of proteins that participate in the export of alginate from *Pseudomonas aeruginosa* and have known 3D structures [33, 34]. In *P. aeruginosa*, AlgK interacts with the peptidoglycan layer and organizes the entire alginate biosynthesis and secretion complex [35, 36], while AlgE forms an outer membrane pore [37, 38]. Accordingly, the TPR-containing N-terminal part of BcsC is believed to interact with peptidoglycan and other BSC components, while its C-terminal β -barrel domain is likely located in the outer membrane, forming a channel that guides the nascent glucan out of the cell [33, 38].

Some type III *bcs* operons encode a different TPR-containing protein, referred to as CelG in *A. tumefaciens* [27] and renamed here BcsK (Table 2). Its functions are probably the same as those of AlgK and the N-terminal part of BcsC, i.e. interacting with the peptidoglycan and organizing the entire cellulose secretion complex.

BcsD and BcsH

BcsD is a periplasmic protein that oligomerizes to form a cylindrical octamer ~90 Å in diameter with a huge central pore that can accommodate up to 4 separate glucan molecules [28]. BcsD seems to be required for the arrangement of the BCS complex along the longitudinal cell axis [29].

Another important protein is the ‘cellulose complementing factor’ CcpA (renamed here BcsH). It is encoded exclusively in the *Komagataeibacter/Gluconacetobacter* lineage and is required for the BCS activity in *K. xylinus* and *Komagataeibacter hansenii* [16–18]. It has been shown to interact with BcsD in the periplasm. This unique organization might account for the extremely high activity of the *K. xylinus* BCS.

BcsQ

In *E. coli*, BCS is localized to the cell pole, which requires the BcsQ protein [24], a predicted ATPase of the MinD/ParA/Soj superfamily. This protein is encoded in most *bcs* operons (Figure 2) and is likely to determine cellular localization of the respective BCS complexes. Insertional inactivation of the *yhjQ* (*bcsQ*) gene caused abnormal cell division, leading to cell filamentation [39]. Based on these observations and its similarity to MinD, BcsQ is often mis-annotated as a cell division protein; the *bcs* operon structures shown in Figure 2 should help in resolving this confusion.

BcsZ and BglX

The widespread presence in the *bcs* operons of the cellulase-encoding *bcsZ* gene (Figure 2) seems counterintuitive and, in *Arabidopsis*, has been aptly referred to as “a cat among the pigeons” [40]. Indeed, BcsZs from several bacteria have been shown to possess an endo- β -1,4-glucanase (cellulase) activity and *E. coli* protein was even crystallized in a complex with the substrate cellopentaose [41]. One clue to its function is the predicted periplasmic and extracellular location of BcsZ, which suggests a role in regulating cellulose production. Disruption of the *K. xylinus bcsZ* gene decreased the overall production of cellulose and caused irregular packing of its fibrils [19]. In *R. leguminosarum*, *bcsZ* mutants produced longer cellulose micro-fibrils but had a reduced ability to form biofilms [42]. A similar proof-reading role could be ascribed to BglX, a β -glucosidase that is occasionally encoded in the *bcs* operons, e.g. in *K. xylinus* (Figure 2). Of note, in *R. leguminosarum*, BcsZ has a function of its own, as it is required to initiate successful symbiosis through selective digestion of the non-crystalline cellulose of the root hair wall at the tip [42].

BcsE and BcsG

BcsE and BcsG are encoded in type II *bcs* operons and are required for optimal cellulose synthesis by the respective enzymes [22, 43]. BcsE has been shown to bind c-di-GMP and thus provide an additional level of regulation of cellulose production [43]. BcsG combines

an N-terminal membrane portion with a C-terminal periplasmic domain that belongs to the alkaline phosphatase superfamily [44]. Its exact role in cellulose biosynthesis remains to be characterized.

BcsO and BcsP

BcsO and BcsP are proline-rich proteins of unknown function encoded in, respectively, enterobacterial (Figure 2, Ib) and β -proteobacterial (Figure 2, Ic) type I *bcs* operons. The N-terminal region of BcsP is similar to BcsR and has been assigned to the same Pfam domain PF10945 [45] (Table 2).

Table 2 lists several more uncharacterized proteins that are encoded within *bcs*-like operons. Their likely involvement in cellulose production and/or modification makes them attractive targets for future experimental studies.

Organization of the BCS holoenzymes

The recent success in solving the crystal structures of the BcsA-BcsB complex from *R. sphaeroides* [31], the BcsD subunit from *K. xylinus*, and of the BcsC-like AlgK protein from *Pseudomonas aeruginosa* [28, 33], makes it possible to imagine the structure of the entire type I BCS holoenzyme (Figure 4a). The structural organization of type II and III BCS enzymes is far less understood. The cartoons on Figure 4b represent proposed positions of their subunits based on the presence of predicted signal peptides and transmembrane segments.

Diversity of cellulose products

The diversity of the *bcs* operons and the respective enzymes might reflect the diversity of their cellulose products. Some *bcs*-like operons encode BcsA-like glucosyltransferases that produce alternative polysaccharides, such as the β -(1 $\rightarrow$ 3)-D-glucan curdlan [46] or the mixed-linkage (1 $\rightarrow$ 3,1 $\rightarrow$ 4)- β -D-glucan [47]. Another example is acetylated cellulose produced by *Pseudomonas fluorescens* [8, 48]. But even if the glucan product is a chemically simple β -1,4-glucose polymer, glucan chains get arranged into complex microfibrils and ribbons, which can have different types of crystallinity. Crystalline cellulose can come in different allomorphs with linear glucan chains packed parallel or anti-parallel and flexibly arranged intra- and intermolecular hydrogen bonds, whereby cellulose I is the predominant allomorph. While the cellulose synthesized by *K. xylinus* has crystalline structure, enterobacteria appear to produce mostly non-crystalline (amorphous) cellulose. One approach to address the question of the structural diversity of cellulose operons and their products is heterologous expression of the BCS complex [49].

Regulation of cellulose biosynthesis

Bacterial cellulose biosynthesis is regulated on both transcriptional and post-translational levels. Expression of the *bcs* genes appears to be controlled by different regulators in different bacteria (e.g. by Fis in *D. dadantii*), but is generally stimulated during biofilm formation, as compared to the log phase [21, 50]. The BCS activity *in vivo* depends on the presence of both BCS subunits and its allosteric regulator c-di-GMP. Accordingly, such

transcriptional regulators as *Salmonella enterica* serovar Typhimurium MlrA and the biofilm regulator CsgD appear to modulate cellulose biosynthesis indirectly by regulating expression of c-di-GMP synthases (diguanylate cyclases, DGCs) and phosphodiesterases (PDEs) [9, 51, 52]. Early studies of the cellulose biosynthesis in *K. xylinus* revealed that c-di-GMP could stimulate it almost 100-fold [3]. The mechanism of this regulation has been uncovered only recently, when the crystal structure of the BcsAB complex revealed the enzyme in an autoinhibitory state with a conserved gating loop interacting with the c-di-GMP-binding PilZ domain [32]. Upon c-di-GMP binding to the PilZ domain, the active site becomes accessible for its UDP-glucose substrate.

In *K. xylinus*, three DGCs provided approximately 80%, 14% and 4% of the total activity [53]. Still, mutants lacking the physiologically dominant DGC1 retained ~85% of cellulose production, indicating that c-di-GMP production by DGC2 and DGC3 was sufficient for activation of the BCS [53]. Accordingly, while most bacteria encode numerous DGCs and PDEs (see http://www.ncbi.nlm.nih.gov/Complete_Genomes/c-di-GMP.html), overexpression of almost any DGC could in principle produce enough c-di-GMP to stimulate cellulose production [51]. However, recent experiments showed that only certain DGCs serve as actual regulators of BCS *in vivo*. Thus, in *E. coli* and *S. Typhimurium*, cellulose biosynthesis is regulated primarily by two DGCs, AdrA (STM0385) and YedQ (STM1987) [51, 54, 55]. DGCs and PDEs that specifically regulate cellulose biosynthesis have also been seen in other bacteria [25, 56–58]. The nature of such dominance of certain specific DGCs is not known. It is possible that AdrA and YedQ are capable of directly binding to the cellulose synthase complex to deliver c-di-GMP straight to its BcsA target or at least produce high local c-di-GMP concentrations at the inner membrane. The recent finding of c-di-GMP binding by BcsE suggests the existence of two different ways of c-di-GMP action: a direct one, through binding to BcsA's PilZ domain, and an indirect one, mediated by the cytoplasmic c-di-GMP receptor BcsE, which then transfers c-di-GMP to BcsA [32, 43].

Cellular c-di-GMP levels are controlled by numerous DGCs and PDEs that are regulated by a variety of extra- or intracellular parameters. While only few of such signals have been identified, oxygen and NO signaling have been demonstrated to specifically affect cellulose biosynthesis (see [9] for a review). In addition, many DGC and PDE domains are fused with the receiver domains, putting these enzymes under the control of the two-component signal transduction systems [25, 56, 59].

Ecology of cellulose biosynthesis

Besides stability and rigidity, bacterial cellulose has a high capacity of water retention. As a common exopolysaccharide component in the extracellular biofilm matrix of ecologically diverse bacteria, from the thermophilic cyanobacterium *T. vulcanus* to the gastrointestinal pathogen *S. enterica* [21, 30], cellulose mediates cell-cell interactions, cell adherence and biofilm formation on biotic and abiotic surfaces [8, 21, 60]. Gradual increase in cellulose production prepares bacteria for surface colonization, as cellulose slows down flagella-associated motility [61]. In mature biofilms, a rigid extracellular matrix termed 'bacterial wood' is formed by the interaction of cellulose with amyloid fimbrial components of the

biofilm matrix [21]. As for resistance mechanisms, cellulose biosynthesis has specifically been shown to protect from chlorine treatment [51].

Cellulose has a specific role in plant-associated bacteria. Plant symbionts such as *P. fluorescens* and *R. leguminosarum* require cellulose production for rhizosphere and phyllosphere persistence and for establishment of tight interactions with host cells [48]. In plant pathogens *A. tumefaciens* and *D. dadantii*, cellulose aids colonization of plant surfaces [50]. Interestingly, cellulose is involved in the interaction of the food-borne pathogen *S. enterica* with the soil fungus *Aspergillus niger* {Balbontin, 2014 #261}, suggesting that cellulose biosynthesis could be a general ecological retention mechanism of soil bacteria via cellulose-chitin interactions.

Food-borne outbreaks due to produce contaminated with microorganisms such as *S. enterica* and *E. coli* increased in the last 20 years. Among other genes relevant for biofilm formation, cellulose biosynthesis genes are consistently found as contributing to the adhesion and colonization of consumer-relevant plants such as tomato, lettuce, and alfalfa sprouts [62–64].

Involvement in pathogenicity

Many enterobacterial species of the human gastrointestinal tract such as *E. coli*, *S. enterica* and *Citrobacter freundii* produce cellulose [65, 66], but cellulose production has not been directly demonstrated in the host. Its significance in bacterial host interaction is indicated as adherence to gut epithelial cell lines is affected in a strain dependent way in *E. coli* [67–69]. Cellulose production inhibits internalization of *E. coli* and *S. Typhimurium* by gastrointestinal epithelial cells and proinflammatory cytokine production of epithelial cells induced by flagellin (which activates the innate immune response) [69, 70]. Thus, as in biofilm formation [71], cellulose partially counteracts the interaction of amyloid curli fimbriae with epithelial cells [21, 69, 70]. Surprisingly, in the probiotic strain *E. coli* Nissle 1917, which does not produce curli fimbriae at body temperature, cellulose biosynthesis has opposite effects as in the commensal *E. coli* strain producing curli [67, 69].

In acute disease situations, cellulose biosynthesis is tightly regulated, while enhanced production leads to a decrease in virulence properties suggesting that cellulose is an anti-virulence factor [72]. For example, in *S. Typhimurium*, invasion of epithelial cells, a key virulence phenotype, is effectively suppressed upon deletion of phosphodiesterases, which can be partially relieved through deletion of BcsA [73]. In the mouse model of urinary tract infection, dysregulation of the c-di-GMP signaling network in an uropathogenic *E. coli* strain, caused by deletion of a negative regulator of the DGC YfiN, resulted in decreased virulence, which could be relieved by combined deletion of BCS and the biofilm regulator CsgD [74]. Cellulose biosynthesis also plays a role in the regulation of growth of intracellular bacteria. Indeed, *S. Typhimurium* produces cellulose inside macrophages, which restricts bacterial growth [72]. Besides providing a trade-off between virulence and persistence in host cells, production of immunologically inert cellulose might mask interaction between bacterial adhesins and host receptors and thereby prevent activation of immune response.

Taken together, these data suggest the existence of both c-di-GMP-dependent and independent pathways to suppress cellulose biosynthesis in bacterial-host interactions. The c-di-GMP signaling network cooperatively regulates suppression of cellulose biosynthesis in acute infections through balanced expression of DGCs and PDEs. Whether cellulose is a virulence factor in chronic infections as part of the biofilm phenotype remains to be established. However, the recent observation of the requirement of the expression of the cellulose-associated *rdar* (red, dry and rough colony morphology [52]) biofilm of *S. Typhimurium* for colonization of solid tumors [75] certainly points in this direction.

Applications of cellulose synthase

From the bioengineering perspective, bacterial cellulose has certain advantages over plant cellulose, including its high purity, high capacity of water retention, and the nano-scale arrangement of the cellulose fibrils. These features make bacterial cellulose an attractive biocompatible material, which is already commercially available as a wound dressing material for complicated wounds such as skin ulcers [76, 77]. Potential applications of bacterial cellulose and its derivatives also include their use as scaffolds for the replacement of small-diameter blood vessels [78, 79] and as drug delivery systems [77, 80], as well as membranes and filters. At this time, only *K. xylinus*-related bacteria produce cellulose in amounts sufficient for industrial use [80]. However, studies of novel bacterial strains and optimization of culture conditions promise to make commercial production of bacterial cellulose and/or its derivatives economically feasible in the near future.

Concluding remarks

Plant-derived cellulose is not just the most abundant biopolymer on Earth, it is also the only fully renewable one. Understanding the mechanisms of its synthesis and using this knowledge to optimize energy generation and production of novel industrial and medical materials has long been the dream of many biologists and engineers. Bacterial cellulose biosynthesis, while taking place on a somewhat smaller scale, is of geochemical, ecological, agricultural and medical importance. The recent structural characterization of BCS provides valuable clues to the organization of the cellulose biosynthesis machinery in bacteria and plants. It also opens new avenues of manipulating these systems towards production of new generation of biopolymers in a renewable and economically sustainable fashion. However, the cellulose biosynthesis and secretion machineries of various bacteria are extremely diverse, and there are still many open questions to be addressed (Box 2).

Box 2

Outstanding questions

- What are the functions of the uncharacterized genes in various *bcs* operons?
- What is the stoichiometry of the subunits in various BCS complexes?
- How is cellulose biosynthesis regulated? What are the input signals?
- What are the mechanisms of cellulose-mediated bacterial adhesion and its role in bacterial-bacterial, bacterial-fungal and bacterial-host interactions?

- Are there substantial differences between cellulose macromolecules produced by different organisms and different cellulose synthase complexes?
- What kinds of additional exopolysaccharides can be made by cellulose synthase-like proteins?
- Will it be possible to produce bacterial cellulose on an industrial scale in an economically efficient way?
- How soon will we see cellulose-based nanomaterials with specifically engineered properties?

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Figure 1. Ecosystems harboring cellulose-producing bacteria

A. The Grand Prismatic Spring in Yellowstone National Park. Sulfur-turf mats of hot springs contain cellulose-producing bacteria [81]. Cyanobacteria such as *Anabaena* and *Nostoc*, which occur in aquatic and terrestrial habitats, also produce cellulose [1, 30].

B. Interaction of cellulose producing bacteria with plant species. Left: In *R. leguminosarum*, the nitrogen-fixing endosymbiont of legumes such as the economically important soybean (in figure), cellulose production aids adhesion and infection of root hairs [82, 83]. Middle: *A. tumefaciens* causes tumors in a broad spectrum of dicotyledonous plants as the preferred host Rosaceae (in figure). Cellulose production stabilizes colonization by the bacteria of the plant surfaces [84]. Right: Fresh produce contaminated with *Salmonella enterica* and *E. coli* is an increasing cause of food-borne outbreaks. Cellulose production is required for *S. Typhimurium* and *E. coli* O157:H7 to optimally adhere to tomato fruits and alfalfa sprouts, respectively [62, 63].

C. *S. Typhimurium* rapidly forms biofilms on the chitinous cell walls of *Aspergillus niger* hyphae [85].

B. and C. In green, purple and red, cellulose producing *R. leguminosarum*, *A. tumefaciens* and *S. Typhimurium* cells.

D. Cellulose production alters the interaction of bacteria with mammalian hosts. Enterobacterial strains isolated from the human gastrointestinal tract produce cellulose [21, 65, 66]. Cellulose production by *S. Typhimurium* and/or *E. coli* affects interaction with epithelial and immune cells in *in vitro* systems [67–69, 72]}.

Gene designations are as in Tables 1 and 2. The gene symbols are drawn approximately to scale. Empty shapes indicate open reading frames of unknown function, the checkered pattern indicates the tetratricopeptide repeat (TPR) domain. The displayed operons are from *Komagataeibacter xylinus* E25 (Ia, locus tags H845_449 -> H845_455), *Dickeya dadantii* Ech703 (Ib, Dd703_3897 -> Dd703_3891), *Burkholderia phymatum* STM815 (Ic, Bphy3259 -> Bphy3253), *Salmonella enterica* serovar Typhimurium (IIa, bcsR -> bcsC, bcsE -> bcsG), *Pseudomonas putida* KT2440 (IIb, Pput_2132 -> Pput_2138), *Burkholderia mallei* ATCC 23344 (IIc, BMAA1590 -> BMAA1584), *Chromobacterium violaceum* ATCC 12472 (IID, CV_2672 -> CV_2673, CV_2679 -> CV_2674), *Agrobacterium fabrum* C58 (IIIa, Atu8187 -> Atu8186, Atu3302 -> Atu3303), *Methylobacterium extorquens* PA1 (IIIb, Mext1366 -> Mext1371), *Azospirillum lipoferum* 4B (IIIc, AZOLI_p30428 -> AZOLI_p30422), *Acidiphilium cryptum* JF-5 (IIId, Acry_0572 -> Acry_0568), *Nostoc punctiforme* PCC 73102 (IVa, Npun_F6499 -> Npun_F6501), and *Nostoc* sp. PCC 7120 (IVb, alr3754 -> alr3757).

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Figure 4. Organization of the cellulose synthase complexes

A. Predicted structure of the type I cellulose synthase holoenzyme, combined 3D structures of BcsA (PDB 4P02, red), BcsB (PDB 4HG6, tan), AlgK (PDB 3E4B), AlgE (PDB 4AZL), and BcsD (PDB 3A8E, blue), see [31–34]. The c-di-GMP molecule bound to BcsA is shown in cyan, the nascent glucan molecule is in green. Abbreviations: IM, inner membrane; PG, peptidoglycan layer; OM, outer membrane.

B – D. Proposed organization of the BCS complexes from *Komagataeibacter xylinus* (B), *Escherichia coli* (C), and *Agrobacterium tumefaciens* (D), see [33, 34, 37, 38] for related models. All BCS subunits are colored the same way as their respective genes in Figure 2. The labels indicate the names of the proteins in Tables 2 and 3.

Table 1

Products of the core genes of various cellulose synthase operons

Protein name	Synonyms	Length, aa	Operon type	UniProt example	Pfam domain name, entry code ^d	Structure ^b	Functional annotation
BcsA	YhjO, CelA	750–870	I, II, III, IV	<u>P37653</u>	Glycos. transf. 2 (PF00535 or PF13641), PilZ (PF07238)	<u>4HG6</u>	Cellulose synthase catalytic subunit A
BcsB	YhjN, CelB	770–800	I, II, III	<u>P37652</u>	BcsB (PF03170)	<u>4HG6</u>	Cellulose synthase subunit B ^c (periplasmic)
BcsC	YhjL, BcsS	1150	I, II	<u>P37650</u>	TPR (PF13414), BcsC_C (PF05420)	<u>3E4B</u> , <u>4AZL</u>	Cellulose synthase subunit C, spans periplasm and outer membrane
BcsD	CesD, AcsD	155	I	<u>P19451</u>	BcsD (PF03500)	<u>3A8E</u>	Cellulose synthase subunit D (periplasmic)
BcsE	YhjS	500–750	II	<u>Q8ZLB5</u>	DUF2819 (PF10995)	–	Cellulose synthase cytoplasmic subunit E, binds c-di-GMP [43] ^d
BcsF	YhjT	60	II	<u>Q7CPI8</u>	DUF2636 (PF11120)	–	Membrane-anchored subunit (1 TM segment)
BcsG	YhjU	550	II	<u>Q7CPI7</u>	DUF3260 (PF11658)	–	Contains 4 TM segments and a periplasmic AlkP domain
BcsQ	YhjQ, WssA	250	I, II	<u>Q8ZLB6</u>	YhjQ (PF06564)	<u>1G3Q</u> ^e	ParA/MinD-related NTPase, may localize BCS at the cell pole ^e
BcsR	YhjR	65	II	<u>P0ADJ3</u>	DUF2629 (PF10945)	–	Likely regulatory subunit
BcsZ	CelA, CelC, YhjM	370	I, II, III	<u>Q8ZLB7</u>	Glyco_hydro_8 (PF01270)	<u>1WZZ</u> , <u>3QXQ</u>	Endo- β -1,4-glucanase (cellulase), periplasmic

^aThe names and codes of the respective protein domains in the Pfam protein families database (<http://pfam.xfam.org/> [45]). In the Pfam release 28 (May 2015), the DUF (domain of unknown function) names have been replaced with the names from the left column.

^bPDB IDs are given for available structures. Structures of homologous proteins are shown in italics.

^cBcsB is often mis-annotated as a c-di-GMP-binding regulatory subunit, divalent ion tolerance protein CutA, or cytochrome *c* biogenesis protein CcmF.

^dBcsE is often mis-annotated as a metalloprotease.

^e1G3Q is a structure of BcsQ-related ATPase MinD. Based on the properties of a *bcsQ* mutant [39], BcsQ is often (mis)annotated as either a cell division protein or an ATPase involved in chromosome partitioning.

Table 2

Products of lineage-specific genes in various cellulose synthase operons

Protein name	Synonyms	Length, aa	Operon type	UniProt example	Pfam domain name, entry code	Functional annotation, comment
BcsH	CcpA, ORF2	340	Ia	Q76KK0	CcpA (PF17040) ^c	Cellulose-complementing protein A, specific for acetobacteria ^a
BglX	BglXa	730	Ia	Q8RTY7	Fn3-like (PF14310), GH3 (PF00933),	β-glucosidase, glycosyl hydrolase family 3, secreted
BcsO	–	200	Ib	D4IFF8	BcsO (PF17037) ^c	Unknown, specific for enterobacterial type Ib <i>bcs</i> operons
BcsP	–	180	Ic	Q2KWO2	BcsP (PF10945) ^c	Unknown, specific for β-proteobacterial type Ic <i>bcs</i> operons
BcsK	CelG	750	III	Q8U4V1	TPR (PF13432)	Likely interacts with peptidoglycan
BcsL	CelD	550	IIla	Q7CS43	Acetyltransf (PF13480), TPR (PF13432)	Acetyltransferase
BcsM	CelE	380	IIIa	Q7CS44	Peptidase_M20 (PF01546)	Zn-dependent amidohydrolase, may deacylate modified glucose residues
BcsN	–	330	III	B9K194	BcsN (PF17038) ^c	Unknown, periplasmic, membrane-anchored (1 TM), specific for α-proteobacteria
BcsS	–	250	III	A9W2G1	BcsS (PF17036) ^c	Unknown, secreted, specific for α-proteobacteria
BcsT	–	620	III	A9CEZ9	GT_21 (PF13506)	Membrane protein (8 TM) with a cytoplasmic glycosyltransferase domain
BcsU	CelJ	170	III	Q7CS50	DUF995 (PF06191)	Unknown, secreted, specific for Rhizobiales
BcsV	CelK	320	III	Q7CS49	GH26 (PF02156)	Beta-mannanase, secreted, specific for Rhizobiales
BcsW	–	440	IV	Q8YQR4	DUF3131 (PF11329)	Unknown, predicted periplasmic
BcsX	WssF	260	–	Q9WX69	SGNH_hydrolase (IPR013830)	Probable cellulose deacetylase ^b
BcsY	–	380	–	Q9WX70	Acyl_transf_3 (PF01757)	Membrane protein (10 TM), probable cellulose acylase

^a BcsH is often mis-annotated as an endoglucanase or β-glucosidase.

^b BcsX is often mis-annotated as cell division protein FisQ.

^c These protein families have appeared in the release 28 (May 2015) of the Pfam database [45].