Cyclic β-Glucans at the bacteria-host cells interphase: one sugar ring to rule them all

Leticia S. Guidolin [1], Vilma Arce-Gorvel [3], Andres A. Ciocchini [1], Diego J. Comerci [1,2] and Jean-Pierre Gorvel[3]*

1- Instituto de Investigaciones Biotecnológicas Dr. Rodolfo A. Ugalde, Instituto Tecnológico de Chascomús, Universidad Nacional de San Martín, IIB-INTECH CONICET, San Martín, Buenos Aires, Argentina.

2- Comisión Nacional de Energía Atómica, CNEA, Grupo Pecuario, Centro Atómico Ezeiza, Buenos Aires, Argentina.

3- Aix Marseille Univ, CNRS, INSERM, CIML, Marseille, France

*To whom correspondence should be addressed. Centre d'Immunologie de Marseille-Luminy, parc scientifique de Luminy, Case 906, 13288 Marseille cedex 9, France, gorvel@ciml.univ-mrs.fr, Tel : +33491269115

Key words: Proteobacteria, Brucella, cyclodextrin, immnuomodulator, cyclic glucan

Running title: Cyclic Beta Glucan host cell interaction

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/cmi.12850

Abstract

Cyclic β-1,2-D-glucans (CβG) are natural bionanopolymers present in the periplasmic space of many *Proteobacteria.* These molecules are sugar rings made of 17 to 25 D-glucose units linked exclusively by β-1,2 glycosidic bonds. CβG are important for environmental sensing and osmoadaptation in bacteria, but most importantly, they play key roles in complex host-cell interactions such as symbiosis, pathogenesis and immunomodulation. In the last years, the identification and characterization of the enzymes involved in the synthesis of CβG allowed to know in detail the steps necessary for the formation of these sugar rings. Due to its peculiar structure, CβG can complex large hydrophobic molecules, a feature possibly related to its function in the interaction with the host. The capabilities of the CβG to function as molecular boxes and to solubilize hydrophobic compounds are attractive for application in the development of drugs, in food industry, nanotechnology and chemistry. More importantly, its excellent immunomodulatory properties led to the proposal of CβG as a new class of adjuvants for vaccine development.

Introduction

The cyclodextrins (CD) are a family of compounds made up of glucose units bound together in a ring (cyclic oligosaccharides). Widely used in the food, pharmaceutical and chemical industries, as well as in agriculture and environmental engineering, cyclodextrins are typically produced by enzymatic conversion of starch and consist of 6 (α CD), 7 (β CD), 8 (γ CD) and 9 (δ CD) α-1,4 linked-D-glucopyranoside units. They form a toroid-like structure that is hydrophobic inside and hydrophilic outside, which can form complexes with hydrophobic compounds improving the solubility of the latter. This property has made these molecules attractive for pharmaceutical and dietary supplement applications in which hydrophobic compounds are delivered. α-, β-, and γ-cyclodextrin are generally regarded as safe by the FDA (Davis *et al.*, 2004).

Unlike those cyclodextrins, CβG are natural bionanopolymers present in the periplasmic space of many *Proteobacteria*. They are also ring-shaped molecules whose building blocks are exclusively D-glucose linked by β-glycosidic bonds. Depending on the species or growth conditions, the sugar ring can be decorated with different chemical groups, also known as substituents, such as sn-1-phosphoglycerol, succinic acid or methylmalonic acid that confer them an anionic character. In all studied cases, these molecules play a key role in environmental sensing and adaptation but most importantly in complex host-cell interactions such as symbiosis, pathogenesis and immunomodulation (Breedveld *et al.*, 1994, Bontemps-Gallo *et al.*, 2017).

Often, the literature refers generically to this group of polymers as cyclic beta glucans. This term, however, can be a source of confusion since it denotes under the same name a variety of molecular structures with diverse mechanisms of synthesis, properties and functions. In order to avoid ambiguities and misunderstandings, this review will follow the classification proposed by Bohin et al. (Bohin, 2000) renaming bacterial beta glucans as

Osmoregulated Periplasmic Glucans (OPGs). This group is divided into four families according to their molecular structure. Family I is composed of linear glucans with β-1,2-glycosidic backbones and β-1,6 ramifications present in many *GammaProteobacteria* such as *E. coli*, *Pseudomonas aeruginosa*, and *Dickeya dadantii*. The remaining three families are composed of cyclic glucans. Family II contains exclusively β-1,2 linked cyclic glucans synthesized by some *AlphaProteobacteria* such as *Agrobacterium*, *Rhizobium* and *Brucella*. Family III is composed of cyclic glucans with a mix of β-1,3 and β-1,6 glycosidic backbones branched with β-1,4 and α-1,3 linkages present in *Bradyrhizobium* and *Azospirillum*. Family IV groups cyclic glucans with backbones containing β-1,2 linkages with a single α-1,6 bond introducing rigidity in the annular structure (Lippens *et al.*, 1998). These polymers are found in some *Xhantomonas*, *Ralstonia* and *Rhodobacter* (Bontemps-Gallo *et al.*, 2017).

Of all of these families, the best characterized at biochemical and genetic levels are that of the cyclic β-1,2 glucans (CβG) also known as cyclosophoraoses or cyclosophorans. The different biological properties of this peculiar sugar ring and the consequence of its action on the immune system are discussed in this review.

Cyclic β -1,2 glucan structure and biochemical properties

CβG were the first OPGs described, found in the supernatant of *Agrobacterium tumefaciens* cultures (McIntire *et al.*, 1942). Following this discovery, CβG were also found in the periplasmic space of other *Agrobacterium*, *Brucella*, *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* species (Abe *et al.*, 1982, Hisamatsu *et al.*, 1983, Bundle *et al.*, 1988). CβG have a sugar ring-like structure with a varied degree of polymerization (DP), with 17-25 glucose units although in some cases, as in *S. meliloti*, it can reach up to 40 glucoses (Fig 1C.) (Koizumi *et al.*, 1984). Depending on the species, they can be neutral or substituted. CβG are stable biopolymers and this is related to two unique structural characteristics. First,

the absence of a reducing end turns the cyclic structure more resistant to enzymatic degradation than the linear structure present in most bacterial polysaccharides. Second, the β-1,2 glycosidic bond is rare in nature, only a few β1,2 glucanases have been described so far (Breedveld *et al.*, 1994, Abe *et al.*, 2017).

CβG are structurally similar to cyclodextrins (CD). The ring-shape of CβG and CD delimits a central hydrophobic cavity and a hydrophilic surface that largely explains the peculiar properties of these molecules including high water solubility and the ability to form inclusion complexes with hydrophobic guest molecules. The solubility of CβG is higher than CD: 250 g/L for CβG versus 145, 18 and 232 g/L for α-, β- and γ-cyclodextrins, respectively.

In contrast to the rigid conformation of CD, structural studies and modeling indicate that CβG can adopt various types of compact and irregular conformations, which means that CβG may be a highly flexible molecule with no single dominant conformation. The bigger central hydrophobic cavity and the higher molecular flexibility of CβG allow them to include and solubilize large hydrophobic molecules such as ergosterol and paclitaxel following an "induced fit" mechanism (Choi *et al.*, 2000, Kwon, 2000, Venkatachalam *et al.*, 2013, Park, 2016, Cho *et al.*, 2018).

Different studies indicate that CβG and derivatives can be utilized as complexing agents to enhance solubility of guest molecules (Park, 2016). Their ability as chiral selectors for various enantiomers, as catalytic carbohydrates for methanolysis, and as morphology-directing agents for the synthesis of selenium nanowires has also been described (Lee *et al.*, 2009, Jeong *et al.*, 2015, Cho *et al.*, 2016). Macrocyclic compounds are very interesting in supramolecular chemistry as their conformation results in high affinity and/or selectivity for specific interactions, useful in the design of artificial host in host-guest chemistry (Cho *et al.*, 2016).

In view of the peculiar structure and its unique properties, it is not surprising that CβG are part of the molecular arsenal used by bacteria for the establishment of complex interactions with the host as in symbiosis or pathogenesis.

CβG Biosynthesis and control of the degree of polymerization

Biological production of CβG occurs at the bacterial cell membrane. It requires the concerted action of at least two enzymatic activities: a synthase and a transporter. In the cases where the CβG is decorated with substituent molecules, an additional enzyme completes the catalytic biosynthetic complex (Fig. 1A).

The CβG synthesis is catalyzed by a particular multimodular and polyfunctional integral inner-membrane enzyme, the Cyclic Glucan Synthase. The first description of this kind of enzyme was done in *A. tumefaciens* (ChvB), *S. meliloti* (NdvB) and *Brucella abortus* (Cgs). (Zorreguieta *et al.*, 1985, Zorreguieta *et al.*, 1986, Castro *et al.*, 1996, Iñón de Iannino *et al.*, 1998). The three enzymes share a high degree of identity and conserved functions allowing heterologous complementation assays (Dylan *et al.*, 1986, Iñón de Iannino *et al.*, 1998). The best characterized of them, both at the biochemical and structural level is the Cgs, the *B. abortus* cyclic glucan synthase.

B. abortus Cgs is a 320-kDa protein with six transmembrane-spanning segments (TMSs), which define three small periplasmic loops and four large cytoplasmic regions (Ciocchini *et al.*, 2004). Cgs itself acts as a protein intermediate and catalyzes the four enzymatic reactions required for the synthesis of CβG: initiation, elongation, phosphorolysis and cyclization.

The initiation reaction starts when Cgs catalyzes the transfer of the first glucose from UDP-glucose (UDP-Glc) to a yet unidentified amino acid residue in the N-terminal third of its structure (Fig 1B). The minimal region necessary for initiation reaction comprises the first 1004 amino acids and thus, it was postulated that the glucosylation should occur at an amino

acid residue located in one of the three Cgs cytoplasmic loops (Ciocchini *et al.*, 2006, Guidolin *et al.*, 2009). Elongation of a polyglucose chain from the non-reducing end of the first enzyme-bound glucose proceeds by successive additions of glucose units provided by UDP-Glc. This process is known as elongation reaction and is catalyzed by the same glycosyltransferase domain responsible for the initiation reaction (Ciocchini *et al.*, 2006). In addition to this domain, Cgs has a β-1,2-glucooligosaccharide phosphorylase domain in the C-terminal half. This domain successively removes glucose residues from the growing oligosaccharide, trimming the non-reducing end of the polyglucose chain and releasing glucose-1-P. The balanced action of β-1,2 glucosyltransferase and β-1,2 glucooligosaccharide phosphorylase activities allows Cgs to efficiently control the CβG polymerization degree. In mutants where phosphorylase activity is absent, Cgs synthesizes CβG with a higher degree of polymerization reaching up to 28 glucose units. The fact that the selection pressure conserves this huge portion of the protein indicates that the size of the CβG is important to their function. From a bioenergetic point of view, the phosphorolysis activity is an efficient way to control the DP since the energy of the β-1,2-glucosidic bond is conserved in the form of glucose-1-P that can be used for the synthesis of UDP-Glc, the sugar donor for the synthesis of CβG. This would not be possible if the DP control would be exerted by a beta-glucosidase activity that releases glucose instead of glucose-1-P (Ciocchini *et al.*, 2007).

The cyclization reaction puts an end to the balance between elongation and phosphorolysis activities when the linear glucose chain bound to Cgs is cyclized and the CβG is released from the enzyme. The cyclization leaves one or more glucose residues linked to Cgs serving as a primer for the next round of CβG synthesis (Guidolin *et al.*, 2009). Once the cyclization and the release of CβG from the synthase have occurred, the newly formed CβG is transported to the periplasmic space by an ABC type transporter that uses ATP hydrolysis as a driving force. The best-studied CβG transporters are Cgt in *B. abortus*, NdvA in

Rhizobium and ChvA in *Agrobacterium*. They share a high degree of similarity, nearly 60% of amino acid identity, and allow heterologous complementation which suggests that they use a common mechanism for transport (Stanfield *et al.*, 1988, Cangelosi *et al.*, 1989, de Iannino *et al.*, 1989, Roset *et al.*, 2004).

Depending on the bacterial species, the periplasmic CβG can be modified with different substituents molecules. Generally, the modification occurs in a fraction of the periplasmic CβG and the degree of substitution is influenced by the growth stage. For example in *B. abortus*, the CβG modifier enzyme (Cgm) is a succinyltransferase adding an average of two O-succinyl residues per sugar ring. This confers an anionic character to the *Brucella* periplasmic cyclic β-1,2-glucans. Cgm is an integral inner membrane protein of 43 kDa with ten predicted TMSs and is part of the CβG enzymatic complex with Cgs and Cgt (Roset *et al.*, 2006, Guidolin *et al.*, 2015). It was postulated that the succinic acid donor in the reaction catalyzed by Cgm might be succinyl-CoA, a molecule only present in the bacterial cytoplasm. Therefore, the succinylation reaction should be concomitant with CβG transport, in a coordinated way facilitated by the Cgs-Cgt-Cgm complex.

Different molecular substitutions have been described in other species. *S. meliloti* and *M. loti* modify CβG with both phosphoglycerol and succinic acid whereas *R. leguminosarum* bv *trifolii* has un-substituted, neutral CβG (Wang *et al.*, 1999, Kawaharada *et al.*, 2010). In *S. meliloti* the phosphoglycerol-transferase is encoded in the gene *cgmB* and the succinyl-transferase is codified in a gene homologous to *opgC*, the succinyl-transferase of *E. coli* OPG (Wang *et al.*, 1999, Bontemps-Gallo *et al.*, 2017). In *A. tumefaciens*, CβG are substituted with phosphoglycerol (Miller *et al.*, 1987), probably by a *cgmB* homologous present in the genome, whereas *A. radiobacter* substitutes CβG with methylmalonic acid (Hisamatsu *et al.*, 1987). In spite of the fact that there is no information about the identity of the

methylmalonyl-transferase, an acyltransferase homologous to *opg*C present in the genome might be the responsible for this modification.

Recently, by means of interactomic, biochemical and cell biology techniques, it was demonstrated that in *B. abortus*, the synthase Cgs, the transporter Cgt and the succinyltransferase Cgm form a discrete multienzymatic cell-membrane complex located at the bacterial pole (Guidolin *et al.*, 2015). Given the homology between *Rhizobium* and *Agrobacterium*, it is probable that similar CβG biosynthesis complexes also exist in those groups. The significance of a multienzymatic membrane complex for CβG biosynthesis is not completely understood, but keeping the three enzymes together (synthesis, transport and modification) could be advantageous to coordinate both temporally and spatially such activities in order to maintain the three dimensional structure of the final product.

CβG in symbiosis and pathogenesis

The CβG fulfill several biological functions, many of them critical for bacterial physiology. This is why the mutants defective in CβG biosynthesis display pleiotropic defects. For example, in *Rhizobium* and *Agrobacterium*, absence of CβG causes a non-motile phenotype due to the inability to assemble the flagella. The mutants also display increased synthesis of exopolysaccharides, membrane instability and are unable to grow under hypo-osmotic conditions (Geremia *et al.*, 1987, Dylan *et al.*, 1990a).

In most species, OPGs play an osmoregulatory function in the periplasm. The same applies for CβG. Their concentration in the periplasm increases to counterbalance a reduction of the environmental osmolarity. An exception is *Brucella*, where this kind of stress instead

of inducing the increase of CβG concentration only affects the proportion of anionic substituted molecules (Roset *et al.*, 2006).

CβG appear to serve both intracellular and extracellular functions, which are non-mutually exclusive. Intracellular functions described involve osmoprotection and structural stabilization of the cell envelope, including supramolecular membrane complex stabilization, for example flagellum assembly.

The extracellular functions seem to be related to the bacteria-host communication, particularly by modulating the host response in those bacteria that interact with plants or animals, where they interfere, alter or activate signaling pathways due to the ability to signal through molecule complexes or activate immune receptors. For example, mutants defective in CβG lose the ability to establish successful symbiotic or pathogenic associations with their animal or plant host. *Agrobacterium* and *Brucella* mutants unable to synthesize CβG are highly attenuated. Even mutants that form CβG but are incapable to transport it to the periplasmic space display defects in symbiosis and pathogenesis thus indicating that not only the synthesis but the right location of CβG is important for the interaction with the host cell (Briones *et al.*, 2001, Roset *et al.*, 2004). These early descriptions led to the idea that CβG play an important role in the bacterial-host cell interaction, either in pathogenic or in the symbiotic relationships with both plants and animals.

Symbiotic members of *Rhizobiaceae* specifically interact with legume roots, inducing the biogenesis of new structures called nodules where atmospheric nitrogen fixation occurs. Nodule development is a complex process triggered by signals secreted by the bacteria that result in root hair colonization, initiation of hair curling, and root cell division to form the nodule primordium. Simultaneously, bacteria are entrapped into the curled root hair, and then proliferate and infect the root hair through a tube-like structure made of plant cell wall called infection threads. The infection threads filled with bacteria grow toward the base of the root

hair where rhizobia enter the primordium cells by endocytosis. Once inside the cells, bacteria differentiate into nitrogen-fixing bacteroids and proliferate inside a new intracellular compartment called symbiosome (Suzaki *et al.*, 2015).

S. meliloti ndvB mutants display defects in the attachment to *Medicago sativa* (alfafa) root cells. They are able to cause the curling of the hair root, but the formation of infection threads is severely impaired. However, some bacteria are capable of reaching the root cortex but are unable to invade the cytoplasm remaining in the extracellular space. As a result, only small and white pseudonodules with no nitrogen-fixing bacteroids are produced (Toro *et al.*, 1986, Geremia *et al.*, 1987). A similar phenotype was described in *Mesorhizobium loti cgs* mutant on *Lotus tenuis* (D'Antuono *et al.*, 2005)**.** It was reported that addition of purified CβG at the micromolar range to wild type *Rhizobium* strains is an effective way to increase the rate of nodule formation and the number of nodules thus enhancing symbiosis (Abe *et al.*, 1982, Dylan *et al.*, 1990b).

In *Bradyrhizobium*, the OPGs belonging to family III are involved in the suppression of the plant defense response necessary for symbiosis (Bhagwat *et al.*, 1999). It was also demonstrated that family IV OPGs from the plant pathogen *Xhantomonas campestris pv campestris* (*Xcc*) induce a systemic suppression of the plant defense response. In this case, the avirulent phenotype of the *Xcc cgs* mutant could be restored by addition of purified *Xcc* OPG (Rigano *et al.*, 2007).

In all these examples, it is not clear if CβG play the same molecular role. In the case of *Bradyrhizobium*, it has been proposed that their OPGs could sequester hydrophobic substances important for plant signaling such as flavonoids. This is a plausible hypothesis since the excellent capacity of the CβG to complex and solubilize flavonoids has been demonstrated (Cho *et al.*, 2016, Park, 2016). Whatever the molecular mechanism underlying

the action of CβG, it is clear that they are necessary for nodule invasion and effective nitrogen fixation.

Agrobacterium tumefaciens is a plant pathogen that provokes neoplastic transformation of dicotyledonous plants called crown gall tumors. The bacteria induce the tumor to synthesize different metabolites required for bacterial growth. An early step in tumor formation is the attachment of *A. tumefaciens* to the plant cell. Subsequently, bacteria inject the Tumor induction (Ti) plasmid into the host cells by means of the VirB Type IV secretion system (T4SS). The Ti plasmid integrates into the plant genome directing the synthesis of different factors necessary for tumorigenesis, crown gall induction and maintenance (Gordon *et al.*, 2014). Comparable to that observed in symbiotic bacteria, *Agrobacterium tumefaciens chvB* and *chvA* mutants have a pleiotropic phenotype and are avirulent. They are defective in attachment to the plant cells and do not induce crown gall tumors when inoculated in wounded plant tissues (Douglas *et al.*, 1982, Puvanesarajah *et al.*, 1985). It was described that incubation of *A. tumefaciens chv*B mutant and plant tissues at low temperature was sufficient to restore motility, attachment and tumorigenesis (Bash *et al.*, 2002). Attachment and virulence was partially restored by incubation in highly osmotic medium with $CaCl_2$, thus suggesting a CβG-mediated effect by osmoadaptation (Swart *et al.*, 1994). The stability of VirB10, a key component of the VirB system necessary for the transfer of Ti plasmid and tumorigenesis, depends on temperature and osmolarity, and this is exacerbated in the *chv*B mutant (Banta *et al.*, 1998). Therefore, the role of CβG in tumorigenesis seems indirect and related to its osmoregulatory properties (Swart *et al.*, 1994). Presumably, it helps to maintain a right periplasmic osmolarity necessary to prevent the miss-assembly of supramolecular machineries required for pathogenesis.

B. abortus is an animal intracellular pathogen, the causative agent of brucellosis, a worldwide distributed zoonosis affecting wild and domestic animals as well as humans

(Moreno, 2014). Upon infection, bacteria are internalized by macrophagic cells and persist into a membrane-bound intracellular compartment called the *Brucella*-containing vacuole (BCV). *Brucella* resists macrophage killing by avoiding fusion of the BCV with lysosomes, redirecting the traffic to the endoplasmic reticulum, from where it coopts membranes to transform the BCV into a new organelle competent to support intracellular replication (Celli *et al.*, 2004, Starr *et al.*, 2008). *B. abortus cgs* mutants display defects in virulence (Briones *et al.*, 2001, Arellano-Reynoso *et al.*, 2005, Roset *et al.*, 2014). Contrary to what happens with wild type *Brucella*, BCV containing *cgs* mutants are defective in preventing phagosome-lysosome fusion. The defect can be reverted by addition of exogenous CβG or even methyl-β-cyclodextrin (MβCD), a structural analog of CβG, indicating that the sugar ring is directly involved in the process probably by disorganizing lysosomal lipid rafts (Arellano-Reynoso *et al.*, 2005). Indeed, CβG can modulate lipid raft organization in eukaryotic membranes by extracting cholesterol and proteins from these signaling platforms, thus this could be the basis of action on the vacuolar membrane. It was observed that the action of CβG on lipid rafts differs from the MβCD being more subtle and differing in the pattern of proteins that can be extracted (Arellano-Reynoso *et al.*, 2005). This is in line with a pathogen such as *Brucella* taking a stealth infection strategy in order to avoid induction of a strong inflammatory response to promote chronic infection.

CβG and immunomodulation

As mentioned before, CβG is expressed in large amounts in the periplasmic space of bacteria and hence when bacteria are killed by the host immune system, CβG is therefore released in the surrounding host cell environment in micromolar concentrations. In the mouse, *Brucella* CβG has been directly linked to spleen inflammation as a consequence of massive cell recruitment of monocytes, dendritic cells (DC) and neutrophils. This results

from the strong induction of pro-inflammatory cytokines secretion such as IL-12 and TNF-α (Roset *et al.*, 2014). In contrast to BtpA, BtpB and WadC virulence factors, which are involved in the inhibition of immune responses (Salcedo *et al.*, 2008, Conde-Álvarez *et al.*, 2012, Salcedo *et al.*, 2013, Zhao *et al.*, 2017), *Brucella* CβG is able to activate DCs and macrophages. It is a potent activator of both human and mouse DC, promoting pro-inflammatory cytokine expression, antigen cross-priming and cross-presentation to specific $CD8^+$ T cells, inducing the secretion of pro inflammatory cytokines, but at the same time it is able to activate specific anti-inflammatory networks, giving a dual response. As it is the case of most known adjuvants such as Poly I:C, MPLA, R848, CβG is capable of inducing local inflammation when injected on mouse ears. There has been an interest in identifying TLR4 agonists with a dampened toxicity. A recent example of lipid A analog is MPLA known to activate immune cells with similar properties to the LPS but being less toxic and non immunogenic. The advantage of CβG over MPLA or Poly I:C is that it is able to induce an early immune response (Martirosyan *et al.*, 2012). This might have a significant impact on the quality of the immune response to vaccines. *In vivo*, purified CβG led to the formation of an edema with cell infiltrates but significantly milder than in *E. coli* LPS-injected ears, suggesting a lower level of inflammation in response to CβG. This reduced inflammation can be explained by a fast and transient neutrophil recruitment at the site of injection and suggests that the consequence of these dual pro- and anti-inflammatory signals triggered by CβG correspond to the induction of a controlled local inflammation (Martirosyan *et al.*, 2012, Degos *et al.*, 2015). Another interesting characteristic of CβG is that it is capable of activating $CD8^+$ T cells. Antigen-specific $CD8^+$ T cell responses were observed *in vivo* in a model of OVA transgenic mice, in a way similar to the adjuvants Poly I:C or MPLA (Martirosyan *et al.*, 2012). These results were confirmed in myeloid DC from human donors (mDC): when the early transcriptional responses of LPS or CβG activated blood mDC from

five donors was assessed using microarray profiling and Ingenuity Pathway Analysis (IPA), the global transcriptional changes that CβG elicit in mDC support the concept that this molecule might enhance DC-dependent T cell responses (Martirosyan *et al.*, 2012). Furthermore, CβG-treated mDC displayed increased transcription of the co-stimulatory molecules CD40, CD80, CD86, CD70 and 4-1BBL, but decreased transcription of the Th2 co-stimulatory molecule OX40-L, giving a strong pro-inflammatory response network. It was observed that CβG activated blood mDC were efficient at inducing allogeneic naıve $CD4^+$ as well as $CD8^+$ T cell proliferation. Activation of mDC loaded with heat inactivated influenza virus with both CβG and *E. coli* LPS resulted in a considerably increased MP specific $CD8^+$ T cell response. This suggests that CβG can enhance secondary $CD8^+$ T cell responses (Martirosyan *et al.*, 2012). It was also observed that LPS and *B. melitensis* CβG-activated mDC polarized $CD4^+$ T cells into IFN-γ-expressing Th1 cells, and that CβG increases $CD4^+$ T memory responses after DC targeting of PBMC in HCV-recovered and acute TB patients.

Concluding remarks

As soon as they were discovered in the middle of the last century, CβG attracted attention due to their peculiar cyclic structure and their abilities to form inclusion complexes. During the 80's and 90's the importance of bacterial CβG in the pathogenic and symbiotic processes with animal and plant cells became evident although the molecular details that govern these processes are not yet clear and deserve to be deeply addressed. Subsequently, the identification of the enzymes involved in the synthesis and the use of genetic and biochemical tools allowed to know in detail the steps involved in the formation of these intriguing sugar rings. The exploration of novel applications for these peculiar molecules in drug development, food industry, nanotechnology and chemistry is a growing field. Of

interest, the immunomodulatory properties of CβG were recently described (this review). CβG are water-soluble and have the capacity to complex and solubilize hydrophobic substances; they are neither immunogenic nor toxic in mice, unable to induce the generation of specific antibodies and induce strong $CD4^+$ and $CD8^+$ T cell responses. Based on these properties and their particular structure, CβG have been proposed as a new class of immunomodulatory molecules for applications in vaccine development. However, many of these potential applications have been hindered by the fact that CβG are natural polymers that are extracted from the producing bacteria, many of them pathogenic, in small quantities. In this way, detailed knowledge of the genetics and biochemistry of CβG synthesis raises the possibility of developing a recombinant and scalable system for the production of CβG with different degrees of polymerization or substituents compatible with industrial applications.

References

Abe, K., Nakajima, M., Yamashita, T., Matsunaga, H., Kamisuki, S., Nihira, T., *et al.* (2017). Biochemical and structural analyses of a bacterial endo-β-1,2-glucanase reveal a new glycoside hydrolase family. *Journal of Biological Chemistry* **292,** 7487-7506.

Abe, M., Amemura, A. and Higashi, S. (1982). Studies on cyclic β-1,2-glucan obtained from periplasmic space of Rhizobium trifolii cells. *Plant and Soil* **64,** 315-324.

Arellano-Reynoso, B., Lapaque, N., Salcedo, S., Briones, G., Ciocchini, A.E., Ugalde, R., *et al.* (2005). Cyclic beta-1,2-glucan is a Brucella virulence factor required for intracellular survival. *Nat Immunol* **6,** 618-625.

Banta, L.M., Bohne, J., Lovejoy, S.D. and Dostal, K. (1998). Stability of the Agrobacterium tumefaciens VirB10 Protein Is Modulated by Growth Temperature and Periplasmic Osmoadaption. *Journal of Bacteriology* **180,** 6597-6606.

Bash, R. and Matthysse, A.G. (2002). Attachment to Roots and Virulence of a chvB Mutant of Agrobacterium tumefaciens Are Temperature Sensitive. *Molecular Plant-Microbe Interactions* **15,** 160-163.

Bhagwat, A.A., Mithofer, A., Pfeffer, P.E., Kraus, C., Spickers, N., Hotchkiss, A., *et al.* (1999). Further studies of the role of cyclic beta-glucans in symbiosis. An NdvC mutant of Bradyrhizobium japonicum synthesizes cyclodecakis-(1-->3)-beta-glucosyl. *Plant physiology* **119,** 1057-1064.

Bohin, J.P. (2000). Osmoregulated periplasmic glucans in Proteobacteria. *FEMS Microbiol Lett* **186,** 11-19.

Bontemps-Gallo, S., Bohin, J.P. and Lacroix, J.M. (2017). Osmoregulated Periplasmic Glucans. *EcoSal Plus* **7**.

Breedveld, M.W. and Miller, K.J. (1994). Cyclic beta-glucans of members of the family Rhizobiaceae. *Microbiol Rev* **58,** 145-161.

Briones, G., Inon de Iannino, N., Roset, M., Vigliocco, A., Paulo, P.S. and Ugalde, R.A. (2001). Brucella abortus cyclic beta-1,2-glucan mutants have reduced virulence in mice and are defective in intracellular replication in HeLa cells. *Infect Immun* **69,** 4528-4535.

Bundle, D.R., Cherwonogrodzky, J.W. and Perry, M.B. (1988). Characterization of Brucella polysaccharide B. *Infect Immun* **56,** 1101-1106.

Cangelosi, G.A., Martinetti, G., Leigh, J.A., Lee, C.C., Theines, C. and Nester, E.W. (1989). Role for [corrected] Agrobacterium tumefaciens ChvA protein in export of beta-1,2-glucan. *Journal of Bacteriology* **171,** 1609-1615.

Castro, O.A., Zorreguieta, A., Ielmini, V., Vega, G. and Ielpi, L. (1996). Cyclic beta-(1,2)-glucan synthesis in Rhizobiaceae: roles of the 319-kilodalton protein intermediate. *Journal of bacteriology* **178,** 6043-6048.

Celli, J. and Gorvel, J.-P. (2004). Organelle robbery: Brucella interactions with the endoplasmic reticulum. *Current Opinion in Microbiology* **7,** 93-97.

Cho, E., Jeong, D., Choi, Y. and Jung, S. (2016). Properties and current applications of bacterial cyclic β-glucans and their derivatives. *Journal of Inclusion Phenomena and Macrocyclic Chemistry* **85,** 175-185.

Cho, E. and Jung, S. (2018). Biotinylated Cyclooligosaccharides for Paclitaxel Solubilization. *Molecules* **23,** 90.

Choi, Y.H., Yang, C.H., Kim, H.W. and Jung, S. (2000). Molecular dynamics simulations of cyclohenicosakis-[(1-->2)-beta-D-gluco-henicosapyranosyl], a cyclic (1-->2)-beta-D-glucan (a 'cyclosophoraose') of DP 21. *Carbohydrate research* **326,** 227-234.

Ciocchini, A.E., Guidolin, L.S., Casabuono, A.C., Couto, A.S., de Iannino, N.I. and Ugalde, R.A. (2007). A glycosyltransferase with a length-controlling activity as a mechanism to regulate the size of polysaccharides. *Proc Natl Acad Sci U S A* **104,** 16492-16497.

Ciocchini, A.E., Roset, M.S., Briones, G., Inon de Iannino, N. and Ugalde, R.A. (2006). Identification of active site residues of the inverting glycosyltransferase Cgs required for the synthesis of cyclic beta-1,2-glucan, a Brucella abortus virulence factor. *Glycobiology* **16,** 679-691.

Ciocchini, A.E., Roset, M.S., Inon de Iannino, N. and Ugalde, R.A. (2004). Membrane topology analysis of cyclic glucan synthase, a virulence determinant of Brucella abortus. *Journal of bacteriology* **186,** 7205-7213.

Conde-Álvarez, R., Arce-Gorvel, V., Iriarte, M., Manček-Keber, M., Barquero-Calvo, E., Palacios-Chaves, L., *et al.* (2012). The Lipopolysaccharide Core of Brucella abortus Acts as a Shield Against Innate Immunity Recognition. *PLoS pathogens* **8,** e1002675.

D'Antuono, A.L., Casabuono, A., Couto, A., Ugalde, R.A. and Lepek, V.C. (2005). Nodule development induced by Mesorhizobium loti mutant strains affected in polysaccharide synthesis. *Mol Plant Microbe Interact* **18,** 446-457.

Davis, M.E. and Brewster, M.E. (2004). Cyclodextrin-based pharmaceutics: past, present and future. *Nature Reviews Drug Discovery* **3,** 1023.

de Iannino, N.I. and Ugalde, R.A. (1989). Biochemical characterization of avirulent Agrobacterium tumefaciens chvA mutants: synthesis and excretion of beta-(1-2)glucan. *Journal of bacteriology* **171,** 2842-2849.

Degos, C., Gagnaire, A., Banchereau, R., Moriyon, I. and Gorvel, J.P. (2015). Brucella CbetaG induces a dual pro- and anti-inflammatory response leading to a transient neutrophil recruitment. *Virulence* **6,** 19-28.

Douglas, C.J., Halperin, W. and Nester, E.W. (1982). Agrobacterium tumefaciens mutants affected in attachment to plant cells. *Journal of bacteriology* **152,** 1265-1275.

Dylan, T., Helinski, D.R. and Ditta, G.S. (1990a). Hypoosmotic adaptation in Rhizobium meliloti requires beta-(1----2)-glucan. *Journal of Bacteriology* **172,** 1400-1408.

Dylan, T., Ielpi, L., Stanfield, S., Kashyap, L., Douglas, C., Yanofsky, M., *et al.* (1986). Rhizobium meliloti genes required for nodule development are related to chromosomal virulence genes in Agrobacterium tumefaciens. *Proc Natl Acad Sci U S A* **83,** 4403-4407.

Dylan, T., Nagpal, P., Helinski, D.R. and Ditta, G.S. (1990b). Symbiotic pseudorevertants of Rhizobium meliloti ndv mutants. *Journal of Bacteriology* **172,** 1409-1417.

Geremia, R.A., Cavaignac, S., Zorreguieta, A., Toro, N., Olivares, J. and Ugalde, R.A. (1987). A Rhizobium meliloti mutant that forms ineffective pseudonodules in alfalfa produces exopolysaccharide but fails to form beta-(1----2) glucan. *Journal of bacteriology* **169,** 880-884.

Gordon, J.E. and Christie, P.J. (2014). The Agrobacterium Ti Plasmids. *Microbiology Spectrum* **2**.

Guidolin, L.S., Ciocchini, A.E., Inon de Iannino, N. and Ugalde, R.A. (2009). Functional mapping of Brucella abortus cyclic beta-1,2-glucan synthase: identification of the protein domain required for cyclization. *Journal of bacteriology* **191,** 1230-1238.

Guidolin, L.S., Morrone Seijo, S.M., Guaimas, F.F., Comerci, D.J. and Ciocchini, A.E. (2015). Interaction network and localization of Brucella abortus membrane proteins involved in the synthesis, transport, and succinylation of cyclic beta-1,2-glucans. *Journal of bacteriology* **197,** 1640-1648.

Hisamatsu, M., Amemura, A., Koizumi, K., Utamura, T. and Okada, Y. (1983). Structural studies on cyclic (1→2)-β-d-glucans (cyclosophoraoses) produced by Agrobacterium and Rhizobium. *Carbohydrate research* **121,** 31-40.

Hisamatsu, M., Yamada, T., Higashiura, T. and Ikeda, M. (1987). The production of acidic, O-acylated cyclosophorans [cyclic (1→2)-β-d-glucans] by Agrobacterium and Rhizobium species. *Carbohydrate research* **163,** 115-122.

Iñón de Iannino, N., Briones, G., Tolmasky, M. and Ugalde, R.A. (1998). Molecular cloning and characterization of cgs, the Brucella abortus cyclic beta(1-2) glucan synthetase gene: genetic complementation of Rhizobium meliloti ndvB and Agrobacterium tumefaciens chvB mutants. *Journal of bacteriology* **180,** 4392-4400.

Jeong, D., Kim, H., Dindulkar, S.D., Lee, J.Y. and Jung, S. (2015). Preparation of a Novel Chiral Stationary Phase Containing Rhizobial Cyclic β-(1 → 2) Glucans for the Chiral Separation of Some Flavonoids. *Bulletin of the Korean Chemical Society* **36,** 2379-2382.

Kawaharada, Y., Kiyota, H., Eda, S., Minamisawa, K. and Mitsui, H. (2010). Identification of the Mesorhizobium loti gene responsible for glycerophosphorylation of periplasmic cyclic β-1,2-glucans. *FEMS Microbiology Letters* **302,** 131-137.

Koizumi, K., Okada, Y., Utamura, T., Hisamatsu, M. and Amenura, A. (1984). Further studies on the separation of cyclic (1→2)-β-d-glucans (cyclosophoraoses) produced by rhizobium meliloti ifo 13336, and determination of their degrees of polymerization by high-performance liquid chromatography. *Journal of Chromatography A* **299,** 215-224.

Kwon, C., Choi, YH., Kim, N, Yoo, JS.,Yang C., Kim, H., Jung, S. (2000). Complex Forming Ability of a Family of Isolated Cyclosophoraoses with Ergosterol and Its Monte Carlo Docking Computational Analysis. *Journal of Inclusion Phenomena and Macrocyclic Chemistry* **36,** 55-65.

Lee, S., Cho, E. and Jung, S. (2009). Periplasmic glucans isolated from Proteobacteria. *BMB reports* **42,** 769-775.

Lippens, G., Wieruszeski, J.M., Horvath, D., Talaga, P. and Bohin, J.P. (1998). Slow Dynamics of the Cyclic Osmoregulated Periplasmic Glucan of Ralstonia

solanacearum As Revealed by Heteronuclear Relaxation Studies. *Journal of the American Chemical Society* **120,** 170-177.

Martirosyan, A., Perez-Gutierrez, C., Banchereau, R., Dutartre, H., Lecine, P., Dullaers, M., *et al.* (2012). Brucella beta 1,2 cyclic glucan is an activator of human and mouse dendritic cells. *PLoS pathogens* **8,** e1002983.

McIntire, F.C., Peterson, W.H. and Riker, A.J. (1942). A polysaccharide produced by the crown-gall organism. *Journal of Biological Chemistry* **143,** 491-496.

Miller, K.J., Reinhold, V.N., Weissborn, A.C. and Kennedy, E.P. (1987). Cyclic glucans produced by Agrobacterium tumefaciens are substituted with sn-1-phosphoglycerol residues. *Biochimica et biophysica acta* **901,** 112-118.

Moreno, E. (2014). Retrospective and prospective perspectives on zoonotic brucellosis. *Frontiers in Microbiology* **5**.

Park, S. (2016). Cyclic Glucans Enhance Solubility of Bioavailable Flavonoids. *Molecules* **21**.

Puvanesarajah, V., Schell, F.M., Stacey, G., Douglas, C.J. and Nester, E.W. (1985). Role for 2-linked-beta-D-glucan in the virulence of Agrobacterium tumefaciens. *Journal of bacteriology* **164,** 102-106.

Rigano, L.A., Payette, C., Brouillard, G., Marano, M.R., Abramowicz, L., Torres, P.S., *et al.* (2007). Bacterial Cyclic β-(1,2)-Glucan Acts in Systemic Suppression of Plant Immune Responses. *The Plant Cell* **19,** 2077-2089.

Roset, M.S., Ciocchini, A.E., Ugalde, R.A. and Inon de Iannino, N. (2004). Molecular cloning and characterization of cgt, the Brucella abortus cyclic beta-1,2-glucan transporter gene, and its role in virulence. *Infect Immun* **72,** 2263-2271.

Roset, M.S., Ciocchini, A.E., Ugalde, R.A. and Inon de Iannino, N. (2006). The Brucella abortus cyclic beta-1,2-glucan virulence factor is substituted with O-ester-linked succinyl residues. *Journal of bacteriology* **188,** 5003-5013.

Roset, M.S., Ibañez, A.E., de Souza Filho, J.A., Spera, J.M., Minatel, L., Oliveira, S.C., *et al.* (2014). Brucella Cyclic β-1,2-Glucan Plays a Critical Role in the Induction of Splenomegaly in Mice. *PloS one* **9,** e101279.

Salcedo, S.P., Marchesini, M.I., Degos, C., Terwagne, M., Von Bargen, K., Lepidi, H., *et al.* (2013). BtpB, a novel Brucella TIR-containing effector protein with immune modulatory functions. *Frontiers in Cellular and Infection Microbiology* **3,** 28.

Salcedo, S.P., Marchesini, M.I., Lelouard, H., Fugier, E., Jolly, G., Balor, S., *et al.* (2008). Brucella Control of Dendritic Cell Maturation Is Dependent on the TIR-Containing Protein Btp1. *PLoS pathogens* **4,** e21.

Stanfield, S.W., Ielpi, L., O'Brochta, D., Helinski, D.R. and Ditta, G.S. (1988). The ndvA gene product of Rhizobium meliloti is required for beta-(1----2)glucan production and has homology to the ATP-binding export protein HlyB. *Journal of Bacteriology* **170,** 3523-3530.

Starr, T., Ng, T.W., Wehrly, T.D., Knodler, L.A. and Celli, J. (2008). Brucella Intracellular Replication Requires Trafficking Through the Late Endosomal/Lysosomal Compartment. *Traffic* **9,** 678-694.

Suzaki, T., Yoro, E. and Kawaguchi, M. (2015) Chapter Three - Leguminous Plants: Inventors of Root Nodules to Accommodate Symbiotic Bacteria. In *International Review of Cell and Molecular Biology*, K.W. Jeon (ed.). Academic Press, pp. 111-158.

Swart, S., Lugtenberg, B.J., Smit, G. and Kijne, J.W. (1994). Rhicadhesin-mediated attachment and virulence of an Agrobacterium tumefaciens chvB mutant can be

restored by growth in a highly osmotic medium. *Journal of Bacteriology* **176,** 3816-3819.

Toro, N. and Olivares, J. (1986). Analysis of Rhizobium meliloti Sym Mutants Obtained by Heat Treatmentt. *Applied and environmental microbiology* **51,** 1148-1150.

Venkatachalam, G., Gummadi, S. and Doble, M. (2013) Cyclic Beta Glucans from Microorganisms. Production, Properties and Applications. In *SpringerBriefs in Microbiology*, Springer (ed.). New York, Springer.

Wang, P., Ingram-Smith, C., Hadley, J.A. and Miller, K.J. (1999). Cloning, sequencing, and characterization of the cgmB gene of Sinorhizobium meliloti involved in cyclic beta-glucan biosynthesis. *Journal of bacteriology* **181,** 4576-4583.

Zhao, Y., Hanniffy, S., Arce-Gorvel, V., Conde-Alvarez, R., Oh, S., Moriyón, I., *et al.* (2017). Immunomodulatory properties of Brucella melitensis lipopolysaccharide determinants on mouse dendritic cells in vitro and in vivo. *Virulence* **9,** 465-479.

Zorreguieta, A. and Ugalde, R.A. (1986). Formation in Rhizobium and Agrobacterium spp. of a 235-kilodalton protein intermediate in beta-D(1-2) glucan synthesis. *Journal of bacteriology* **167,** 947-951.

Zorreguieta, A., Ugalde, R.A. and Leloir, L.F. (1985). An intermediate in cyclic beta 1-2 glucan biosynthesis. *Biochem Biophys Res Commun* **126,** 352-357.

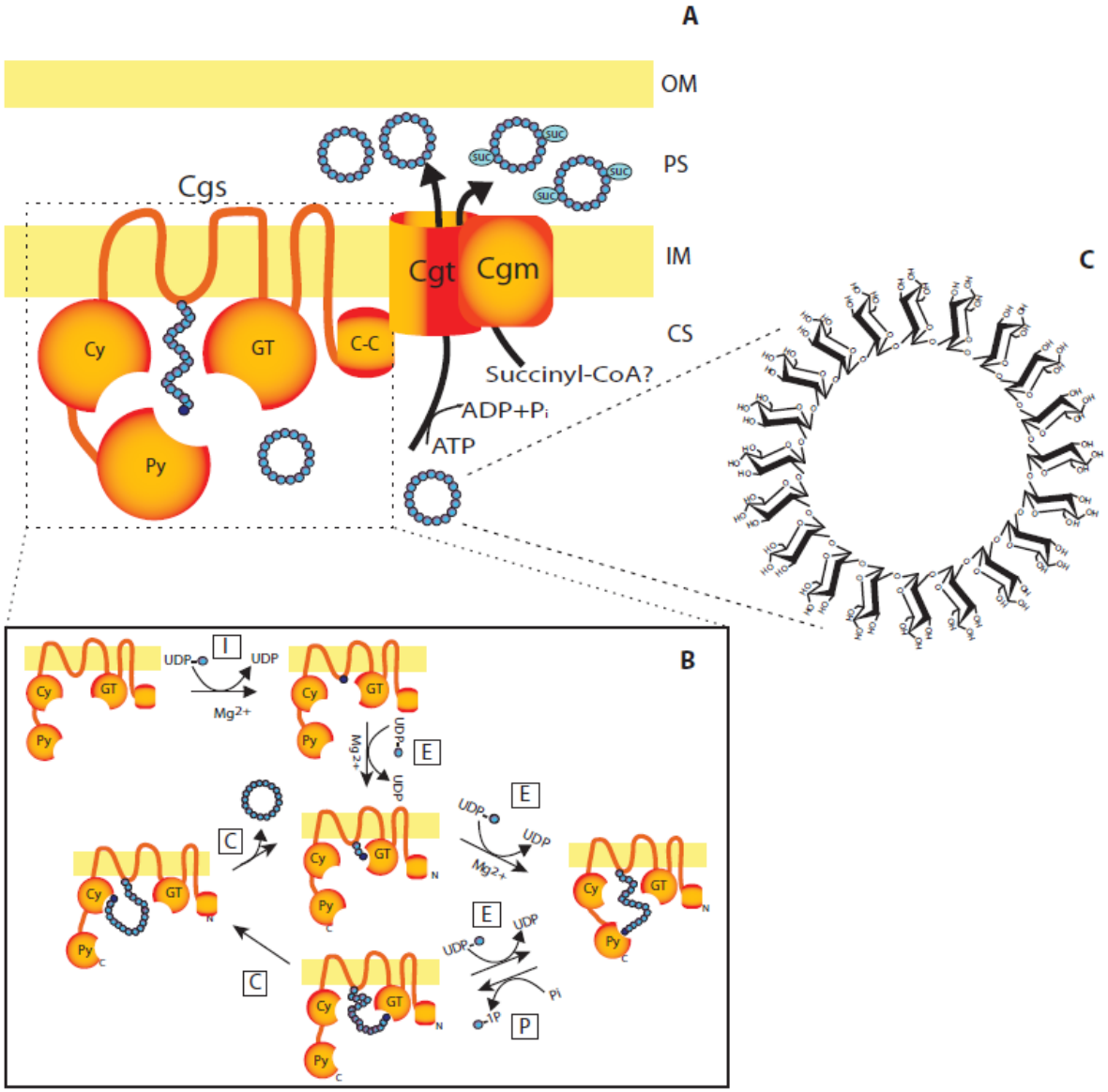

Figure 1: The CβG biosynthetic complex

A. The multienzymatic inner-membrane complex responsible for biosynthesis of CβG in *Brucella abortus* is composed of the CβG synthase (Cgs), the CβG transporter (Cgt) and CβG modifier (Cgm) enzymes. CβG synthesis is catalyzed by Cgs, a multimodular and polyfunctional enzyme.

B. The proposed mechanism for CβG synthesis. Cgs catalyzes the four enzymatic reactions necessary for the synthesis: initiation (I), elongation (E), phosphorolysis (P), and cyclization (C). During the initiation reaction Cgs catalyses the transfer of

the first glucose from UDP-glucose to a yet unidentified amino acid of its structure. The Cgs glycosyl-transferase domain catalyzes the elongation reaction by adding glucose units to the non-reducing end of the growing linear polyglucose chain. The Cgs phosphorylase domain controls the length of the growing oligosaccharide by trimming the non-reducing end and releasing glucose-1-P. Once the linear polysaccharide bounded to Cgs reaches a length of between 17 to 24 glucose units, the cyclization reaction forms the ring structure releasing the CβG from the enzyme. The cyclization leaves a short enzyme-bound oligoglucosaccharide serving as a primer for the next round of synthesis. Cgt, an ABC transporter, catalyzes ATP hydrolysis to transport the released CβG from the cytoplasm to the periplasmic space. Concomitant with the transport, Cgm catalyzes the addition of O-succinyl groups to the C6 of CβG probably from succinyl-CoA.

C. Schematic representation of the neutral (unsubstituted) CβG.

Light blue circles, glucose; dark blue circles, nonreducing end glucose; Pi, inorganic phosphate. GT, glycosyl-transferase domain; Cy, cyclization domain; Py, phosphorylase domain; C-C, Coiled-coil interaction domain.