Chapter

Biosynthesis of the Immunomodulatory Molecule Capsular Polysaccharide A from *Bacteroides fragilis*

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Abstract

Capsular Polysaccharide A (CPSA) is a polymer of a tetrasaccharide unit found on the surface of the symbiotic gut bacteria *Bacteroides fragilis*. CPSA has been suggested to be important for maintaining a natural equilibrium between Th1 and Th2 cell levels in the normal immune system of mammals. If this equilibrium is disrupted, the human body can develop different autoimmune disorders. The gene locus responsible for CPSA biosynthesis has been previously identified. The locus was proposed to encode one glycosyl-1-phosphate transferase (WcfS) and three glycosyltransferases (WcfN, -P and -Q), three sugar modifying enzymes (WcfM, WcfR and WcfO), a flippase (Wzx) and a polysaccharide polymerase (Wzy) based on homology tools. A route for the complete biosynthesis of CPSA has been elucidated. The initiating sugar transferase, WcfS has been previously identified and characterized. An *in vitro* method was used to enzymatically synthesize CPSA, which was assembled on a fluorescent analogue of the native bactoprenyl diphosphate anchor one sugar at a time. Function of the hypothesized pyruvyltransferase WcfO was also determined. This is the first study to characterize a pyruvyltransferase involved in polysaccharide biosynthesis from a prokaryote. The biosynthesis of the polysaccharide was achieved in a single pot, compared to multiple steps involved in chemical synthesis, displaying an enormous leap in the biosynthesis of complex molecules like CPSA.

Keywords: *Bacteroides fragilis*, pyruvyltransferase, glycosyltransferase, capsular polysaccharide A, biosynthesis

1. Introduction

B. fragilis is an obligate anaerobic bacterium which colonizes the intestinal tract of the human gut, and essentially all other mammals. It is an integral component of the normal gastrointestinal flora [1, 2]. It is classified as a Gram-negative, non-spore forming and anaerobic bacilli. This mammalian symbiont and opportunistic pathogen depends on its capsular layer for virulence as well as for symbiosis in the mammalian gut [3, 4]. Eight capsule polysaccharides can be expressed on its surface, depending on the environmental niche of the organism, designated as CPSA through CPSH [5–10]. Capsular polysaccharide A is one of the eight polysaccharides

found on the surface of *B. fragilis,* and is the most abundant. CPSA plays a role in abscess formation when the bacterium localizes outside of its normal niche in the gastrointestinal tract or during surgical procedures [11]. However, this view has been challenged when it was found that treating the animal with the CPSA and then introducing the abscess-inducing bacteria resulted in the immune system of the animal protecting itself against the production of abscesses. Furthermore, few studies have also claimed that the abscess formation by *B. fragilis* actually prevents infection in the wound by other pathogenic bacteria [12, 13].

CPSA is a unique polymer. It has both negatively and positively charged motifs present on each repeating monomer, making it a zwitterionic molecule [7, 14] (**Figure 1**). The presence of this zwitterionic character has been attributed to the novel immunologic activity displayed by CPSA. The zwitterionic character has been shown to modulate the mammalian immune system by interacting with the adaptive immune system [15]. Elimination of either charge group in CPSA results in a lack of *in vivo* activation of the T-cells [16, 17].

CPSA modulates the immune system by its stimulation of a T-cell dependent form of immunity that provides protection against the formation of the intraabdominal abscesses. At the molecular level, CPSA interacts with the MHCII pathway similar to traditional protein antigens [18]. The first step is endocytosis of CPSA by the antigen-presenting cells like dendritic cells. Once in the endosome, CPSA is depolymerized based on the chemical reaction, deaminative cleavage [19]. This cleaving is mediated by nitric oxide, that has been generated by the upregulation of inducible nitric oxide synthase (iNOS). The 130 kDa CPSA is processed

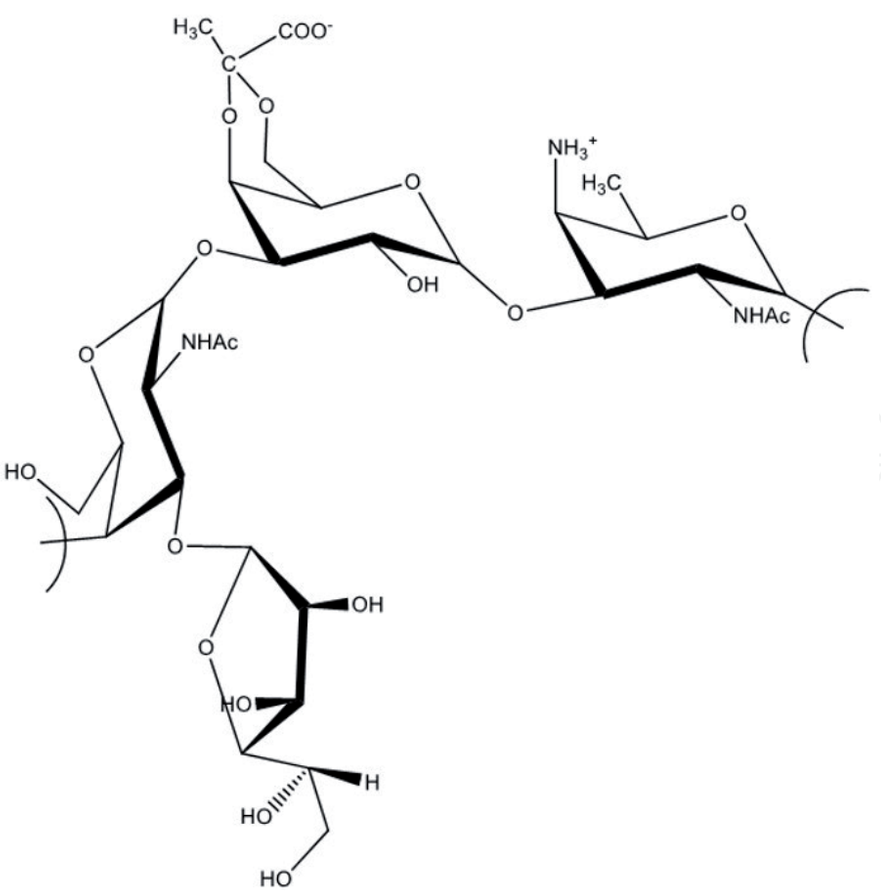

Figure 1.
Tetrameric repeat unit of the CPSA found on B. fragilis*. It consists of an acetamido-4-amino-6-deoxygalactopyranose (AADGal), 4,6-pyruvate galactose (4,6-pyr-gal), N-acetylgalactosamine (GalNAc), and a galactofuranose (Galf) sugar.*

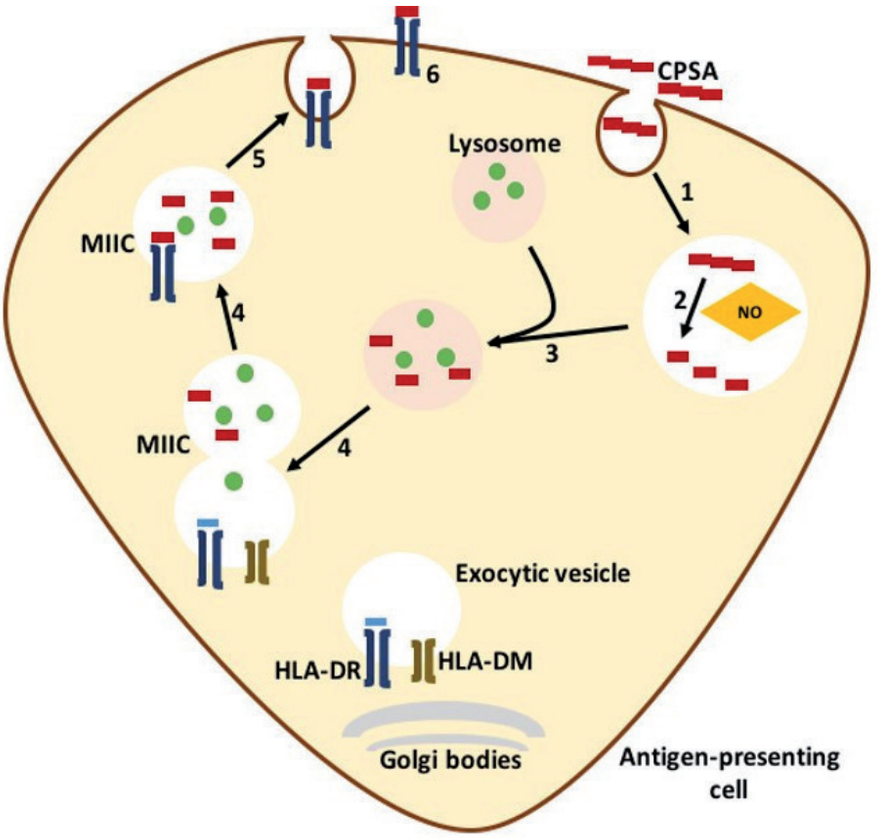

Figure 2.
Depolymerization of CPSA in antigen presenting cell. 1. Internalization of CPSA in an endosome. 2. iNOS upregulation produces NO, which cleaves 130 kDa CPSA to ~15 kDa units. 3. Endosome fuses with the lysosome. 4. Endo-lysosome fuses with exocytic vesicle to form MIIC vesicle which has HLA-DR, HLA-DM and processed polysaccharide. In here, processed polysaccharide is loaded on HLA-DR with the help of HLA-DM. 5, 6. The loaded HLA-DM is presented on the surface of the antigen presenting cell to be recognized by alpha beta TCR present on CD4+ T-cell.

to 15 kDa units. After being processed, the endosomes fuse with lysosomes and exocytic vesicles to form MIIC vesicle carrying HLA-DR and the accessory molecule HLA-DM. HLA-DM catalyzes the binding of MHCII to CPSA fragments, which is then presented to the CD4+ T cell receptor (**Figure 2**). This leads to the proliferation of the CD4+ T cell population, that produces IL-10, which is responsible for providing protection against the formation of intra-abdominal abscesses [15, 20].

CPSA can restore the immune system from a variety of autoimmune disorders, making it a promising candidate for a therapeutic drug. Colonization of nude mice with wild type *B. fragilis*, that produces the zwitterionic capsular polysaccharide A, protected animals from antibiotic induced experimental autoimmune encephalomyelitis (EAE), while animals infected with mutant *B. fragilis* deficient in the production of the polysaccharide were not protected [12, 21]. In germ free animal models of Inflammatory Bowel Disease (IBD), it was found that CPSA alone without the bacterial carrier was enough to stimulate normal immune system function and prevent intestinal inflammatory disease [22, 23]. CPSA has been given therapeutically to decrease pro-inflammatory cytokine production in an experimental model of colonic irritation [24].

2. CPSA gene locus

CPSA is a polymer of a tetramer repeated approximately 160 times. Its size is estimated to be 110 kDa [25]. The CPSA tetrameric repeat unit consists of an

acetamido-4-amino-6-deoxygalactopyranose (AADGal), 4,6-pyruvate galactose (4,6-pyr-Gal), N-acetylgalactosamine (GalNAc), and a galactofuranose (Galf) sugar (**Figure 3**) [26]. The structure of CPSA has previously been well investigated using total correlated spectroscopy and NOESY NMR [27]. Three-dimensional structure of a highly related PSA2 molecule shows a right-handed helix with two repeating units per turn, and a pitch of 20 Å. The zwitterionic motif is formed with alternating anionic carboxylate lying in repeated grooves and the cationic-free amines exposed on the outer surface of the carbohydrate [12, 28].

Although the chemical composition of CPSA is known, yet the biochemical pathway involved in its production is poorly documented [29, 30]. The location of the proposed CPSA locus was knocked out, making a mutant *B. fragilis* which did not express CPSA on its surface, thereby confirming the location of the biosynthetic locus (**Figure 4**). Within the CPSA locus, there are eleven genes, of which nine express proteins similar to other proteins involved in various other polysaccharide biosynthesis (**Table 1**).

2.1 Initiating the CPSA biosynthesis

The function of the nine genes have been elucidated and a pathway has been constructed (**Figure 5**). The identity of the genes present in the CPSA gene locus

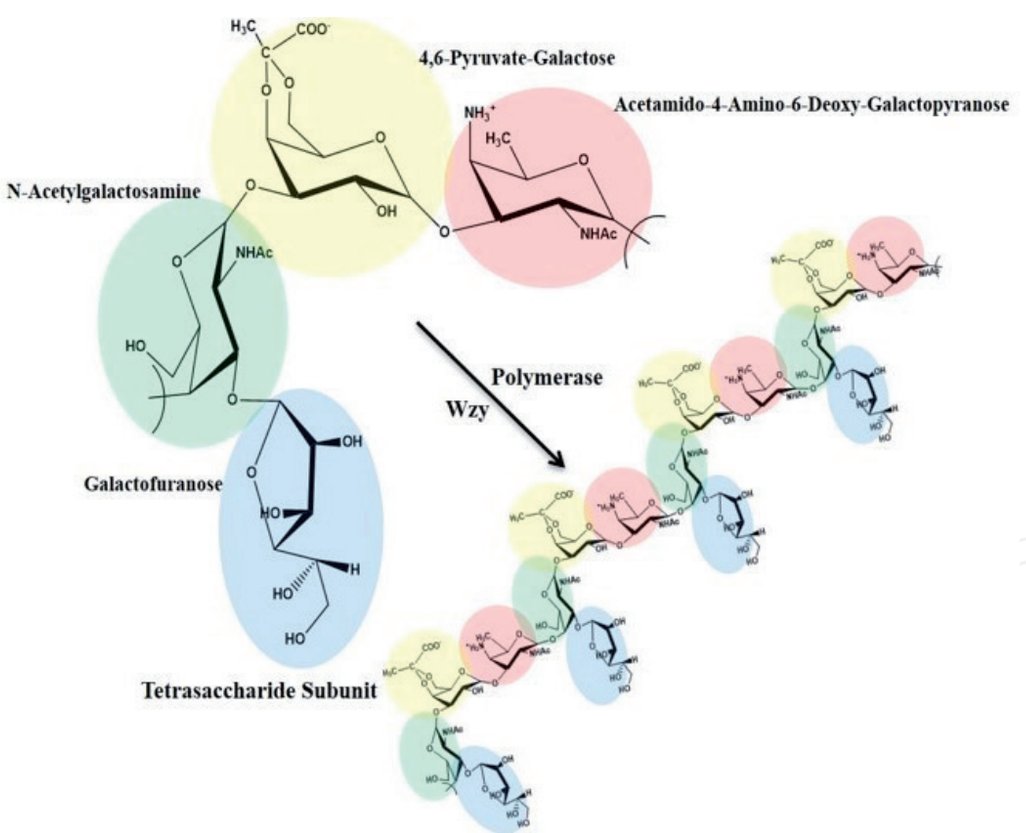

Figure 3.
Tetrameric repeat of CPSA.

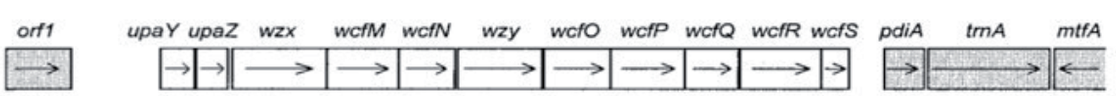

Figure 4.
CPSA locus in the B. fragilis *genome.*

ORF	Size (aa)	Size (kDa)	Role	Accession no.
wzx	482	56	flippase	AAK68914.1
wcfM	364	43	galactopyranose mutase	AAK68915.1
wcfN	291	34	glycosyltransferase	AAK68916.1
wzy	434	43	polymerase	AAK68917.1
wcfO	357	40	pyruvyltransferase	AAK68918.1
wcfP	378	44	glycosyltransferase	AAK68919.1
wcfQ	268	32	glycosyltransferase	AAK68920.1
wcfR	407	45	aminotransferase	AAK68921.1
wcfS	195	23	glycosyltransferase	AAK68922.1

Table 1.
Functions of the gene products in the CPSA biosynthesis operon.

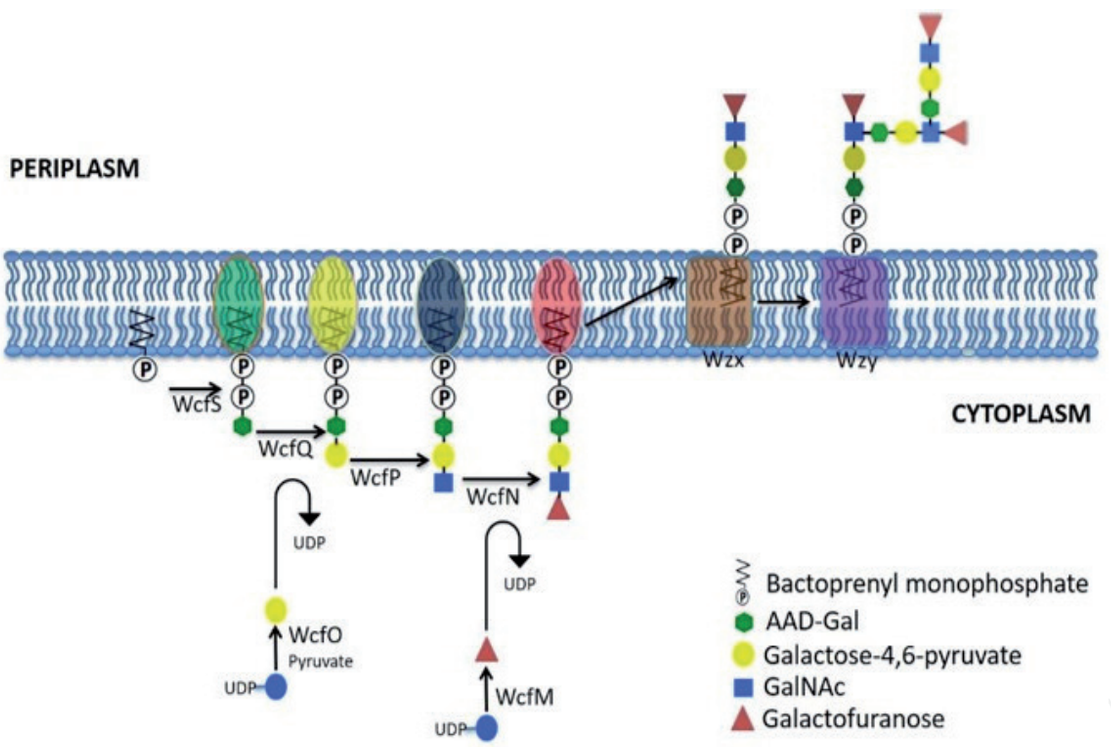

Figure 5.
Pathway of CPSA biosynthesis.

suggests that the most likely route for assembling the complex bacterial polysaccharide is a Wzy-dependent pathway in which the repeat unit oligosaccharides are assembled one sugar at a time on the cytosolic face of the bacterial inner membrane [31]. Assembly of the oligosaccharide takes place on a C55 isoprenoid bactoprenol [32]. It is a hydrophobic anchor which holds the growing polymer in the cell membrane.

The enzymes responsible for the synthesis of the first sugar, AADGal, in the tetrameric repeat, and the enzyme that catalyzes the transfer of this sugar to the bactoprenol anchor have been well characterized [33]. AADGal is synthesized by the sequential action of a dehydratase and an aminotransferase, which is then transferred to the bactoprenyl anchor by a hexose phosphate initiating transferase.

Within the CPSA biosynthesis locus, there is a predicted aminotransferase gene, *wcfR*, and a hexose phosphate initiating transferase, *wcfS*, but no predicted dehydratase was found. However, a gene encoding a potential dehydratase, *ungD2*, has been identified elsewhere in the *B. fragilis* genome. When this gene was knocked out by Coyne *et al*, they found out that, synthesis of the seven out of the eight capsular polysaccharides was stopped. Initial studies with UngD2 and WcfR did not show any promise in the synthesis of AADGal. Hence a previously well characterized dehydratase, PglF [34], from *Campylobacter jejuni* was used to provide the substrate needed for WcfR function. The coupling of these enzymes together led to the production of AADGal (**Figure 6**) [35, 36]. This also points to the notion that depending on homology alone for functional assignment of genes, is not always right, and wet lab results are needed to confirm the function of the gene product.

The synthesized UDP-AADGal was further used as a potential substrate for WcfS, identified as the initiating hexose phosphate transferase. Studies done by Mostafavi et al. demonstrated that WcfS was indeed the initiating hexose phosphate transferase, which lead to the formation of the bactoprenyl linked monosaccharide (**Figure 7**) [33].

As mentioned previously, assembly of the polysaccharides in bacterial cells is done on a C55 bactoprenyl anchor. It is produced by the condensation of farnesyl diphosphate (FPP) to eight units of isopentenyl diphosphate (IPP), done by the enzyme undecaprenyl diphosphate synthase (UPPS). A major drawback of using this compound in in vitro assays is that, it does not have easily distinguishable chromophores associated to it, hence very few rapid assays are available to detect and quantify the activity of enzymes associated with polysaccharide synthesis. To circumvent this problem, the Troutman lab developed fluorescent analogues of the native bactoprenyl, which are easily traceable [25, 37]. Assays done using these analogues take a short time to reveal valuable information about the enzymes when compared to traditional assays, which follow the more tedious route of using radioactive labeled substrates. Mostafavi et al. used a p-nitroaniline bactoprenyl phosphate analogue to find out the function of WcfS (**Figure 7**) [33].

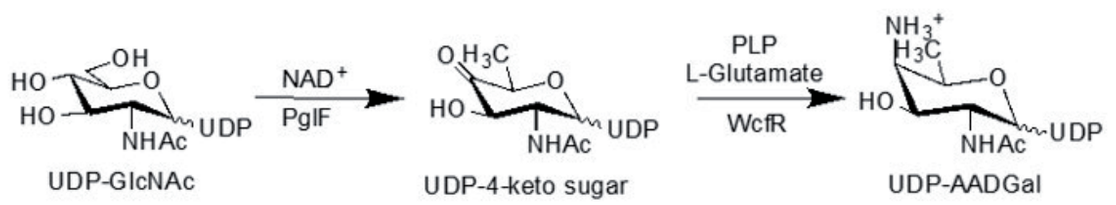

Figure 6.
Biosynthesis of AADGal.

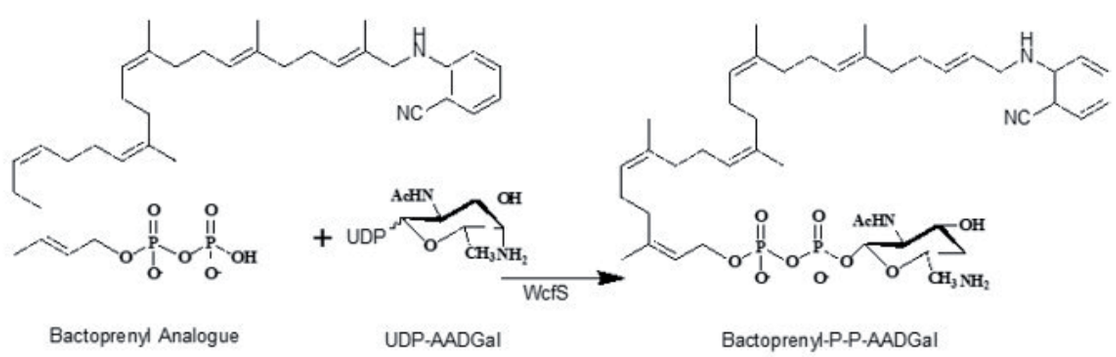

Figure 7.
Biosynthesis of bactoprenyl linked monosaccharide.

2.2 Glycosyltransferases involved in CPSA biosynthesis

Cell surface polysaccharides are nothing but complex carbohydrates. They play important roles in a number of biological processes such as cell growth, cell to cell interactions, immune response, and inflammation. The polysaccharides are synthesized by a class of enzymes known as glycosyltransferases [38]. Glycosyltransferases are an enzyme superfamily responsible for the attachment of carbohydrate moieties to a wide array of acceptors that include nucleic acids, polysaccharides, proteins, lipids, and carbohydrates. The majority of glycosyltransferases are sugar nucleotide-dependent enzymes, and utilize nucleoside diphosphate sugars (NDP-sugars) as donors for the glycosidic bond formation. In other cases, the sugar donors can also be lipid phosphates and unsubstituted phosphate [39].

The glycosyltransferases have been classified by sequence homology into 96 families in the Carbohydrate Active enZyme database (CAZy), each of which catalyze the reaction as shown in **Figure 8** [40]. Chain elongation of the oligosaccharide units in complex carbohydrates is achieved by the addition of monosaccharide units through the action of different glycosyltransferases in a specific sequence. The CAZy database provides a highly powerful predictive tool, as the structural fold and mechanism of action are invariant in most of the families [22]. Therefore, where the structure and mechanism of a glycosyltransferase member for a given family has been reported, some assumptions about other members of the family can be made. Substrate specificity, however, is more difficult to predict, and requires experimental characterization of individual glycosyltransferases.

Determining both the sugar donor and acceptor for a glycosyltransferase of unknown function can be challenging, and it is one of the reasons there are significantly fewer well characterized isoprenoid linked sugar glycosyltransferases when compared to the glycosyltransferases responsible for synthesizing disaccharides or the oligosaccharides [40]. The less reports on isoprenoid linked sugar transferases can be attributed to the fact that, a high throughput method has not yet been developed which will enable for faster characterization. Another challenge in characterizing the glycosyltransferases is the availability of rare sugars, as most of the bacterial polysaccharides contain rare sugars. Rare sugars, such as rhamnose or fucose, may provide the bacterial polysaccharides with additional biological properties compared to those composed of more common sugar monomers [23, 41]. Rare sugars are monosaccharides that are not commonly found in nature, in comparison to D-glucose, D-galactose, D-fructose, D-xylose, D-ribose, and L-arabinose which are more abundant [23]. Moreover, the traditional methods like radioisoptopic

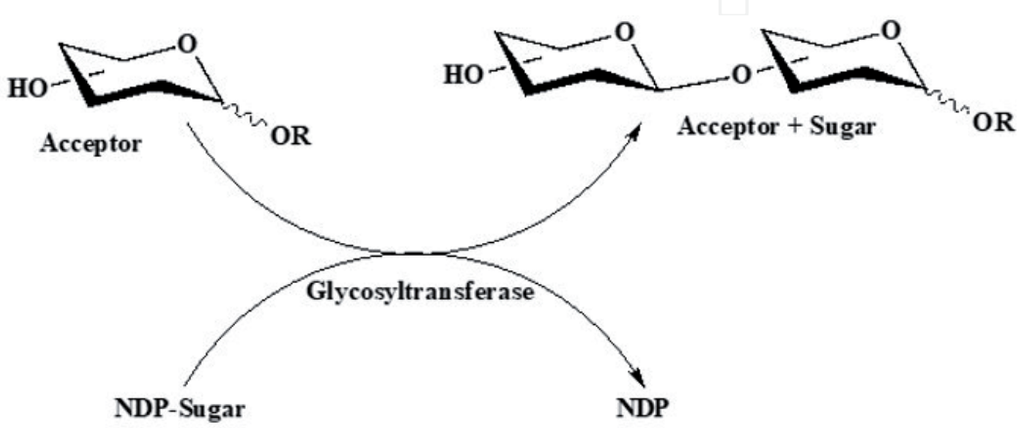

Figure 8.
General reaction scheme for a glycosyltransferase (GTs).

labelling, thin-layer chromatography (TLC) used to characterize the glycosyltransferase, often tends to be tedious and challenging in tracking the product.

Glycosyltransferases catalyze glycosidic bond formation with either overall retention or inversion of anomeric configuration when compared to the stereochemistry in the sugar donor (**Figure 9**). Inverting glycosyltransferases are generally believed to proceed via a single displacement SN2 mechanism with concomitant nucleophilic attack by the acceptor at the anomeric carbon, facilitated by proton transfer to the catalytic base, and leaving group departure [22]. Structural data have shown that several inverting glycosyltransferases, contain no obvious candidate catalytic base indicating these enzymes use an alternative mechanism [38, 39].

The reaction coordinate employed by retaining glycosyltransferases has been much debated, and it could be possible the mechanism is not conserved for all retaining enzymes. One possibility is a double displacement mechanism via a covalent mechanism, analogous to that used by glycoside hydrolases [22]. A report by Soya et al. provided mass spectrometry evidence for the formation of a covalent intermediate between the donor substrate and a cysteine, which had been substituted for the candidate catalytic nucleophile, on two retaining glycosyltransferases [42]. The more favored mechanism in the field is an SN1 or SN1-like mechanism, which involves interaction between the leaving group and attacking nucleophile on the same face. This mechanism is supported by kinetic isotope effect studies to analyze the structure of the transition state and by computational modeling [38, 39].

The CPSA gene locus has three genes, wcfQ, wcfP and wcfN, that putatively encode for glycosyltransferases [29, 30]. Each of these glycosyltransferases is expected to transfer a sugar moiety to the bactoprenyl linked monosaccharide, the disaccharide and the trisaccharide. Based on the CAZy database, and homology studies, WcfQ and WcfN are hypothesized to belong to the glycosyltransferase superfamily A, which follows the inverting mechanism in the sugar transfer. Whereas WcfP is proposed to belong to the glycosyltransferase superfamily B, which follows the retaining mechanism [40].

WcfQ, identified as the first glycosyltransferase, transfers galactose to the isoprenoid linked monosaccharide, even though it was observed by authors that, WcfQ could also transfer glucose to the bactoprenyl linked monosaccharide. This is because WcfQ required glucose in much excess when compared to galactose. It was also found out that even though WcfP had the capability of transferring galactose, WcfQ was more efficient in it, hence it was identified as the galactosyltransferase

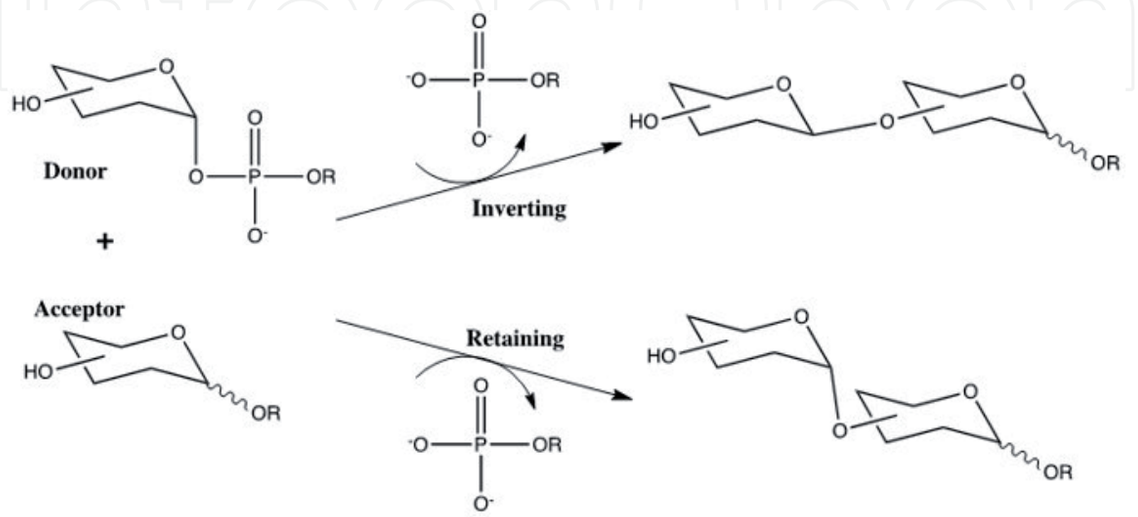

Figure 9.
Glycosyltransferases catalyze glycosyl group transfer with either inversion or retention of the anomeric stereochemistry with respect to the donor sugar.

in the CPSA biosynthetic pathway. Moreover, based on the Carbohydrate-Active enZYmes (CAZY) database the WcfQ sequence matched the GT_2 family of glycosyltransferases which invert the configuration of the anomeric carbon of the donor, while WcfP was similar to a GT_4 family glycosyltransferase, which retains the anomeric stereo-configuration of the donating sugar [43, 44]. The published structure of the CPSA tetrasaccharide unit suggests that the linkage should be in a beta configuration [27]. This supported the conclusion that WcfQ is the protein responsible for introducing galactose, and that it introduces the sugar in the appropriate beta configuration [45].

As stated before, WcfP is related to the GT_4 family of proteins suggesting that it is a retaining glycosyltransferase, it was therefore more likely that WcfP catalyzed UDP-GalNAc transfer to the galactose, but it was not known if it transferred UDP-GalNAc to the unpyruvylated disaccharide or the pyruvylated disaccharide. Both WcfN and WcfP were analyzed with the pyruvylated and the unpyruvylated disaccharides, it was demonstrated that WcfP transfers only UDP-GalNAc to the pyruvylated disaccharide.

In homology studies, WcfN was predicted to be a member of the GT_2 family, whose members have been identified to transfer furanose residues. WcfN was also hypothesized to be an inverting transferase, which inverts the stereochemistry of the anomeric carbon. Since the linkage between the third and the fourth sugar in the tetrasaccharide repeat unit is in the beta configuration, WcfN fitted the role of being the last glycosyltransferase. WcfN was found to transfer the galactofuranose to the trisaccharide, hence completing the mapping of the pathway of synthesis of the tetrasaccharide.

2.3 WcfM as the galactopyranosemutase

Polysaccharides composed of furanosyl residues are important constituents of many bacteria, protozoa, fungi, plants and archaebacteria [46, 47]. The furanosyl constituents have also been identified in glycopeptides, glycolipids as well as nucleotide sugars. D-Galactose is by far the most widespread hexose in the furanose form in naturally occurring polysaccharides, and the most impressive examples of these glycans are encountered in mycobacteria [48–50]. Galactofuranose, (Galf), which is thermodynamically less stable than galactose, is essential for the viability of several pathogenic species of bacteria and protozoa. It is absent in this form in mammalian cell structure, hence the biochemical pathways by which galactofuranose containing glycans are assembled have been attractive sites for drug action [47, 51]. This potential has led to an increased interest in the synthesis of molecules containing galactofuranose residues, and their subsequent use in studies directed towards understanding of the enzymes that process these residues and the identification of potential inhibitors of these pathways [46].

The enzyme UDP-galactopyranose mutase is central to galactofuranose metabolism. Most organisms cannot use exogenous galactofuranose, and UDP-galactofuranose appears to be the biological source of galactofuranose residues in polysaccharides [46]. The major structural component of the *Mycobacterium tuberculosis* cell wall contains a galactan chain of approximately thirty-five galactofuranose units, and the biosynthesis of the galactan is essential for viability [47]. The O-antigens of both *Escherichia coli* and *Klebsiella pneumoniae* contain galactofuranose as a component of lipopolysaccharide [47]. Several galactofuranose containing glycoconjugates have been found in *Trypanosoma cruzi*, the causative agent of Chagas disease, including glycoinositolphospholipids, lipopeptidophosphoglycans and mucin-like proteins. The galactomannan of *Aspergillus fumigatus* also contains galactofuranose, and this polysaccharide is used for clinical detection

of fungal infections. Finally, it is also known that stopping galactofuranose biosynthesis in *Leishmania major* attenuates its virulence [46–48, 51]. The above-mentioned pathogenic organisms all use the same building block for synthesizing galactofuranose-containing polysaccharides: uridine diphosphogalactofuranose (UDP-galactofuranose). This sugar nucleotide is produced from UDP-Glcp by the enzymes UDP-Glucose 4-epimerase (generating UDP-Galp,) and UDP-galactopyranose mutase (UGM), which catalyzes the transformation of UDP-Galp to UDP-galactofuranose. The gene encoding UGM was first identified in *E. coli* in 1996, followed shortly by its identification in *K. pneumoniae* and *M. tuberculosis* [48, 49, 52] . More recently, UGM was identified in the eukaryotes *A. fumigatus, Cryptococcus neoformans, L. major* and *T. cruzi*.

In the past several years' major milestones have been achieved, which include an in-depth understanding of the mechanism of UDP-galactopyranose mutase (UGM), the enzyme which produces UDP-galactofuranose, and is the donor species used by galactofuranosyltransferases. A number of methods for the synthesis of galactofuranosides have also been developed [50]. UDP-galactofuranose has also been prepared by a number of approaches, and currently it appears that a chemoenzymatic approach is the most viable method for producing multi-milligram amounts of this important rare sugar [46, 50].

The biosynthetic gene operon of CPSA encodes a wcfM gene, which was found to be homologous to other galactopyranose mutases. It is homologous to two known UDP-galactopyranose mutases, one from *Streptococcus pneumonia* (Cps33fN: 66% identity and 82% similarity) and the other from *E. coli* (59% identity and 79% similarity). The gene encodes a 43 kDa protein with one potential N-terminal transmembrane domain. Like other galactopyranose mutases, the protein is hypothesized to catalyze the reaction as shown in **Figure 10**. The product of WcfM is required for the final step in the synthesis of the CPSA tetrasaccharide repeat unit. The last glycosyltransferase transfers UDP-galactofuranose to the trisaccharide.

2.4 WcfO as the pyruvyltransferase

Pyruvyltrasferases and pyruvylation have been less studied in prokaryotes, despite a burgeoning evidence of its presence in bacteria. Addition of pyruvate moiety gives a negative charge to the polymer and is utilized by the bacteria in various functions [53]. An example of this is the pyruvylation of ManNAc residue by the enzyme CsaB in the secondary cell wall polymer of *Bacillus anthracis* and *Paenibacillus Alvei* [54, 55]. This pyruvylated residue comes in use in anchoring the S-layer proteins in Gram positive bacteria by binding to the SLH domains of the S-layer proteins [56]. Knocking out the CsaB has led to a lethal phenotype, which suggests that, pyruvylation of the secondary cell wall polymer is essential to the

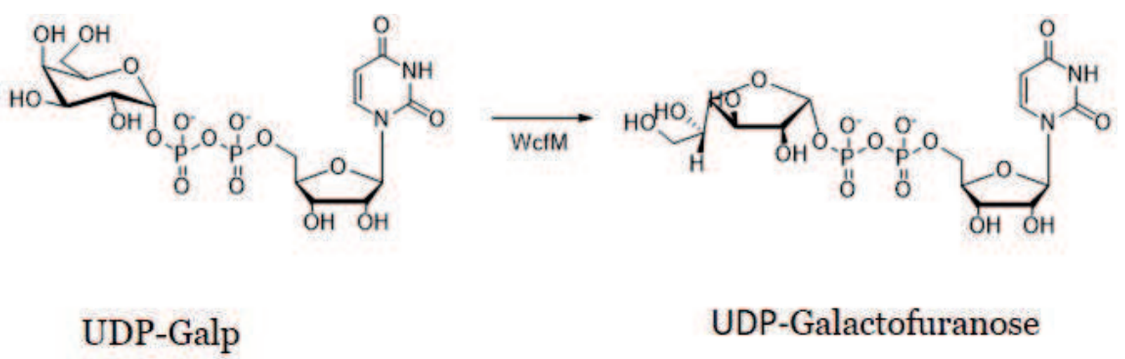

Figure 10.
Reaction catalyzed by UGM.

growth and survival of the bacteria [55]. CsaB was recently characterized by the Schaffer group [57]. Including WcfO, a total of three pyruvyltransferases have now been functionally characterized. Pvg1b is from an eukaryote, and whose crystal structure has been solved [58, 59].

Polysaccharides of various prokaryotes are covalently linked with variable combinations of sulfates and pyruvates, for example, *Rhizobium leguminosarum*: 4,6-pyrGalactose and 4,6- pyrGlucose, *Bacillus anthracis*: 4,6-pyrManNAc, and *Xanthomonas campestris*: 4,6-pyrMannose. These modifications provide a highly negative charge of these polysaccharides, which is often essential for function [60]. For example, when the pyruvyltransferase PssM, responsible for the pyruvate modification in the *R. leguminosarum* exopolysaccharide was deleted, the bacterium was found to be ineffective in infecting pea plants to initiate the formation of root nodules. This led to formation of aberrant root nodules, which were unable to fix nitrogen [61, 62]. Moreover, some studies have linked the pyruvic acetals in oligo- and polysaccharides to their immunological properties [63, 64].

Among the eleven proteins encoded in the CPSA gene operon, one of the genes transcribes a hypothesized pyruvyltransferase based on homology studies performed using pBLAST [31]. There is little sequence similarity to other known proteins with the wcfO gene product. WcfO has very minimal sequence identity to the two characterized pyruvyltransferases Pvg1p from *S. pombe* and PssM from *R. leguminosarum*. The activity of CPSA is dependent on its zwitterionic character in which the –AADGal amino group is positively charged while the pyruvate is negatively charged [16]. Due to the fact that all other sugar modifying enzymes and glycosyltransferases required for CPSA biosynthesis have been located in the CPSA biosynthesis operon, it was proposed by the authors that the wcfO gene product was likely responsible for the pyruvylation modification required for the formation of the second sugar in the CPSA tetrasaccharide repeat unit. WcfO is capable of modifying galactose or glucose when they are linked to the isoprenoid lipid carrier. This points to the direction that, there may be sub-families within the pyruvyl-transferase family that utilize different substrates. Kinetic evaluation of WcfO was performed by the authors to test if discriminated between glucose and galactose, and it apparently utilized both the substrates with equal vigor.

3. Significance of capsular polysaccharide A

Previous studies on the CPSA molecule have revealed it to be effective as a therapeutic molecule, the tetrasaccharide repeat needs to be a polymer of ten repeat units or longer. If shorter than that, it fails to activate the immune system [64, 65]. CPSA operon encodes for a flippase wzx, which takes the repeat unit and flips it from the cytoplasmic space to the periplasmic space, where the polymerase wzy, utilizes the repeat unit and polymerizes it till it reaches a length of approximately 130 repeat units [65, 66].

Recent successes in cancer vaccines and in monoclonal antibody cancer immunotherapy have given the impetus towards development of vaccines targeting cancer-associated carbohydrates. The Andreana group have been developing carbohydrate immunogens to elicit a T-cell dependent immune response. CPSA is known to stimulate a strong T-cell mediated response. They have successfully linked CPSA to the tumor-associated carbohydrate antigen (TACA), Sialyl Thomsen-nouveau (STn) and were able to obtain a robust immune response to the antigen [67–71]. They have further reported total synthesis of the CPSA unit in 19 steps with a final yield of 5% [67]. Chemoenzymatic assembly is a faster and scalable approach, that can be used as an alternative or in combination with chemical synthesis. CPSA

obtained in this way, can then be linked to the antigen. The chemoenzymatic method has also been used to create capsule polysaccharide based glycoconjugates for *Neisseria meningitidis* serotypes A, C and X [72–74]. In some cases, recombinant glycosyltransferases can be used to assemble non-native carbohydrate antigens in compliant host organisms like *Escherichia coli*. This method has been successfully used by the Brendan W. Wren lab for the in vivo assembly of capsular polysaccharide from several serotypes of *Streptococcus pneumoniae*. A similar approach is also currently being applied with respect to CPSA, wherein the whole CPSA biosynthesis and assembly will be done inside *E. coli*. This will allow to have access to longer oligomers of CPSA, which can be helpful in studies towards size requirement in eliciting immune response. So far there have been no reports of CPSA unit being polymerized synthetically.

4. Conclusion

CPSA molecue has a very common modification on its surface. Pyruvylation of sugars is fairly common yet an extensive search of the literature reveals little on successful isolations of an enzyme responsible for this sugar modification. However, very recently a family of genes has been identified that appear to be involved in pyruvate transfer reactions in prokaryotes. A publication in 2013 showed successful purification of pyruvyltransferase Pvg1p from the eukaryote *Schizosaccharomyces pombe*. This group demonstrated the activity of Pvg1p on beta-nitrophenyl galactose, a substrate analogue of galactose [54]. Apart from this eukaryotic pyruvyltransferase Pvg1p and the prokaryotic pyruvyltransferase PssM from *R. leguminosarum*, no other pyruvyltransferases have been characterized [55]. More studies are needed in uncovering this family of enzymes, and also a path needs to be elucidated towards the polymerization of CPSA, to reap its full therapeutic benefits.

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Conflict of interest

The authors declare no conflict of interest.

Acronyms and abbreviations

AADGal	2-N-acetylamido-4-amino-6-deoxy-galactopyranose
BPP	bactoprenyl diphosphate
BP	bactoprenyl phosphate
CAZY	Carbohydrate-Active Enzymes database
CE	capillary electrophoresis
CPS	capsular polysaccharide
CPSA	capsular polysaccharide A
E. coli.	*Escherichia coli*
EAE	experimental autoimmune encephalomyelitis
HLA-DM	human leukocyte antigen DM

HLA-DR	human leukocyte antigen DR
HR-MS	high resolution mass spectrometry
GAG	glucosaminoglycan
Galf	galactofuranose
GalNAc	N-acetylgalactosesamine
GlcNAc	N-acetylglucosamine
Galp	galactopyranose
iNOS	inducible nitric oxide synthase
IPTG	isopropylthiogalactopyranoside
LC/MS	Liquid chromatography mass spectrometry
MALDI-MS	Matrix assisted laser desorption/ionization mass spectrometry
MHCII	major histocompatibility class II
PglF	a dehydratase
PHYRE2	Protein Homology/analogy Recognition Engine v 2.0
TACA	tumor-associated carbohydrate antigen
TCR	T-cell receptor
2AA-BP	2-amideaniline bactoprenyl monophosphate
2CNA-BP	2-nitrileaniline bactoprenyl monophosphate
4,6-pyr-Gal	4,6-pyruvate-galactose
4,6-pyr-Glc	4,6-pyruvate-glucose

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