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Unravelling the intricate biosynthetic pathways of cyanobacterial extracellular polymeric substances (EPS)

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UNRAVELLING THE INTRICATE BIOSYNTHETIC PATHWAYS OF CYANOBACTERIAL EXTRACELLULAR POLYMERIC SUBSTANCES (EPS)

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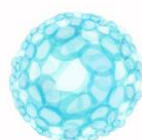
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“To be a scientist is to be naïve.”

— Mazin, *Chernobyl* (2019)

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List of publications

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Gonçalves J, Marcos B, Silva MB, Conceição I, **Pissarra J**, Roque J, Tamagnini P, Melo P, Pereira R, Honrado J. Spectral monitoring of a system for the rehabilitation of burned soils based on inoculation with cyanobacteria and microalgae. In *Advances in Forest Fire Research* 2022, 381-384. Imprensa da Universidade de Coimbra, **2022**. doi: 10.14195/978-989-26-2298-9_60

Brito Â, Rocha M, Kaššovský J, Vieira J, Vieira PC, Ramos V, Correia M, Santos M, Mota R, Roque J, **Pissarra J**, Melo P, Tamagnini P. A new cyanobacterial species with a protective effect on lettuce grown under salinity stress: Envisaging sustainable agriculture practices. *J Appl Phycol* **2022** 34, 915–928. doi: 10.1007/s10811-022-02692-4

List of abbreviations

ABC	ATP-binding cassette
Abs ₅₀₉	Absorbance at 509 nm
ADP	Adenosine diphosphate
Amp	Ampicillin
Ara	Arabinose
ATP	Adenosine triphosphate
bp	Base pairs
cDNA	Complementary DNA
CDP	Cytidine diphosphate
Chl <i>a</i>	Chlorophyll <i>a</i>
Cm	Chloramphenicol
CPS	Capsular polysaccharides
Cq	Quantification cycle
CRISPR	Clustered regularly interspaced short palindromic repeats
CRISPRi	CRISPR interference
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
dTDP	Deoxythymidine diphosphate
EPS	Extracellular polymeric substances
fps	Frames per second
Fru	Fructose
Fuc	Fucose
Gal	Galactose
GalN	Galactosamine
GC-FID	Gas chromatography with a flame ionization detector
GDP	Guanosine diphosphate
GFP	Green Fluorescent protein
Glc	Glucose
GlcN	Glucosamine
GTP	Guanosine Triphosphate
GTs	Glycosyltransferases
Hyp.	Hypothetical
Km	Kanamycin

LEGE	Blue Biotechnology and Ecotoxicology Culture Collection
LPS	Lipopolysaccharides
Man	Mannose
MT	Mutant
NAD ⁺	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
NADP ⁺	Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
nd.	Not detected
OD ₇₃₀	Optical Density at 730 nm
P	Phosphate
PCC	Pasteur Culture collection of Cyanobacteria
PCR	Polymerase chain reaction
PHB	Polyhydroxybutyrate
PPi	Inorganic pyrophosphate
px	Pixels
Rha	Rhamnose
Rib	Ribose
RNA	Ribonucleic acid
rpm	Rotations per minute
RPS	Released polysaccharides
RT-qPCR	Reverse transcription quantitative PCR
SB	Southern blot
SD	Standard deviation
SDS	Sodium dodecyl sulfate
sp.	Species
spp.	Species pluralis
SSC	Saline-sodium citrate buffer
TEM	Transmission electron microscopy
TES	N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid
TTP	Thymidine triphosphate
UDP	Uridine diphosphate
Unk.	Unknown
v	Volume
w	Weight
WT	Wild type
Xyl	Xylose

Abstract

Cyanobacteria are a monophyletic group of Gram-negative bacteria that are distinguished for their ability to perform oxygenic photosynthesis, a characteristic that makes them responsible for a significant part of the global atmospheric oxygen and also important primary producers, namely in the oceans. These microorganisms have great metabolic plasticity that not only allows them to fill and thrive in virtually every ecological niche but also confers on them the ability to produce a vast array of metabolites. Due to their simple nutritional requirements, capacity to use water as an electron donor and directly convert carbon dioxide into biomass using solar energy, and for being generally amenable to genetic manipulation, cyanobacteria emerge as ecofriendly cell factories capable of producing biocompatible and biodegradable compounds of commercial value. Among these natural products are extracellular polymeric substances (EPS) that are mainly composed of heteropolysaccharides and can be classified as released polysaccharides (RPS) or capsular polysaccharides (CPS), depending on whether they remain attached or not to the cell surface. These EPS have been gaining attention for several industrial applications due to their unique properties, composition, versatility and bioactivities. However, most of what is known regarding biosynthesis, assembly and export of cyanobacterial EPS is inferred from other organisms. As such, the EPS-related pathways remain poorly understood hindering the optimization of production and selection of polymers' desired characteristics.

This work aimed to better understand the biosynthesis of cyanobacterial EPS, in particular the pathways for the production of the deoxyhexoses fucose and rhamnose. To that end, using the model unicellular cyanobacterium *Synechocystis* sp. PCC 6803, single and double knockout strains were characterized in an attempt to elucidate the role of putative key enzymes on the composition and amount of the EPS produced. As previously observed, deletion of *sll1213* ($\Delta fucS$) resulted in a phenotype of fast sedimentation, absence of S-layer, significant growth impairment and RPS without fucose or rhamnose. Moreover, it was also demonstrated here that this strain is incapable of producing any of the deoxyhexoses revealing that rhamnose biosynthesis must be in some way dependent on FucS. Furthermore, *Synechocystis* does not seem to possess a mechanism for the incorporation of exogenous fucose and rhamnose into the nucleotide sugar metabolism. In contrast, when targeting genes putatively encoding for RfbC1 and RfbC2 (*slr0985* and *slr1933*), all the knockout strains ($\Delta rfbC1$, $\Delta rfbC2$ and $\Delta rfbC1\Delta rfbC2$) had impaired RPS production. $\Delta rfbC1$ and $\Delta rfbC1\Delta rfbC2$ showed an increase in CPS, fast sedimentation and altered S-layer, while $\Delta rfbC2$ and $\Delta rfbC1\Delta rfbC2$ showed an increase in total carbohydrates. Only the double knockout strain $\Delta rfbC1\Delta rfbC2$ exhibited a growth impairment. Additionally, while deletion of *rfbC* genes affected carbohydrate production, it did not affect the

monosaccharidic composition of RPS, clearly showing that the putative RfbC1 and RfbC2 are not essential for the biosynthesis of rhamnose. A transcriptional analysis revealed that *slr1072* (putative *gmd* of the fucose pathway) was upregulated in $\Delta fucS$, $\Delta rfbC1$ and $\Delta rfbC1\Delta rfbC2$ strains and that three EPS-related genes in the same operon as *rfbC1* (*slr0982*, *slr0983*, and *slr1610*) were upregulated in both $\Delta rfbC1$ and $\Delta rfbC1\Delta rfbC2$.

Overall, this study contributed to further elucidating the pathways for fucose and rhamnose in *Synechocystis* sp. PCC 6803, and how putative key enzymes may affect the biosynthesis of EPS regarding amount and composition. Additionally, this work emphasizes that these cyanobacterial pathways are more intricate than previously thought, potentially crosstalk, and are still far from fully resolved. Furthermore, several features possibly relevant for industrial and commercial applications were identified in the knockout strains.

Keywords: cyanobacteria, *Synechocystis*, deoxyhexoses, nucleotide sugars, fucose, rhamnose, extracellular polymeric substances (EPS)

Resumo

As cianobactérias são um grupo monofilético de bactérias Gram-negativas que se destacam pela sua capacidade de realizar fotossíntese oxigénica. Esta característica faz com que sejam responsáveis por uma parte significativa do oxigénio atmosférico sendo, também, importantes produtores primários, sobretudo nos oceanos. Estes microrganismos possuem grande plasticidade metabólica, o que lhes permite não só ocupar e prosperar em praticamente todos os nichos ecológicos, mas também lhes confere a capacidade de produzir uma vasta gama de compostos. As cianobactérias estão cada vez mais a emergir como fábricas celulares ecológicas capazes de produzir compostos biocompatíveis e biodegradáveis de elevado valor comercial devido aos seus requisitos nutricionais simples, à capacidade de usar água como dador de eletrões e de converter diretamente dióxido de carbono em biomassa utilizando energia solar e ao facto de poderem ser facilmente manipuladas geneticamente. Entre os produtos naturais que estes microrganismos produzem estão as substâncias poliméricas extracelulares (EPS – *extracellular polymeric substances*), compostas maioritariamente por heteropolissacarídeos e que podem ser libertadas para o meio (RPS - *released polysaccharides*) ou permanecer ligadas à superfície celular (CPS – *capsular polysaccharides*). Estes polímeros são atrativos para diversas aplicações industriais devido às suas propriedades únicas, composição, versatilidade e bioatividades. No entanto, grande parte do que se sabe sobre a biossíntese, montagem e exportação das EPS cianobacterianas é inferido a partir do conhecimento de outros organismos. Assim, as vias relacionadas com a síntese destes polímeros ainda estão pouco definidas, dificultando a otimização da produção e a seleção das características desejadas para um determinado fim.

Este trabalho teve como objetivo compreender melhor a biossíntese das EPS cianobacterianas, em particular as vias de produção das desoxihexoses fucose e ramnose. Para atingir este objetivo utilizou-se a cianobactéria modelo *Synechocystis* sp. PCC 6803, e foram gerados e caracterizados vários mutantes de deleção tendo em vista elucidar o papel de presumíveis enzimas-chave na quantidade e composição do polímero produzido. Como observado anteriormente, a deleção de *sll1213* ($\Delta fucS$) resultou num fenótipo de rápida sedimentação, ausência de camada S do invólucro celular, comprometimento significativo do crescimento e produção de RPS sem fucose nem ramnose. Além disso, foi demonstrado que esta estirpe, para além de sintetizar RPS sem ramnose, é também incapaz de produzir qualquer das desoxihexoses. Isto implica que a síntese de ramnose deve depender, de algum modo, da enzima FucS. *Synechocystis* também não parece possuir um mecanismo para incorporação de fucose e ramnose exógenas no metabolismo de açúcares nucleotídicos. Em contraste, quando foram visados os genes que codificariam

as proteínas RfbC1 e RfbC2 (*slr0985* e *slr1933*), todas as estirpes geradas ($\Delta rfbC1$, $\Delta rfbC2$ e $\Delta rfbC1\Delta rfbC2$) apresentaram uma redução na produção de RPS. $\Delta rfbC1$ e $\Delta rfbC1\Delta rfbC2$ mostraram aumento de CPS, sedimentação rápida e alterações na camada S, enquanto $\Delta rfbC2$ e $\Delta rfbC1\Delta rfbC2$ tiveram os carboidratos totais aumentados. Apenas a estirpe com a deleção dupla $\Delta rfbC1\Delta rfbC2$ apresentou uma diminuição no crescimento. Embora a deleção dos genes *rfbC* tenha afetado a produção de carboidratos, não afetou a composição monossacarídica dos RPS, mostrando claramente que as putativas RfbC1 e RfbC2 não são essenciais para a síntese de ramnose. Uma análise transcricional revelou que o gene *slr1072* (putativo *gmd* da via da fucose) é sobre-expresso em $\Delta fucS$, $\Delta rfbC1$ e $\Delta rfbC1\Delta rfbC2$, e que três outros genes, situados no mesmo operão do *rfbC1* e relacionados com a produção de EPS (*slr0982*, *slr0983* e *slr1610*), são também sobre-expressos tanto em $\Delta rfbC1$ como em $\Delta rfbC1\Delta rfbC2$.

Em suma, este estudo contribuiu para a elucidação das vias de síntese da fucose e ramnose em *Synechocystis* sp. PCC 6803, e como determinadas enzimas-chave podem afetar a biossíntese das substâncias poliméricas extracelulares (EPS), quer em termos quantitativos quer em termos qualitativos. Para além disto, este trabalho destaca a complexidade destas vias cianobacterianas, possíveis interligações, e o facto de permanecerem, em grande parte, por elucidar. Nas estirpes de deleção geradas foram, ainda, identificadas várias características que podem ser relevantes para aplicações industriais e comerciais.

Palavras-chave: cianobactérias, *Synechocystis*, desoxihexoses, açúcares nucleotídeos, fucose, ramnose, substâncias poliméricas extracelulares (EPS)

Chapter I

Introduction

Introduction

1. Cyanobacteria

1.1. Evolution and diversity

Cyanobacteria are prokaryotic organisms that stem from an ancient lineage of life with evidence of stromatolites dating back to 3500 million years ago, with origin at least during the early Proterozoic Eon (Knoll, 2008; Sánchez-Baracaldo, 2015). They are classified as Gram-negative bacteria since they possess a cell wall with an outer membrane with lipopolysaccharides, but generally they also exhibit a thick and highly cross-linked peptidoglycan layer typical of Gram-positive bacteria (Hoiczyk & Hansel, 2000; Stewart et al., 2006). These microorganisms were the earliest life forms able to produce molecular oxygen through water oxidation (Schopf, 2000), shaping life on Earth with the rapid oxygenation of the oceans during what is known as the Great Oxygenation Event over 2350 million years ago (Blaustein, 2016; Soo et al., 2017). This transformation of Earth's geochemistry was only possible since cyanobacteria were adapted to survive in aerobic and anaerobic environments (Soo et al., 2017), with some strains being able to perform anoxygenic photosynthesis through the use of hydrogen sulfide (Cohen et al., 1986). Many cyanobacterial strains have also the capacity to fix atmospheric nitrogen (Herrero et al., 2001; Stal, 2015) which makes them primary producers responsible for a significant part of the global fixation of carbon dioxide and dinitrogen, essential for the establishment and maintenance of the carbon and nitrogen cycles (Canfield et al., 2010; Nawaz et al., 2025; Raven et al., 2012). Most cyanobacteria are able to produce extracellular polymeric substances (EPS) that can be released to the surroundings or remain attached to the cell surface, as sheaths, capsules or slime (Mota et al., 2021; Pereira et al., 2009; Rossi & De Philippis, 2016) and their biological functions are mainly related to protection, biofilm formation and nutrient sequestration (Kehr & Dittmann, 2015; Rossi & De Philippis, 2015, 2016).

Cyanobacteria are classically organized in five Orders/Subsections based on the type of reproduction, cell differentiation, and other ecological adaptations (Castenholz et al., 2001; Rippka et al., 1979). Unicellular cyanobacteria are categorized into subsections I and II that are distinguished mainly by their type of cellular division, with strains from Subsection I resorting to binary fission and the ones from Subsection II being capable of reproduction by multiple fission. An example of a unicellular cyanobacterium belonging to Subsection I is the first photosynthetic organism to have its genome fully sequenced (Kaneko et al., 1996), *Synechocystis* sp. PCC 6803, that became a model cyanobacterium (Figure 1A).

Subsections III to V include the filamentous cyanobacteria (Figure 1B and C). Strains from Subsection III are filamentous strains composed of vegetative cells only, such as *Arthrospira* spp., popular in the food industry as *Spirulina* (Lafarga et al., 2020). Strains from Subsections IV and V are capable of cellular differentiation, but while cyanobacteria from the Subsection IV only form non-branching filaments (cell division in a single plane), Subsection V strains divide in more than one plane, forming true-branching filaments (Castenholz et al., 2001; Rippka et al., 1979). Examples of cellular differentiation are heterocysts (cells specialized in nitrogen fixation), akinetes (dormant cells important for the survival in austere conditions) and hormogonia (short filaments useful in dispersal and colonization) (Garcia-Pichel, 2009; Kumar et al., 2010; Singh et al., 2020). However, while being a monophyletic group within bacteria, cyanobacteria's morphological diversity does not always have phylogenetic meaning. Recent molecular evidence shows high levels of cryptic diversity and convergence of morphotypes, highlighting a complex evolution with several events of horizontal gene transfers and paralogous duplications, leading to multiple attempts to redefine their taxonomy (Garcia-Pichel et al., 2020; Komárek et al., 2014; Strunecký et al., 2023; Willis & Woodhouse, 2020).



Figure 1. Micrographs highlighting cyanobacterial morphological diversity. (A) Unicellular *Synechocystis* sp. PCC 6803, (B) filamentous *Microcoleus* sp. LEGE 191162, (C) and filamentous heterocystous *Nostoc* sp. LEGE 191164 with a heterocyst indicated by the arrow.

The metabolic flexibility, simple nutritional requirements, complex evolution and sophisticated morphological differentiation acquired throughout the millennia allowed cyanobacteria to become nearly omnipresent, conquering and thriving in multiple distinct habitats from fresh to sea water, soil and extreme environments, such as hot springs, hot and cold deserts and hypersaline areas (Bahl et al., 2011; Whitton, 2012). Cyanobacteria's ubiquity, further extends beyond abiotic habitats as they form strong symbiotic relationships with several organisms such as fungi, plants and animals (Álvarez et al., 2023; Chen et al., 2025; Mutalipassi et al., 2021). At the extreme, the prime example of these symbioses is the endosymbiotic event between a free-living cyanobacterium and a phagotrophic organism believed to have led to the origin of the organelles known as chloroplasts around

1500 million years ago. The engulfment of a cyanobacterium by an ancestral Archaeplastida resulted in the birth of the first photosynthetic eukaryote, originating a lineage that would give rise to algae and higher plants (Maréchal, 2018).

1.2. Bioproducts and cyanobacteria as ecofriendly cell factories

Cyanobacterial biomass has already been used or have shown potential to be used in a wide range of applications (Abed et al., 2009). This includes human consumption, with the prominent example of *Spirulina* (Asowata-Ayodele et al., 2024); livestock and aquaculture feed, with proven nutritional/nutraceutical benefits (Becker, 2013; Dineshbabu et al., 2019); organic fertilizers, with many terrestrial strains acting as biostimulants and plant growth promoters (Brito et al., 2022; Roque et al., 2023; Santini et al., 2021); remediation and improvement of soil, such as stabilization of burned soils by inoculation of cyanobacterial strains (Chamizo et al., 2020a); and wastewater treatment, such as the removal of arsenic contamination by bioaccumulation (Naveed et al., 2020). Other novel approaches include the use cyanobacterial cells for the development of biosensors for health monitoring (Shavelkina et al., 2025) and of microbial biophotovoltaic cells that work as a bio-solar panel during the day and as a bio-battery during the night (Sawa et al., 2017). Moreover, the high metabolic plasticity of cyanobacteria also reveals itself on the capacity to produce a vast array of metabolites such as lipids, proteins, vitamins, EPS, pigments, compatible solutes and toxins (Abed et al., 2009; Brito et al., 2015, 2025; Deepika et al., 2023; Dittmann et al., 2015; Mota et al., 2013). Many of these biomolecules have been gaining attention for the development of added-value products in a multitude of industries because of their unique structural properties, composition, versatility and bioactivity. Regarding pharmacological/biomedical applications, noteworthy examples include analogs of dolastatin (initially isolated from *Caldora penicillata*) that are used in the formulation of anticancer drugs like Brentuximab-Vedotin (Kallifidas et al., 2024; Singh, 2022), and several others undergoing clinical trials (Luesch et al., 2025; Zhang et al., 2024). Additionally, there are many other promising cyanobacterial products, such as polyketides, alkaloids, nonribosomal peptides and terpenes, due to their antibacterial, antiviral and antitumor bioactivities (Carpine & Sieber, 2021; Gonçalves et al., 2024; Tan, 2007). Pigments, namely carotenoids and phycobilins, besides being used as natural colorants in food industry, have proven anti-inflammatory and antioxidant properties useful in the prevention and treatment of chronic diseases and in the cosmetic industry (Chen et al., 2012; Gonçalves et al., 2024; Paz-Montelongo et al., 2024). Cyanobacterial EPS are also proven as active ingredients, as well as rheological modifiers and useful for cosmetic formulations and food applications (Mota et al., 2020). Also, the production of biofuels and bioplastics, such as alcohols and

polyhydroxybutyrate (PHB), respectively, is industrially/commercially relevant (Kitchener & Grunden, 2018; Markl et al., 2018; Meixner et al., 2018).

Cyanobacteria-derived products have advantages over synthetic and other natural products already implemented in the market. For example, biofuels and bioplastics present considerable benefits when compared to the less sustainable and pollutant fossil fuel-based alternatives currently in use, making them increasingly attractive as the demand for products with a smaller ecological footprint and the implementation of regulations to promote environmental conservation increases (Lea-Smith & Howe, 2017). Cyanobacteria, like plants and algae, have relatively simple nutritional requirements and capacity to directly convert carbon dioxide and water into biomass using solar energy, allowing for a reduction of production related costs since, unlike heterotrophs, cultivation of these organisms does not require the use of carbon sources (Freitas et al., 2011; Quijano et al., 2024; Rehm, 2010). Moreover, the use of cyanobacteria as biotechnology platforms for industrial settings presents key advantages over the use of other photosynthetic organisms. In particular, cyanobacteria's use of solar energy for the production of biomass is generally more efficient (3–9% reported for cyanobacteria compared with up to 3.7% for terrestrial plants), allowing for faster growth and subsequently faster production (Dismukes et al., 2008). Additionally, large scale production of cyanobacteria does not require the use of arable land and potable fresh water, and is not restricted by laws regarding ethics, animal protection and deforestation prevention (Dismukes et al., 2008; Ducat et al., 2011). Furthermore, these organisms are generally more amenable to genetic manipulation than eukaryotes, allowing them to be engineered in order to optimize production and obtain/enhance the desired properties of the end products. In particular, well-studied cyanobacteria model organisms such as *Synechocystis* sp. PCC 6803, *Synechococcus* fast-growing strains, and *Nostoc* sp. PCC 7120 are particularly suitable for bioengineering and synthetic biology approaches since there are *in silico* genome-scale metabolic models (essential to predict behavior, production rates, and aid in the design of complex devices) and sets of well characterized molecular tools available (Andrews et al., 2024; Carroll et al., 2018; Hudson, 2021; Malatinszky et al., 2017; Pacheco et al., 2021; Santos-Merino et al., 2019). These toolboxes include self-replicative vectors, reporter genes, customized regulatory elements such as promoters, ribosome binding sites, terminators and riboswitches (Ferreira et al., 2018; Pacheco et al., 2021; Vavitsas et al., 2019), as well as clustered regularly interspaced short palindromic repeat (CRISPR)-based techniques (Du et al., 2025; Hudson, 2021; Yao et al., 2016). These tools not only allow for the optimization and/or redirection of existing pathways, but have also allowed the implementation of heterologous devices/pathways for the production of various compounds of interest like columbamide, ethanol, glycine betaine, hydroxylated testosterone and ϵ -caprolactone (Ferreira et al., 2022; Hudson, 2021;

Malihan-Yap et al., 2025; Mascia et al., 2022; Taton et al., 2022; Velmurugan & Incharoensakdi, 2020).

As such, considering the immense potential of cyanobacteria-derived products, the simple and efficient cultivation, the environmental advantages and the ease of prokaryotic genetic manipulation, cyanobacteria can be considered prime candidates as ecofriendly microbial cell factories for the synthesis of customizable added-value compounds of interest for many industrial sectors (Luan & Lu, 2018; Yu et al., 2013).

2. Extracellular polymeric substances (EPS)

2.1. Biological functions and composition of EPS

Cyanobacterial extracellular polymeric substances (EPS) are complex structures mainly composed by heteropolysaccharides and peptides (Rossi et al., 2018; Rossi & De Philippis, 2016). These polymers have been mostly described as being involved in cell protection against harmful biotic and abiotic stress factors, such as desiccation, heat, ultra-violet light, high salinity, predation, and infection (Adessi et al., 2018; Harbaoui et al., 2025; Jittawuttipoka et al., 2013; Kashi Zenouzi et al., 2024; Soule et al., 2016; Tamaru et al., 2005). Additionally, these polymers have also been associated with interactions with the surroundings like cell-cell communication, aggregation, adhesion to substrates, sequestration of nutrients and gliding motility, as well as a form to store excess energy, acting as a carbon sink (Chamizo et al., 2020b; Fisher et al., 2013; Jittawuttipoka et al., 2013; Li et al., 2021; Otero & Vincenzini, 2004; Wilde & Mullineaux, 2015; Zilliges et al., 2008). These EPS can remain attached to the cell surface, being commonly designated as capsular polysaccharides (CPS), or can be released into the environment/medium, being referred as released polysaccharides (RPS). The cell attached polysaccharides can be categorized according to their thickness, consistency and appearance, being classified as sheaths (usually a thin, dense layer loosely covering cells or groups of cells), capsules (a thick and structurally coherent layer tightly associated with the cell surface) or slime (a mucilaginous material dispersed around the cells but not reflecting their distinct shape) (Pereira et al., 2009; Rossi & De Philippis, 2015, 2016). It has been reported that RPS and CPS produced by the same cyanobacterial strain can have distinct compositions, strongly suggesting that they may result from a divergent biosynthetic process (Micheletti et al., 2008; Pereira et al., 2013). Even though EPS production is not unique to cyanobacteria, EPS from other bacteria are usually less complex and contain up to four different monosaccharides, while the ones from cyanobacteria can be particularly complex as they usually have molecular masses that can reach more than 2 MDa, and consist of repeating

units composed by up to 13 different monosaccharides (Debnath et al., 2024; Delattre et al., 2016; Laroche, 2022; Pereira et al., 2009; Rossi & De Philippis, 2016). The most common/abundant monomer observed in cyanobacterial EPS is glucose. However, many others have been found such as other hexoses (galactose, mannose and fructose), pentoses (ribose, xylose and arabinose), deoxyhexoses (fucose, rhamnose), amino sugars (glucosamine, galactosamine), and acidic hexoses (glucuronic and galacturonic acids). In addition, the presence of other constituents such as acetyl, methyl, pyruvyl and sulphate groups, besides peptides, have been reported (Delattre et al., 2016; Laroche, 2022; Mota et al., 2021; Rossi & De Philippis, 2016). Notably, the presence of two different uronic acids and sulphate groups are characteristics of cyanobacterial EPS that are unusual in other bacterial EPS (Pereira et al., 2013). The high number of different building blocks observed in the composition of cyanobacterial EPS leads to a variety of linkages within these macromolecules, which, in turn, result in complex polymeric structures and architectures that are hard to decipher (Leemhuis et al., 2013; Pereira et al., 2009; Srikanth et al., 2015).

2.2. Properties and applications of EPS

Depending on the cyanobacterial strain, several factors can interfere in the amount, composition and structure, and therefore the functional properties, of EPS (Pereira et al., 2009). Environmental and nutritional conditions, as well as stress factors have been strongly associated to alterations in EPS production (Delattre et al., 2016; Madsen et al., 2023), although the specific triggers and mechanisms that result in the alterations in production and characteristics of the polymers remain mostly unknown. As such, it is paramount to optimize the culture conditions in order to maximize the quantity and quality of the polymer to be produced. High light intensity, continuous light exposure, or specific wavelengths have been correlated to carbohydrate synthesis and shown to contribute to higher EPS production and sometimes altered monosaccharide composition (Han et al., 2018; Khattar et al., 2010; Otero & Vincenzini, 2003). Deviations to the optimal temperature for cell growth, even if small, have also been shown to modify polymer yield, probably due to alterations in nutrient uptake, cell membranes and/or enzymatic activity (Gris et al., 2017; Trabelsi et al., 2009). Harvesting during different growth phases has also resulted in different productivity and EPS quality (Delattre et al., 2016). Significant alterations to the carbon:nitrogen ratios and limitations to the nutrients available in the culture media confer alterations in the total sugar content and amount of specific monosaccharides. In particular, carbon excess, nitrogen source and/or nitrogen starvation can increase EPS production (Otero & Vincenzini, 2003, 2004; Qian et al., 2022). Among others, phosphorus, sulfur, magnesium, calcium and sodium quantities present in the medium have also been shown to regulate

amount and composition of the polymers produced (Kharwar et al., 2021; Madsen et al., 2023; Singh et al., 2016; Zevin et al., 2015). Aeration and agitation can also contribute to cell productivity and therefore better polymer yield by promoting physical EPS shedding and improved light penetration and nutrient availability (Mota et al., 2013; Pereira et al., 2009).

Due to their diversity, complexity and physicochemical properties, EPS secreted by cyanobacteria have multiple potential applications (Abed et al., 2009; Cruz et al., 2020; De Philippis et al., 2001; Debnath et al., 2024; Delattre et al., 2016). The hydrophilic constituents of cyanobacterial EPS, such as sulphate groups, uronic acids, and ketal-linked pyruvyl groups, give the polymer an anionic nature that confers it a “sticky” behavior and affinity to positively charged molecules like metal ions as well as minerals, nutrients, and water (Han et al., 2014; Kauser et al., 2024; Ohki et al., 2014; Okajima et al., 2008, 2010; Pereira et al., 2013; Rossi & De Philippis, 2016). While the hydrophobic fractions like the ester-linked acetyl groups, peptidic moieties and deoxy sugars allow for better cell adhesion and emulsifying properties of the polymers (Bai et al., 2016; Chamizo et al., 2019; Han et al., 2014; Pereira et al., 2013; Rossi & De Philippis, 2016). The unusual composition of cyanobacterial EPS often results in amphiphilic properties, that allied to the high molecular mass of these polymers, confer them interesting rheological and jellification properties (Han et al., 2014; Pereira et al., 2009; Rossi & De Philippis, 2016; Sun et al., 2020). More so, bioactivity properties of polymers like the anticoagulant activity of *Synechocystis aquatilis*’ EPS; the antioxidant activity of polymers from three *Anabaena* spp., *Tolypothrix tenuis*, and *Arthrospira platensis*; and the antiviral, antibacterial and inhibition of cell proliferation of EPS from *Arthrospira platensis* have been associated with the presence of sulphate groups (Challouf et al., 2011; Kaji et al., 2004; Parwani et al., 2014; Raposo et al., 2013; Reichert et al., 2017; Volk et al., 2006). Several cyanobacterial polymers have shown potential as antioxidants, antibacterial, antifungal, antivirals, antitumors and as drug delivery systems, among others (Flores et al., 2019a; Gacheva et al., 2013; Han et al., 2014; Leite et al., 2017; Mota et al., 2016; Pereira et al., 2011; Raposo et al., 2013; Xu et al., 2013). This vast array of properties of cyanobacterial EPS are most likely related to specific structural and compositional characteristics, however only in a few cases has this association been clearly demonstrated. As such, the cause of these properties is mostly inferred from the characteristics of simpler EPS from other organisms such as macroalgae and other bacteria (Mota et al., 2021; Singh et al., 2019). Nevertheless, the described properties of cyanobacterial EPS can be useful for the development of natural products for a variety of industrial sectors such as wastewater treatment, soil remediation and stabilization, food supplementation and conservation, cosmetics, textiles, detergents, painting, coatings, goldsmithing, drugs, nanocarriers and tissue engineering (Abed et al., 2009; Pierre et al.,

2019; Raposo et al., 2013; Singh et al., 2019). In fact, many cyanobacterial polymers have multiple potential applications, and some noteworthy examples are included in Table 1.

Table 1. Examples of cyanobacterial EPS and their main biological activities and/or applications.

Organism	Polymer	Bioactivities and Putative applications	References
<i>Aphanothece sacrum</i>	Sacran	Drug delivery Wound healing Bioelectronics	Goto et al., 2019 Wathoni et al., 2016 Matura et al., 2025
<i>Crocospaera chwakensis</i>	Cyanoflan	Heavy metal remediation Drug delivery Wound healing Anti-adhesive coating Emulsification	Mota et al., 2016 Leite et al., 2017 Costa et al., 2021 Costa et al., 2019, 2020 Mota et al., 2020
<i>Arthrospira platensis</i>	Immulan	Immunostimulatory and pro-inflammatory	Løbner et al., 2008 Pugh et al., 2001
	Spirulan	Antiviral Anticoagulant Inhibitor of cell proliferation	Mader et al., 2016 Hayakawa et al., 2000 Kaji et al., 2004
<i>Phormidium J-1</i>	Emulcyan	Flocculation and emulsification	Fattom & Shilo, 1985
<i>Nostoc flagelliforme</i>	Nostoflan	Flocculation and emulsification Antiviral Antitumor	Han et al., 2014 Kanekiyo et al., 2005, 2007 Yue et al., 2012

2.3. Biosynthetic pathways

The machinery responsible for the production/assembly and export of bacterial EPS has already been extensively studied and has been shown to be particularly complex (Schmid, 2018). While most studies focus on pathogenic bacteria, both Gram-positive and Gram-negative, it has been demonstrated that some of the involved mechanisms are relatively conserved among organisms (Cuthbertson et al., 2009; Schmid, 2018; Whitfield et al., 2020). The biosynthesis and export of EPS involves three major steps in different cellular regions. First, sugar precursors are activated and modified into nucleotide sugars in the cytoplasm. Secondly, carbohydrate modifying enzymes alter the sugar moieties and glycosyltransferases (GTs) promote the assembly of the monosaccharides in the plasma membrane. Finally, the repeating units of the EPS are polymerized together and exported through the cell envelope by proteins belonging to the Wzy-, ATP-binding cassette (ABC) transporter-, or synthase-dependent pathways (Mota et al., 2021; Schmid, 2018) (Figure 2). Both the ABC transporter- and Wzy-dependent pathways are involved in the assembly and export of complex heteropolysaccharides, with the latter being the most common and present in a large number of bacterial strains (Cuthbertson et al., 2010; Islam & Lam, 2014; Schmid, 2018). In contrast, the synthase-dependent pathway is involved in the export of simple homopolysaccharides like cellulose and alginate (Schmid, 2018; Whitney & Howell,

2013). In some cases, homopolymers like dextrans and levans can be synthesized directly on the exterior of the cell by outer membrane enzymes that cleave sucrose and polymerize glucose or fructose (Schmid, 2018; Srikanth et al., 2015). In the Wzy-dependent pathway, the polysaccharide bound to the lipid acceptor is flipped by the protein Wzx to the periplasmic space where further polymerization occurs catalyzed by the polymerase Wzy in conjugation with a Wzz protein (Islam et al., 2013; Islam & Lam, 2014). Then, translocation of the polysaccharide from the periplasm to the exterior of the cell happens via a scaffold complex composed by a polysaccharide co-polymerase (Wzc) and an outer membrane export (Wza), which is mediated by cycles of phosphorylation and dephosphorylation of Wzc regulated by the phosphatase Wzb (Islam & Lam, 2014; Obadia et al., 2007; Paiment et al., 2002). Regarding the ABC transporter-dependent pathway, a series of proteins (KpsC, KpsS, KpsF and KpsU) are necessary to link the polysaccharide to a specific lipid acceptor in the inner surface of the plasma membrane (Doyle et al., 2023; Lanz et al., 2021). There, the heteropolysaccharides are fully polymerized by action of GTs, with the complexity and diversity of the resulting polymer usually being directly related to the number and type of GTs. The polysaccharide is then translocated to the periplasm by the ABC transporter complex composed by KpsM and KpsT (Cuthbertson et al., 2010; Kuklewicz & Zimmer, 2024; Willis & Whitfield, 2013). Afterwards, a protein scaffold similar to the one from the Wzy-dependent pathway composed by a polysaccharide co-polymerase (KpsE) and an outer membrane export (KpsD) facilitates the export to the cell surface (Cuthbertson et al., 2010; Kuklewicz & Zimmer, 2024; Willis & Whitfield, 2013). In contrast, the synthase-dependent pathway does not require a lipid acceptor, and the polymer is simultaneously assembled and translocated to the periplasm by a multi-protein complex comprised of a polysaccharide synthase and co-polymerase (often Alg8 and Alg44) (Hay et al., 2013; Low & Howell, 2018; Whitney & Howell, 2013). As the polysaccharide passes through the periplasm, it can suffer modifications catalyzed by multiple enzymes such as AlgI, AlgF, AlgG and AlgL. Finally, complete export is mediated by a tetratricopeptide (often AlgK) in association to a β -barrel porin (AlgE) (Hay et al., 2013; Low & Howell, 2018; Whitney & Howell, 2013).

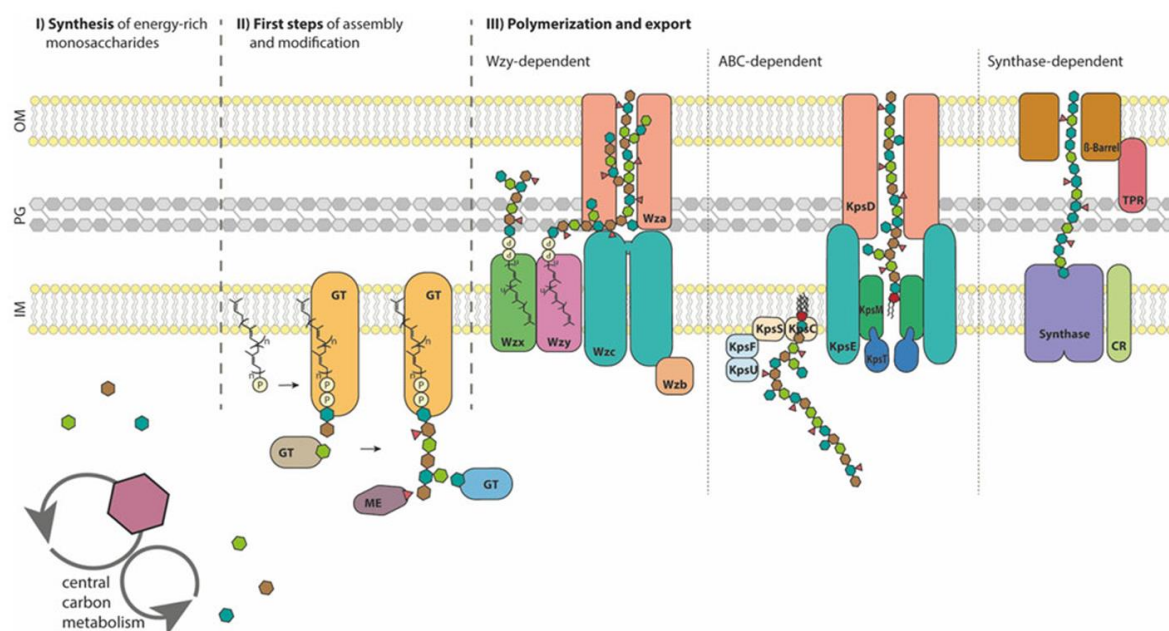


Figure 2. Schematic representation of the main bacterial EPS biosynthetic pathways (adapted from Mota et al., 2021). (I) Nucleotide sugar precursors are formed in the cytoplasm; (II) the monosaccharides start to be assembled onto a lipid carrier by glycosyltransferases (GT) and modified by enzymes such as epimerases and other carbohydrate modifying enzymes (ME); (III) the polymerization of the repeating units and export of the polymer through the cell envelope may occur by one of the three main mechanisms (Wzy-, ABC-, or synthase-dependent). In the synthase-dependent pathway, a lipid carrier may not be present, and the synthase accumulates the functions of GT and transporter. IM inner membrane, PG peptidoglycan, OM outer membrane, TPR tetratricopeptide repeat (TPR)-containing scaffold lipoprotein, CR c-di-GMP receptor.

In cyanobacteria, EPS biosynthesis begins with glucose and/or fructose typically synthesized during the Calvin cycle from the fixation of carbon dioxide, but also from direct import or the catabolism of glycogen (Kato et al., 2024; Mills et al., 2020; Zhang et al., 2023). These simple sugars are then modified by multiple enzymes of the sugar metabolism and are activated into nucleotide sugars by several cytosolic nucleosyltransferases not exclusive to EPS biosynthesis. The resulting activated monosaccharide units are then further modified by a vast array of enzymes such as epimerases, dehydratases and synthases (Drula et al., 2022; Mota et al., 2021; Pereira et al., 2013). After, several specific carbohydrate-active enzymes such as GTs catalyze the formation of glycosidic bonds and promote the transfer of the saccharide chain onto acceptor molecules in the inner face of the plasma membrane, defining the backbone of the polymer. The vast majority of these enzymes, involved in sugar activation, modification and transfer of the nucleotide sugar moieties, are highly strain specific and play a determinant role in the composition, and therefore properties, of the resulting EPS (Mota et al., 2021; Pereira et al., 2013). Further polymerization/assembly and export to the cell surface is catalyzed by proteins in the cell envelope that tend to be

conserved among bacteria and usually belong to the three previously mentioned export mechanisms (Wzy-, ABC transporter-, or Synthase-dependent pathways) (Kehr & Dittmann, 2015; Mota et al., 2021; Pereira et al., 2013). However, while most of the cyanobacterial strains harbour genes encoding proteins related to the three pathways, it has been demonstrated in a phylum-wide genomic analysis comprising of 124 genomes that they usually do not have the complete set of genes for each pathway and may not strictly follow one of the pathways previously characterized in other bacteria (Pereira et al., 2015). Notably, in the Wzy-dependent pathway, the Wzz protein seems to be missing in many cyanobacterial strains, while the Wzc kinase is apparently ubiquitous. Several proteins of the ABC transporter-dependent pathway, typically essential for capsule export (KpsS, KpsC, KpsF, KpsU), rarely possess homologues in cyanobacteria (Pereira et al., 2015). Interestingly, the synthase-dependent pathway is almost entirely absent in cyanobacteria with the exception of the synthase Alg8 that seems to be present in all strains analyzed (Pereira et al., 2015). Therefore, it is evident that cyanobacterial EPS assembly and export diverges from the mechanisms observed in other bacteria, being controlled by a highly intricate regulatory network with multiple other players involved. These multiple factors include phosphorylations, inorganic carbon availability, sigma factors, and countless other proteins whose precise mechanism of action remains largely unknown (Eisenhut et al., 2007; Flores et al., 2019b; Herrero et al., 2016; Pereira et al., 2019; Yoshimura et al., 2007). Furthermore, the complex evolution and typical polyploidy of cyanobacteria led to several events of horizontal gene transfers and paralogous duplications that resulted in an unusual amount and distribution of EPS-related genes in cyanobacterial genomes (Pereira et al., 2013, 2015). Gene copies tend to be more abundant in larger genomes, and these genes are frequently scattered throughout the genomes or in small clusters, as opposed to what is observed in other bacteria (Dimopoulou et al., 2014; Pereira et al., 2009, 2015). Altogether, the intricate EPS-related pathways, regulation mechanisms and genetic redundancy further exacerbate the mystery surrounding cyanobacterial EPS pathways.

A good example is the model cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) that – despite being the most studied cyanobacterium – its EPS-related pathways still remain largely unknown. In this microorganism, several presumed pathways for the biosynthesis of nucleotide sugars have been identified, such as deoxythymidine diphosphate-L-rhamnose (dTDP-L-Rha) and uridine diphosphate-D-glucose (UDP-D-Glc) from glucose-1-phosphate (Glc-1P), and guanosine diphosphate-D-mannose (GDP-D-Man) and its derivative guanosine diphosphate-L-fucose (GDP-L-Fuc) from fructose-6-phosphate (Fru-6P) (Figure 3) (Kanehisa et al., 2023; Moore et al., 2024). However, few of these presumed nucleotide sugar pathways seem to be complete in this strain as only a fraction of the associated genes/proteins have been identified (Table 2). Moreover this

identification relies mostly on the often-small similarity of the aminoacidic sequence of proteins compared to previously characterized enzymes from bacteria (Mills et al., 2020).

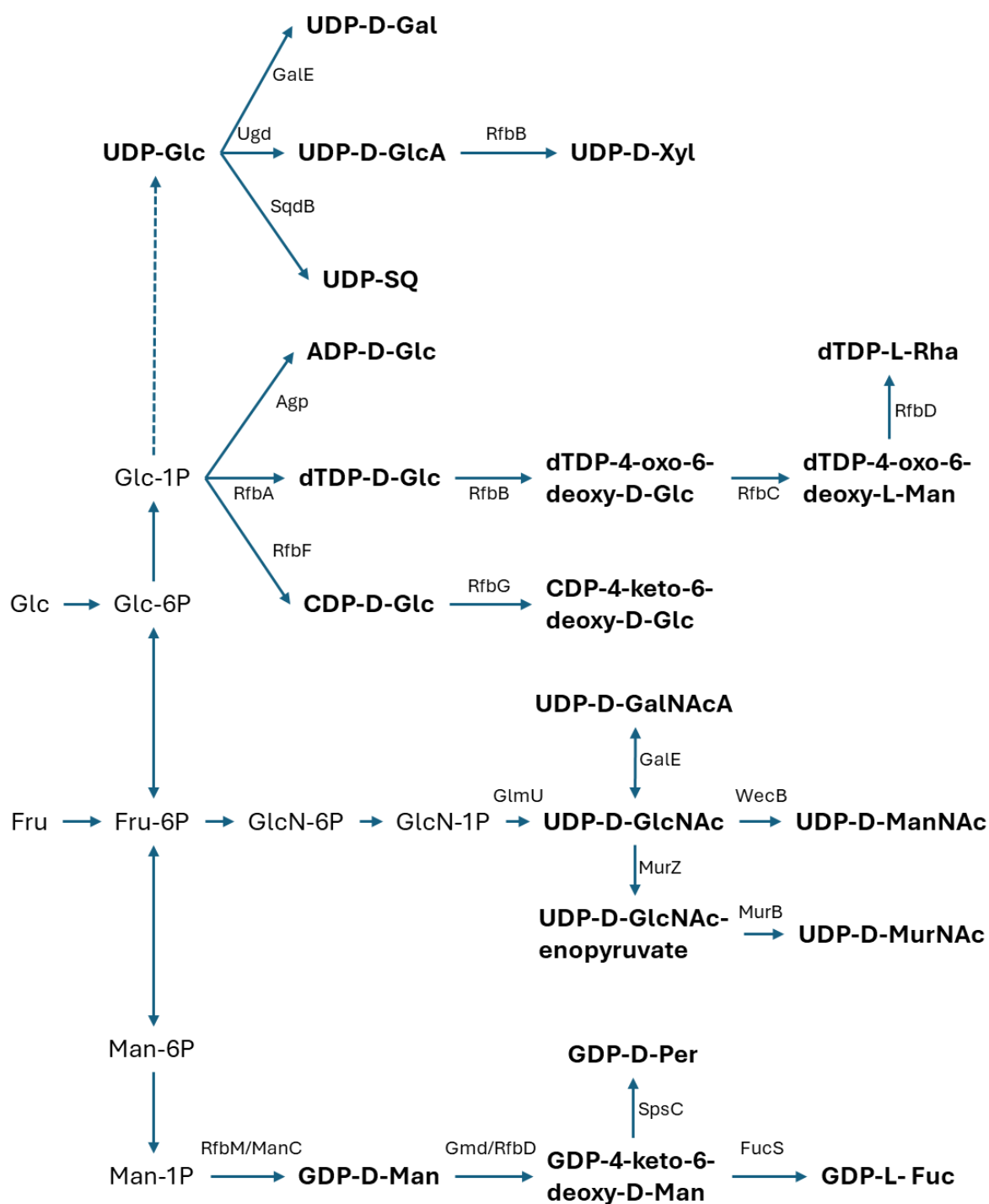


Figure 3. Expected nucleotide sugar pathways in *Synechocystis* sp. PCC 6803 based on the predicted function of gene products.

Table 2. *Synechocystis* sp. PCC 6803 genes predicted to encode enzymes in the biosynthetic pathways of nucleotide sugars.

ID	Gene Product	Gene Name(s)	Biosynthetic Pathway
<i>slr1176</i>	glucose-1-phosphate adenylyltransferase	<i>agp</i>	ADP-D-glucose
<i>slr0983</i> <i>slr0984</i>	glucose-1-phosphate cytidylyltransferase CDP-glucose 4,6-dehydratase	<i>rfbF</i> <i>rfbG</i>	CDP-4-dehydro-6-deoxy-D-glucose
<i>slI0207</i> <i>slr0836</i> <i>slr0985</i> <i>slr1933</i> <i>slI1395</i>	glucose-1-phosphate thymidylyltransferase dTDP-glucose 4,6-dehydratase dTDP-4-dehydrorhamnose 3,5-epimerase dTDP-4-dehydrorhamnose 3,5-epimerase dTDP-6-deoxy-L-mannose-dehydrogenase	<i>rfbA</i> <i>rfbB</i> <i>rfbC</i> <i>rfbC</i> <i>rfbD</i>	dTDP-L-rhamnose
<i>slI1558</i> <i>slI1496</i> <i>slI1370</i>	mannose-1-phosphate guanylyltransferase mannose-1-phosphate guanylyltransferase mannose-1-phosphate guanylyltransferase	<i>rfbM</i> <i>rfbM</i> <i>rfbM, manC</i>	GDP-D-mannose
<i>slr2114</i>	perosamine synthetase	<i>spsC</i>	GDP-D-perosamine
<i>slI1213</i> <i>slI1212</i> <i>slr1072</i>	GDP-fucose synthetase GDP-mannose 4,6-dehydratase GDP-D-mannose dehydratase	<i>fucS, wcaG</i> <i>gmd, rfbD</i> <i>rfbD, yefA</i>	GDP-L-fucose
<i>slr1078</i> <i>slr1067</i> <i>slI0244</i>	UDP-glucose 4-epimerase UDP-glucose 4-epimerase UDP-glucose 4-epimerase	<i>galE</i> <i>galE</i> <i>galE, galD</i>	UDP-D-galactose and/or UDP-N-acetyl-D-galactosamine
<i>slr0809</i> <i>slr1299</i>	dTDP-glucose 4,6-dehydratase UDP-glucose 6-dehydrogenase	<i>rfbB</i> <i>ugd</i>	UDP-D-xylose
<i>slI0899</i>	UDP-N-acetylglucosamine pyrophosphorylase	<i>glmU</i>	UDP-N-acetyl-D-glucosamine
<i>slr0624</i>	UDP-N-acetylglucosamine 2-epimerase	<i>wecB</i>	UDP-N-acetyl-D-mannosamine
<i>slr1424</i> <i>slr0017</i>	UDP-N-acetylmuramate dehydrogenase UDP-N-acetylglucosamine 1-carboxyvinyltransferase	<i>murB</i> <i>murZ</i>	UDP-N-acetyl-D-muramate
<i>slr1020</i>	UDP-sulfoquinovose synthase	<i>sqdB</i>	UDP-sulfoquinovose

Regarding the EPS assembly and export pathways in *Synechocystis*, several different genes have been identified as coding proteins with the same predicted function such as Wzy, KpsE and Alg8, while other proteins seem to be coded by a single gene or even absent (Pereira et al., 2015). Despite the efforts that have been made to elucidate the role of these putative EPS-related genes (Dai et al., 2013; Fisher et al., 2013; Foster et al., 2009; Jittawuttipoka et al., 2013; Pereira et al., 2019; Planchon et al., 2013; Santos et al., 2021a, 2021b), a lot more remains to be uncovered. The vast majority of genes associated with EPS biosynthesis and export are scattered throughout the genome of *Synechocystis* (Maeda et al., 2021; Pereira et al., 2015). However, several important clusters of genes relevant for the biosynthesis and export of EPS have already been identified, such as the *slI1722-26* cluster, believed to be important for stress protection (Foster et al., 2009); the *slr0977* gene cluster, with several genes experimentally demonstrated to be involved in EPS production (Fisher et al., 2013; Jittawuttipoka et al., 2013); and the Synechan cluster located in the megaplasmid pSYSM (Maeda et al., 2021) (Figure 4).

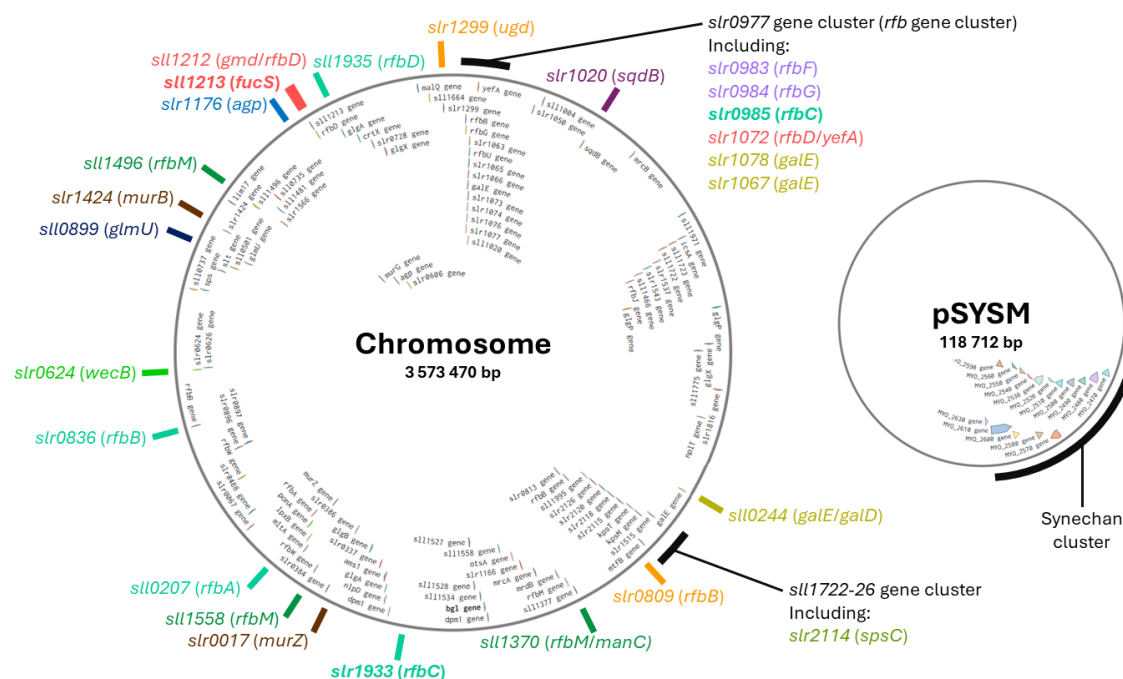


Figure 4. Locus of genes encoding enzymes putatively involved in EPS biosynthesis and export, including sugar activation and modification, glycosyltransferases, and EPS assembly and export mechanisms in the chromosome and the megaplasmid pSYSM of *Synechocystis* sp. PCC 6803. Highlighted are relevant gene clusters (black) and genes encoding enzymes of nucleotide sugar pathways (colored). Genes represented with the same color encode proteins of the same putative pathway.

3. Aims

The main goal of this project was to disclose fundamental steps in the biosynthesis of cyanobacterial EPS, focusing on the pathways for the production of the nucleotide deoxyhexoses fucose and rhamnose, and by establishing the role of key enzymes on the composition and amount of the EPS produced. For that, the model unicellular cyanobacterium *Synechocystis* sp. PCC 6803 was used to:

- (i) Generate single and double knockout strains on selected genes putatively encoding key enzymes of the pathways for the synthesis of nucleotide deoxyhexoses.
- (ii) Characterize the generated strains in terms of growth/fitness and carbohydrates production.
- (iii) Analyze the polymers produced regarding its monosaccharidic composition, and total sugar and protein contents.

Chapter II

Rhamnose biosynthesis is not impaired by the deletion of putative *rfbC* genes, *slr0985* and *slr1933*, in *Synechocystis* sp. PCC 6803

Work published in:

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Rhamnose biosynthesis is not impaired by the deletion of putative *rfbC* genes, *slr0985* and *slr1933*, in *Synechocystis* sp. PCC 6803

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ABSTRACT Cyanobacterial extracellular polymeric substances (EPS) mainly composed of heteropolysaccharides can be attached to the cell wall as capsular polysaccharides (CPS) or released to the environment as released polysaccharides (RPS). These polymers have an unusually high diversified monosaccharidic composition, making them attractive for biotechnological/biomedical applications. However, their production is still poorly understood, hindering their optimization for industrial needs. This work aimed to better understand the biosynthesis of the 6-deoxy sugars, fucose and rhamnose, in the model cyanobacterium *Synechocystis* sp. PCC 6803. To that end, genes encoding proteins putatively involved in the biosynthesis of GDP-L-fucose [*slr1213* (*fucS*)] and dTDP-L-rhamnose [*slr0985* (*rfbC1*) and *slr1933* (*rfbC2*)] were deleted. As previously observed, $\Delta fucS$ had significant growth impairment, and its RPS did not contain any fucose or rhamnose. Here, we also showed that both deoxyhexoses' pathways are completely impaired in $\Delta fucS$. In contrast, both $\Delta rfbC1$ and $\Delta rfbC1\Delta rfbC2$, although producing significantly less RPS and more CPS than the wild type, did not show major differences regarding the RPS monosaccharidic composition. These results strongly suggest that their gene products are not essential for rhamnose biosynthesis. Transcriptional analysis revealed that one of the *gmd* genes (*slr1072*) putatively encoding a GDP-mannose 4,6-dehydratase was upregulated in all the knockout strains and that the three EPS-related genes in the same operon as *rfbC1* (*slr0982*, *slr0983*, and *slr1610*) were upregulated in both $\Delta rfbC$ strains. Altogether, our results reveal that rhamnose biosynthesis in *Synechocystis* depends on FucS but not on the putative RfbC enzymes, underlining the need to further elucidate the mechanisms involved in the biosynthesis of this deoxyhexose.

IMPORTANCE This study contributes to the overall knowledge of deoxyhexoses' biosynthesis in *Synechocystis* sp. PCC 6803. Here, we demonstrated that the $\Delta fucS$ strain not only produces EPS without fucose and rhamnose, but that both pathways are completely impaired. Furthermore, we also showed that the deletion of both putative *rfbC* genes does not affect rhamnose biosynthesis despite having an impact on carbohydrate production/export, shifting RPS to CPS production. Altogether, our results suggest that the *rfbC* genes are not correctly annotated and highlight the intricacies and/or potential crosstalk between the two deoxyhexose pathways, yet to be completely unraveled in *Synechocystis*. The understanding of the cyanobacterial EPS assembly and export is crucial for the optimization of their production and tailoring for industrial/commercial applications.

KEYWORDS cyanobacteria, deoxyhexoses, extracellular polymeric substances (EPS), fucose, rhamnose, *Synechocystis*

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Many cyanobacteria can produce extracellular polymeric substances (EPS) that are mainly composed of heteropolysaccharides. EPS can remain attached to the cell surface as capsular polysaccharides (CPS) or be released to the extracellular medium as released polysaccharides (RPS) (1). Several biological functions can be attributed to EPS, such as protection against several external factors, nutrient sequestration, water retention, carbon reservoirs, intercellular interactions, formation of biofilms, motility, and cell adhesion (2-4). The interest in cyanobacterial EPS for biotechnological applications—mostly in high-value markets like the cosmetic and pharma industries—is increasing as an alternative to synthetic polymers, as well as due to their potential bioactivities (5-10). However, cyanobacterial polymers must compete with polymers already established in the market (11-14), and their potential will only be fully exploited when their biosynthetic pathways are better understood, paving the way to tailoring these polymers to industrial needs (9, 15).

In contrast to other bacterial polymers, cyanobacterial EPS can be composed of up to 13 different monosaccharides, including hexoses (glucose, mannose, galactose, and fructose), deoxyhexoses (fucose and rhamnose), pentoses (arabinose, ribose, and xylose), acidic (glucuronic and galacturonic acids), and amino sugars (glucosamine and galactosamine), as well as sulfate, methyl, and acetyl groups, and even peptides and several other non-carbohydrate constituents, resulting in complex and highly variable structures (2, 6, 9, 15-20). In several organisms, the 6-deoxy sugars play an important role in maintaining the integrity of the cell wall and capsule and are incorporated into glycoproteins and several glycosylated metabolites, such as antibiotics (21-25). These sugars may also confer bioactive properties to EPS and other metabolites since antioxidant, antibacterial, and biofilm-inhibiting properties have been associated with fucose- or rhamnose-rich EPS or glycosides (26-33). In cyanobacterial EPS, fucose and rhamnose are mostly assumed to confer hydrophobic properties that can contribute to cell adhesion and polymers emulsifying properties (4, 9). Deoxyhexoses are often only biologically available in activated forms with nucleotide groups, two of the most common being guanosine diphosphate-L-fucose (GDP-Fuc) and deoxythymidine diphosphate-L-rhamnose (dTDP-Rha) (23, 34). Despite being extensively characterized in several organisms, most of what is known regarding the activation and conversion of sugar nucleotides in cyanobacteria is inferred (35-37). The *de novo* biosynthetic pathway of GDP-Fuc is highly conserved among bacteria, plants, fungi, and animals and entails a three-step reaction catalyzed by two enzymes, GDP-mannose 4,6-dehydratase (Gmd) and GDP-L-fucose synthase (FucS, also known as WcaG or GMER). In this reaction, GDP-D-mannose is oxidized and dehydrated into GDP-4-keto-6-deoxy-D-mannose that is subsequently epimerized into GDP-4-keto-6-deoxy-L-galactose and finally reduced into GDP-Fuc (23, 38, 39). The genes encoding Gmd and FucS are generally clustered and nearly ubiquitous (23), with cyanobacteria being no exception (24, 40, 41). According to the biosynthetic pathways described for *Synechocystis* sp. PCC 6803 in the databases Kyoto Encyclopedia of Genes and Genomes and CyanoCyc (42-45), and as reviewed by Mills et al. (36), there are two candidate genes, *slr1212* and *slr1072*, encoding the GDP-mannose 4,6-dehydratase (Gmd), while FucS is encoded by *slr1213*. It has also been shown that *fucS* deletion leads to a distinct phenotype, including impaired growth, cell aggregation, lack of S-layer, altered organization of the thylakoid membranes, EPS without fucose and rhamnose, lipopolysaccharides with truncated O-antigen portions, abnormal vesicle size, and hypervesiculation (24, 46, 47). The biosynthetic pathway of dTDP-Rha is highly conserved and consists of four steps, each catalyzed by a different enzyme, namely, glucose-1-phosphate thymidyltransferase (RfbA or RmlA), dTDP-glucose 4,6-dehydratase (RfbB or RmlB), dTDP-4-dehydrorhamnose 3,5-epimerase (RfbC or RmlC), and dTDP-6-deoxy-L-mannose-dehydrogenase (RfbD or RmlD). First, the nucleotide thymidylmonophosphate is transferred onto the substrate glucose-1-phosphate. Then, the D-glucose residue is oxidized and dehydrated, leading to the formation of dTDP-4-keto-6-deoxy-D-glucose, followed by a double epimerization resulting in dTDP-4-keto-6-deoxy-L-mannose. Finally, the C4 keto group is reduced,

forming dTDP-Rha (23, 34, 48). The genes encoding RfbA, RfbB, RfbC, and RfbD are often scattered, and while many putative orthologues have been identified in several cyanobacterial strains (36, 49, 50), others seem to lack the pathway for the biosynthesis of dTDP-Rha (51, 52). In *Synechocystis* sp. PCC 6803, RfbA is likely encoded by *sll0207*, RfbB by *slr0836*, and RfbD by *sll1395*. However, there are two *rfbC* candidates, *slr0985* and *slr1933* (36, 42–45).

In this study, we aimed at better understanding the pathways involved in the biosynthesis of the deoxyhexoses GDP-Fuc and dTDP-Rha in *Synechocystis* sp. PCC 6803, contributing to unveiling the intricate mechanisms of the cyanobacterial EPS assembly and export. For this purpose, we generated Δ *slr0985* (Δ *rfbC1*) and Δ *slr0985* Δ *slr1933* (Δ *rfbC1* Δ *rfbC2*) *Synechocystis* knockout strains and characterized them in relation to the previously generated Δ *sll1213* (Δ *fucS*, 47) and the wild-type strain. Characterization included growth analyses, EPS production and monosaccharidic composition, ultrastructure of the cell envelope, as well as analysis of the transcript levels of target genes.

RESULTS

Genomic context of *sll1213* (*fucS*), *slr0985* (*rfbC1*), and *slr1933* (*rfbC2*) in *Synechocystis* sp. PCC 6803 and deletion strains' generation

This work aimed at better understanding the biosynthesis of the 6-deoxy sugars, fucose and rhamnose, in the model cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) and the impact of the absence of putative key enzymes, namely, the GDP-L-fucose synthase (*FucS*) and the dTDP-4-dehydrorhamnose 3,5-epimerase (*RfbC*), on EPS production (schematic representation of the putative pathways and locus of the respective genes are depicted in Fig. 1). For this purpose, three strains were used: Δ *sll1213* (Δ *fucS*, 47), Δ *slr0985* (Δ *rfbC1*), and Δ *slr0985* Δ *slr1933* (Δ *rfbC1* Δ *rfbC2*). The last two strains were generated within this study, and their segregation was confirmed by PCR and Southern blot (Fig. S1).

Growth, S-layer, and carbohydrate production

To understand how the deletion of the selected genes affects *Synechocystis* features and carbohydrate production, the strains were grown in liquid BG11 medium. Remarkably, the Δ *fucS* strain displayed an accentuated clumping phenotype at low optical densities ($OD_{730} < 1$), while the Δ *rfbC* strains did not exhibit this obvious phenotype (Fig. 2A). In addition, all strains showed a faster sedimentation index compared with the wild type (Fig. 2B). However, while 85% of the Δ *fucS* cells and 81% of the Δ *rfbC1* Δ *rfbC2* cells have sedimented after 24 h, only 35% of the Δ *rfbC1* cells had sedimented at this timepoint. Nonetheless, all knockout strains exhibit an almost total sedimentation after 48 h (Fig. 2B).

Transmission electron microscopy confirmed the absence of the typical S-layer in the Δ *fucS* strain (Fig. 2C). However, in some cells, it was still possible to observe a string-like structure with a similar electron density as the S-layer, suggesting that in Δ *fucS*, the S-layer can be partially formed but is unable to remain attached to the cell wall (Fig. 2C, asterisk). Regarding Δ *rfbC1* and Δ *rfbC1* Δ *rfbC2*, both strains have a similar S-layer that seems to be slightly less electron dense and more uneven compared with the wild type.

Besides the obvious clumping phenotype and lack of an S-layer, Δ *fucS* also exhibits a significant growth impairment compared with the wild type at 21 days: 14 and 23% lower OD_{730} and chlorophyll *a*, respectively (Fig. 3A). The Δ *rfbC1* strain grows similarly to the wild type, while Δ *rfbC1* Δ *rfbC2* exhibits a significant growth impairment at 21 days: 13 and 32% lower OD_{730} and chlorophyll *a*, respectively. Concerning the total carbohydrate content, neither Δ *fucS* nor Δ *rfbC1* showed differences compared with the wild type, while Δ *rfbC1* Δ *rfbC2* showed an increase of 35% (Fig. 3B). Regarding the relative amount of RPS, Δ *fucS* had no significant differences compared with the wild type, while Δ *rfbC1* and Δ *rfbC1* Δ *rfbC2* produced 35 and 27% less than the wild type, respectively, at the same timepoint (Fig. 3C). Additionally, both Δ *rfbC1* and Δ *rfbC1* Δ *rfbC2* produced 14 and 22% more CPS than the wild type, respectively (Fig. 3D).

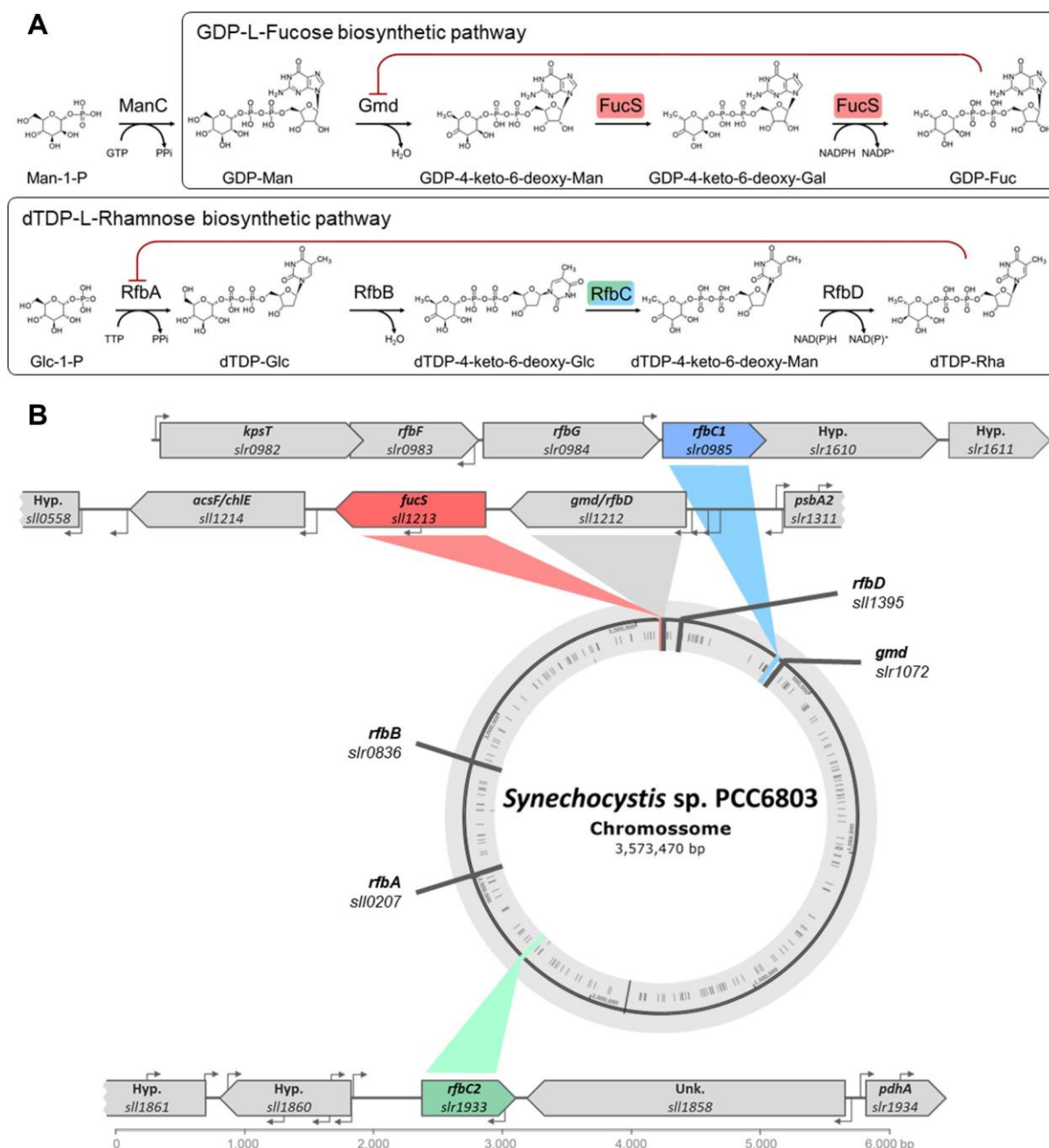


FIG 1 Biosynthetic pathways and genomic context of genes involved in the synthesis of fucose and rhamnose in *Synechocystis* sp. PCC 6803. (A) Schematic representation of the putative biosynthetic pathways of guanosine diphosphate-L-fucose (GDP-Fuc) and deoxythymidine diphosphate-L-rhamnose (dTDP-Rha). The red line indicates feedback inhibition. (B) Locus of the genes encoding enzymes putatively involved in the biosynthetic pathways of GDP-Fuc and dTDP-Rha, with the gene knockouts in this study highlighted in red, blue, and green. Both the locus tag and the gene name/symbol are provided when available. Unk./Hyp.—genes encoding unknown or hypothetical proteins.

Monosaccharidic profiles of RPS and biomass

To evaluate if the RPS composition also varies, the isolated polymers were hydrolyzed, and their monosaccharidic profile were analyzed. The $\Delta fucS$ RPS contains neither fucose nor rhamnose but had approximately 18% more hexoses, predominantly glucose. Another remarkable feature is the decrease in xylose: ~53% less than the RPS from the wild type. Unexpectedly, the RPS from $\Delta rfbC1$ and $\Delta rfbC1\Delta rfbC2$ still contain rhamnose, with both polymers quite similar to that of the wild-type strain (Table 1).

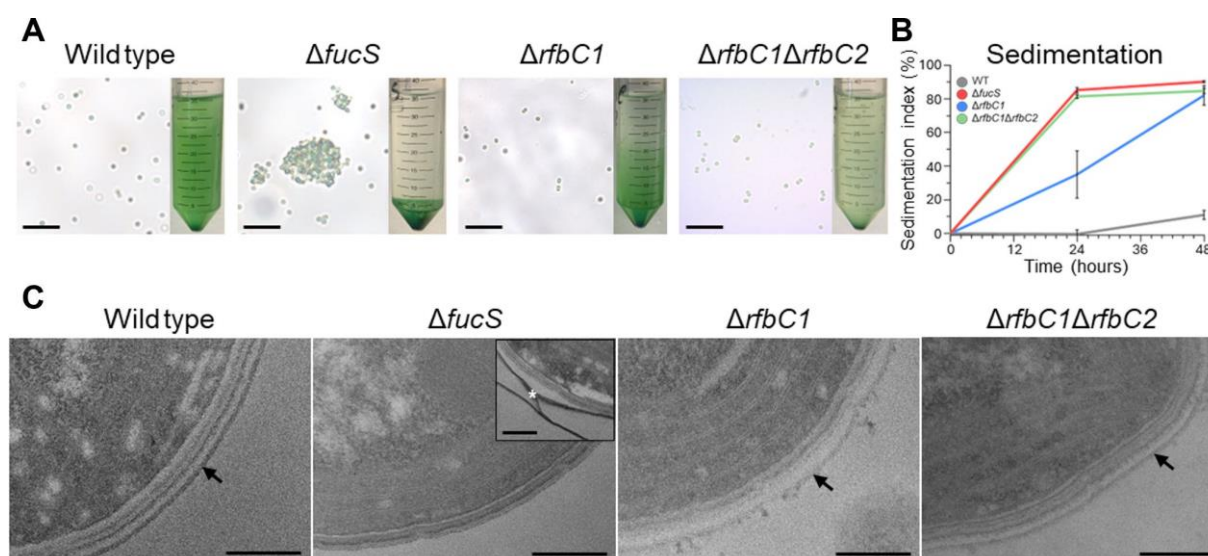


FIG 2 Aggregation/sedimentation and cell wall ultrastructure of *Synechocystis* sp. PCC 6803 wild type (WT) and $\Delta fucS$, $\Delta rfbC1$, and $\Delta rfbC1\Delta rfbC2$ strains. (A) Micrographs of the strains at low cell densities highlighting the clumping phenotype of $\Delta fucS$, scale bar: 20 μm . Insets highlight differences in sedimentation. (B) Sedimentation index (%) at 0, 24, and 48 h. Data represent means $\pm$ SD ($n = 3$). (C) Ultrastructure of the cell wall with the S-layer highlighted (arrow) and inset with the putative detached S-layer in $\Delta fucS$ also highlighted (asterisk). Scale bars: 200 nm. Cells were grown in BG11 medium at 30°C under a 12 h light (25 $\mu E m^{-2} S^{-1}$)/12 h dark regimen, with orbital shaking at 150 rpm.

To understand if rhamnose was present/absent in the CPS and/or lipopolysaccharides of the knockout strains, in particular in $\Delta fucS$, a GFP-tagged protein that specifically binds to rhamnose was used to evaluate the amount of rhamnose attached to its cell wall relative to the wild type. In the micrographs, the difference between the wild type and all the knockout strains is clearly visible (Fig. 4A). However, the results obtained in the 96-well plate measurements showed a striking ~60% reduction in the GFP signal for $\Delta fucS$ compared with the wild type, while $\Delta rfbC1$ showed a reduction of only ~28%, and for $\Delta rfbC1\Delta rfbC2$, no statistical differences could be detected (Fig. 4B). Taking into account the micrographs, and that there is no significant difference between the single and double $\Delta rfbC$ strains, the result obtained for the double mutant is most probably due to the variation in the different measurements.

To determine if $\Delta fucS$ is only impaired in the incorporation of rhamnose into the EPS or also in the biosynthesis of this sugar, the monosaccharidic composition of the whole biomass was determined. The results obtained revealed no cytosolic fucose or rhamnose in the $\Delta fucS$ strain (Table 2). Since rhamnose was never detected by gas chromatography with a flame ionization detector (GC-FID) in the $\Delta fucS$ strain, it seems reasonable to consider that the signal detected in Fig. 4B corresponds to the background. The biomass sugar profile of $\Delta rfbC1$ is very similar to the wild type, while $\Delta rfbC1\Delta rfbC2$ biomass shows a small decrease in glucose and an increase in all other sugars, except the deoxyhexoses (Table 2). Once again, our results show no impairment in the rhamnose biosynthesis for the two $\Delta rfbC$ strains.

Transcriptional analysis of genes putatively involved in fucose and rhamnose biosynthesis and/or EPS production

To better understand the complex pathways involved in the synthesis of the deoxyhexoses, the transcript levels of the *slr1072*, *slr1212* (putative *gmd*, necessary for the biosynthesis of GDP-Fuc), *slr1395* (putative *rfbD*, essential for the last step of the dTDP-Rha biosynthesis), *slr1213* (*fucS*), *slr0985* (*rfbC1*), and *slr1933* (*rfbC2*) were analyzed in the wild type and $\Delta fucS$, $\Delta rfbC1$, and $\Delta rfbC1\Delta rfbC2$ strains (Fig. 5; Fig. S2). In addition, the transcript levels of other putative EPS-related genes present in the *rfbC1* operon were also analyzed, namely, *slr0982* (encoding a putative KpsT protein involved in EPS export),

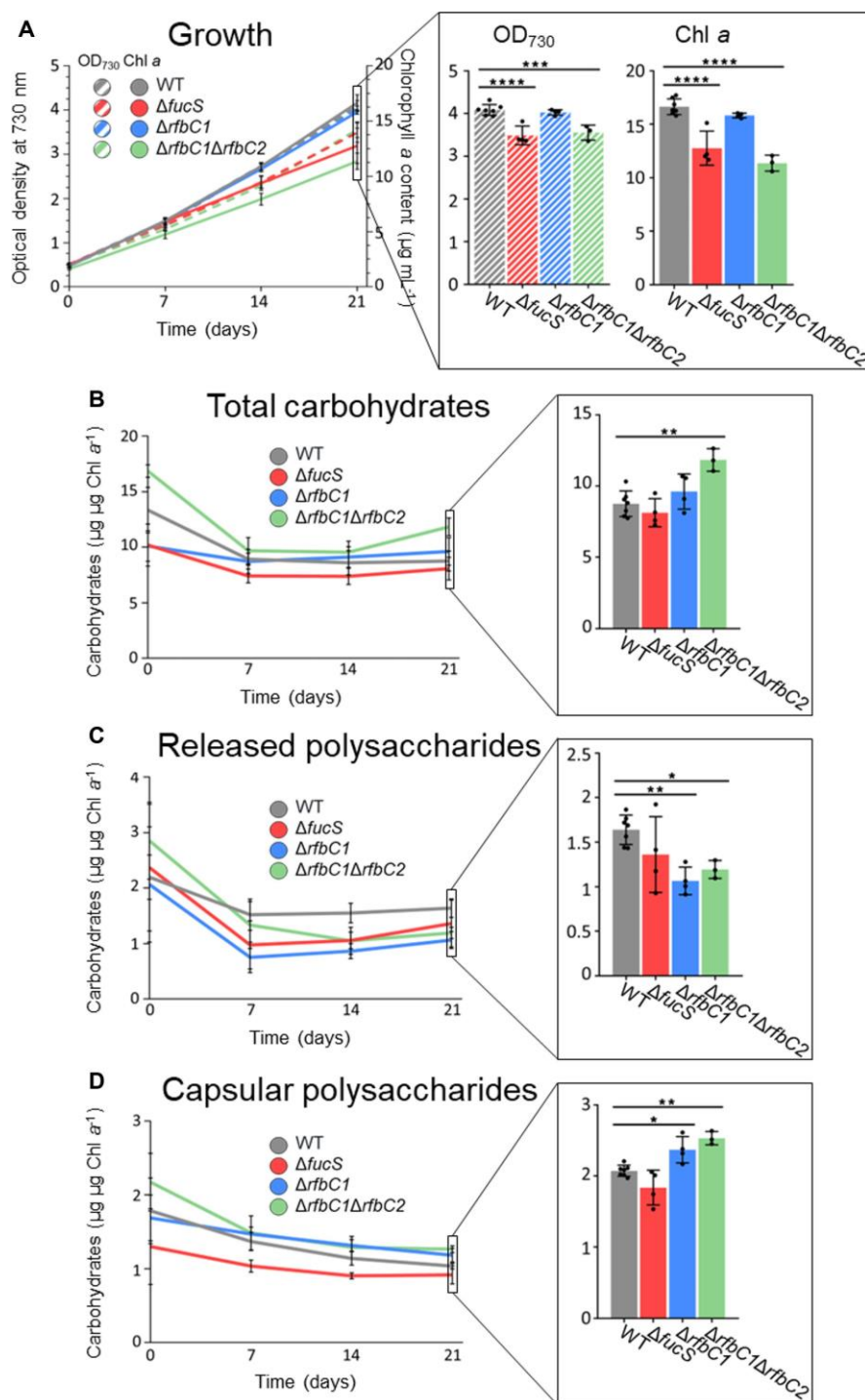


FIG 3 Growth, total carbohydrates, released polysaccharides, and capsular polysaccharides of *Synechocystis* sp. PCC 6803 wild type (WT) and $\Delta fucS$, $\Delta rfbC1$, and $\Delta rfbC1\Delta rfbC2$ strains. (A) Growth (optical density at 730 nm) and micrograms of chlorophyll *a* per milliliter of culture and production of (B) total carbohydrates, (C) released polysaccharides, and (D) capsular polysaccharides expressed as micrograms of carbohydrates per microgram of chlorophyll *a*. Data represent means $\pm$ SD ($n \geq 3$), and individual measurements are shown. Statistical analysis performed using one-way analysis of variance, followed by Dunnett's multiple comparisons, is shown for the last timepoint. Significant differences are identified: * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$), and **** ($P \leq 0.0001$).

TABLE 1 Monosaccharidic composition of the RPS from *Synechocystis* sp. PCC 6803 wild type and $\Delta fucS$, $\Delta rfbC1$, and $\Delta rfbC1\Delta rfbC2$ strains expressed as molar %^b

Monosaccharide ^a	Wild type			$\Delta fucS$		$\Delta rfbC1$		$\Delta rfbC1\Delta rfbC2$	
	% mol	SD	% mol	SD	% mol	SD	% mol	SD	
Rha	9.3	0.3	nd.	0.0	7.7	0.4	10.4	1.1	
Fuc	10.6	0.5	nd.	0.0	9.5	0.5	11.7	1.8	
Rib	Traces	0.1	1.1	0.1	Traces	0.1	Traces	0.1	
Ara	Traces	0.1	Traces	0.1	Traces	0.1	Traces	0.1	
Xyl	7.2	0.2	3.4	0.1	6.8	0.6	7.9	0.6	
Man	9.4	1.0	7.6	0.4	9.3	0.4	8.6	1.1	
Gal	3.1	0.2	6.6	0.7	4.5	1.6	3.1	0.1	
Glc	52.6	1.5	68.9	0.5	53.3	0.8	47.7	2.1	
GalN	1.8	0.0	1.9	0.1	2.7	0.0	Traces	0.0	
GlcN	1.2	0.4	2.0	1.0	1.0	0.1	4.4	0.3	
Uronic acids	4.0	0.5	8.0	1.1	4.7	0.6	6.8	0.7	

^aRha: rhamnose; Fuc: fucose; Rib: ribose; Ara: arabinose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose; GalN: galactosamine; GlcN: glucosamine.

^bTraces, <1%; not detected (nd.), 0%. Numbers in bold highlight changes in the amount of a specific monosaccharide. Sums may not be exactly 100% due to rounding.

slr0983 (encoding a putative RfbF transferase responsible for the activation of glucose-1-phosphate to cytidine diphosphate-D-glucose in an alternative sugar pathway), and *slr1610* (encoding a hypothetical methyltransferase potentially involved in EPS methylation) (46).

Among all the transcripts analyzed, the levels of *sll1212* and *sll1395* (putative *gmd*/ *rfbD* and *rfbD*, respectively) were stable in all strains and therefore used as reference genes (Fig. S2). In contrast, the results obtained showed that the transcript levels of the other putative *gmd* gene (*slr1072*) increased in all the knockout strains compared with the wild type. In $\Delta fucS$ and $\Delta rfbC1$, the increase was by 2.4- and 2.1-fold, respectively, at 21 days (Fig. 5A and B), while in the double-*rfbC* knockout strain, the increase is more evident and over time (2.0-, 2.5-, and 7.3-fold at 0, 7, and 21 days, respectively; Fig. 5C). In the $\Delta rfbC1$ and $\Delta rfbC1\Delta rfbC2$ strains, all the analyzed genes in the same operon as *rfbC1* have increased expression, more pronounced, and were at all timepoints in the double mutant. In $\Delta rfbC1$, *kpsT* (*slr0982*), *rfbF* (*slr0983*), and *slr1610* showed increased expression at 21 days of 3.0-, 4.0-, and 3.1-fold, respectively (Fig. 5B). In the $\Delta rfbC1\Delta rfbC2$ strain, these three EPS-related targets were significantly upregulated with fold changes ranging from 2.3 to 5.5 (Fig. 5C). Furthermore, in the $\Delta fucS$ strain, a decrease in the transcript levels of *rfbC2* was detected (Fig. 5A).

DISCUSSION

In this work, we aimed to better understand the inferred pathways for fucose and rhamnose biosynthesis in *Synechocystis* and how the deletion of genes encoding putative key enzymes impacts EPS composition and production. For that, we targeted the epimerization step of each deoxyhexose pathway. The GDP-Fuc pathway, in particular the deletion of the *fucS* gene, had already been explored by others, who have described, among other things, impaired growth, cell aggregation, sedimentation, absence of the S-layer, and EPS lacking fucose and rhamnose (24, 46, 47, 53). Our findings are in agreement with those of previous studies, but while the absence of the S-layer surrounding the cells was verified in the $\Delta fucS$ strain, sometimes, it was possible to observe what appears to be an S-layer detaching from the cell surface (Fig. 2C). This observation, together with the results presented by Trautner and Vermaas (53) that detected the S-layer protein (Sll1951) in a $\Delta fucS$ culture supernatant, strongly suggests that this strain is capable of generating an S-layer but incapable of keeping it attached most likely due to a fucose- or rhamnose-dependent anchor point. Regarding the other putative deoxyhexose pathway (dTDP-Rha), the deletion of *rfbC1* resulted in minor phenotypic alterations, such as a faster sedimentation rate and a slightly altered S-layer,

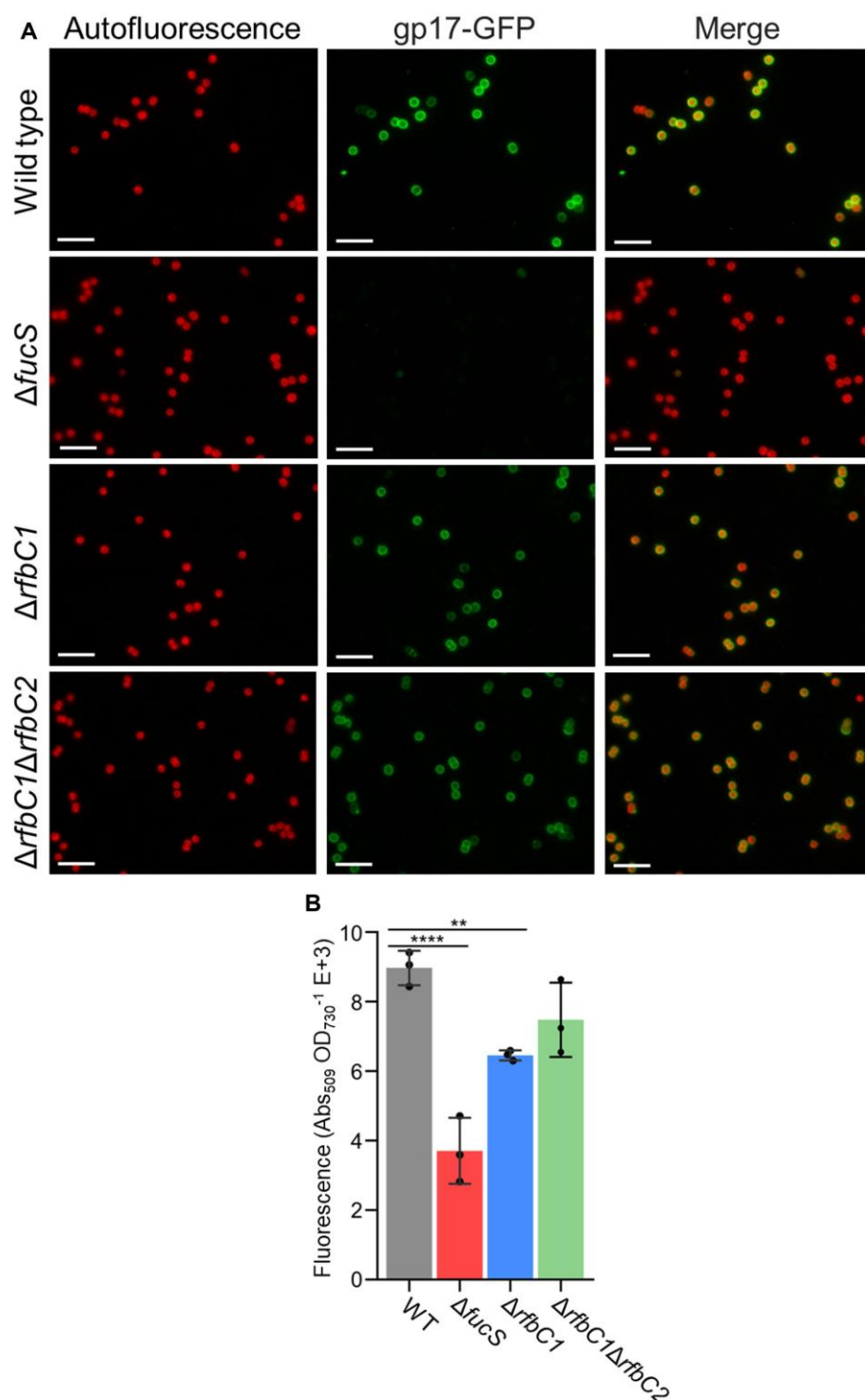


FIG 4 Detection of rhamnose on the cell surface of *Synechocystis* sp. PCC 6803 wild type (WT) and $\Delta fucS$, $\Delta rfbC1$, and $\Delta rfbC1\Delta rfbC2$ strains using a rhamnose-binding protein tagged with GFP (gp17-GFP). (A) Micrographs depicting the red autofluorescence signal and the green signal from the gp17-GFP. The right panels show the merging of the other two micrographs. Scale bar: 10 μ m. (B) Quantification of fluorescence associated with gp17-GFP bound to rhamnose at the surface of the cells measured in 96-well microplates. Data represent means $\pm$ SD ($n = 3$), and individual measurements are shown. Statistical analysis consists of one-way analysis of variance, followed by Dunnett's multiple comparisons. Significant differences are identified: **($P \leq 0.01$) and ****($P \leq 0.0001$).

TABLE 2 Monosaccharidic composition of the biomass of *Synechocystis* sp. PCC 6803 wild type and $\Delta fucS$, $\Delta rfbC1$, and $\Delta rfbC1\Delta rfbC2$ strains expressed as molar %^b

Monosaccharide ^a	Wild type		$\Delta fucS$		$\Delta rfbC1$		$\Delta rfbC1\Delta rfbC2$	
	% mol	SD	% mol	SD	% mol	SD	% mol	SD
Rha	3.3	0.6	nd.	0.0	2.9	0.3	3.2	0.3
Fuc	2.5	0.9	nd.	0.0	2.7	0.1	2.6	0.4
Rib	4.4	0.7	5.8	0.8	4.6	0.5	7.8	0.7
Ara	Traces	0.2	Traces	0.0	Traces	0.1	Traces	0.1
Xyl	2.0	0.9	Traces	0.1	2.5	0.1	3.1	0.2
Man	3.0	0.6	3.6	0.2	3.9	0.6	6.1	2.6
Gal	7.7	1.6	13.9	2.0	12.2	0.4	11.7	1.4
Glc	68.8	2.5	65.4	3.6	61.4	1.9	51.4	3.5
GalN	Traces	0.1	Traces	0.1	Traces	0.0	1.5	1.4
GlcN	4.1	0.4	5.5	0.6	5.3	0.3	7.0	1.8
Uronic acids	3.5	0.6	4.0	0.4	3.7	0.4	5.2	1.2

^aRha: rhamnose; Fuc: fucose; Rib: ribose; Ara: arabinose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose; GalN: galactosamine; GlcN: glucosamine

^bTraces, < 1%; not detected (nd.), 0%. Numbers in bold highlight changes in the amount of a specific monosaccharide. Sums may not be exactly 100% due to rounding.

while the deletion of both putative copies ($\Delta rfbC1\Delta rfbC2$) had a stronger impact with an accentuated growth impairment and increase in sedimentation (at 24 h) compared with both the wild type and the single-deletion $\Delta rfbC1$ strain. Notably, and in contrast with the wild type, all the knockout strains sediment after 48 h. These higher sedimentation rates might be due to the loss of hydrophobicity and/or the loss or alteration of a diversity of surface structures, as postulated by Zedler et al. (54). More so, $\Delta rfbC1\Delta rfbC2$ produced more total carbohydrates (per units of chlorophyll *a*). The *rfbC* deletions seem to affect, even if indirectly, the EPS assembly/export since both *rfbC* knockout strains have a shift in the production of EPS, with a decrease in RPS and an increase in CPS (Fig. 3C and D). It is reasonable to consider that this change is linked to the observed upregulation of the transcriptional unit of *rfbC1* since all the targeted genes in the *rfbC1* operon become upregulated in the *rfbC* knockout strains (Fig. 5B and C). This could indicate that *rfbC1* and *rfbC2* gene products have redundant catalytic function or are similarly regulated. Among these upregulated genes are *kpsT* (*slr0982*) encoding an ATP-binding component of an ATP-binding cassette transporter and *slr1610* encoding a methyltransferase, whose deletion has been shown to reduce EPS export (46).

Strikingly, fucose and rhamnose are absent not only from the EPS of the $\Delta fucS$ strain, as previously shown (46) but also from its biomass, indicating that this strain is indeed incapable of synthesizing rhamnose. It is intriguing, however, how the deletion of *fucS* leads to the inability of *Synechocystis* to produce rhamnose. To the best of our knowledge, there is no naturally occurring system for rhamnose biosynthesis that has fucose as a precursor, raising the question of how these two sugar pathways are linked. Many monosaccharide pathways, in particular the respective nucleosyltransferases, have been shown to be under the regulation of a myriad of molecules, such as end-product sugars of the pathway, other sugars, and other nucleoside-derived compounds (55-58). An example of this is RfbA, the nucleosyltransferase of the dTDP-Rha pathway, which in several organisms has been shown to be allosterically regulated by a variety of mechanisms, such as analogue sugars and compounds derived from dTDP (55, 58). With this in mind, one can hypothesize that the absence of *fucS* leads to an accumulation of GDP-4-keto-6-deoxy-D-mannose (not too dissimilar to dTDP-4-keto-6-deoxy-L-mannose, precursor of dTDP-Rha), which in turn could interfere with the catalytic function of RfbA culminating in the absence of rhamnose. However, it is important to notice that GDP-D-rhamnose (GDP-Rha) has been shown not to inhibit RfbA in other bacteria, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Mycobacterium tuberculosis* (58).

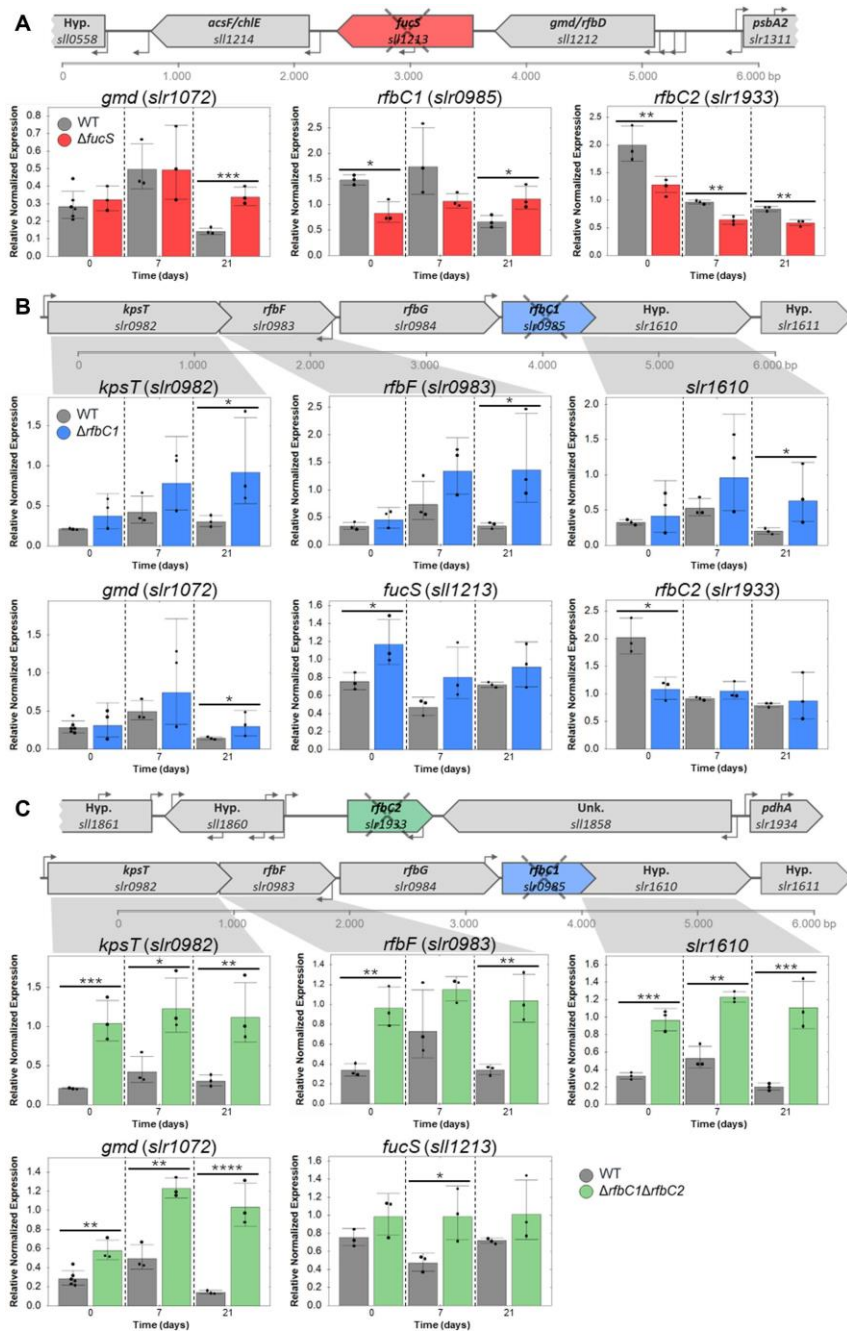


FIG 5 Analysis of the relative normalized expression of genes putatively related to the biosynthesis of deoxyhexoses and EP5 in *Synechocystis* sp. PCC 6803 wild type (WT) and $\Delta fucS$, $\Delta rfbC1$, and $\Delta rfbC1\Delta rfbC2$ strains. RNA was extracted from cells of the different strains collected at three timepoints: 0, 7, and 21 days. RT-qPCR analysis of (A) *gmd* (*slr1072*), *rfbC1* (*slr0985*), and *rfbC2* (*slr1933*) expressions in *Synechocystis* sp. PCC 6803 wild type and $\Delta fucS$, (B) *rfbB* (*slr0982*), *rfbF* (*slr0983*), *slr1610* (putative methyltransferase), *gmd* (*slr1072*), *fucS* (*slr1213*), and *slr1933* (*rfbC2*) expressions in wild type and $\Delta rfbC1$, and (C) *rfbB* (*slr0982*), *rfbF* (*slr0983*), *slr1610* (putative methyltransferase), *gmd* (*slr1072*), and *fucS* (*slr1213*) expressions in wild type and $\Delta rfbC1\Delta rfbC2$. The normalized fold expression of the target genes relative to wild type is represented at each timepoint. Data from three biological and three technical replicates were normalized against two reference genes (*slr1212* and *slr1395*), error bars represent the standard deviations, and individual measurements are shown. Statistical analysis was performed using *t*-test, and significant differences are identified: * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$), and **** ($P \leq 0.0001$).

Additionally, and contrary to what was expected, rhamnose residues are present in both RPS and biomass of the $\Delta rfbC1$ and $\Delta rfbC1\Delta rfbC2$ strains at similar amounts to the ones observed for the wild type. Under the assumption that *Synechocystis* produces dTDP-Rha, these results indicate that there must be another *rfbC* gene yet to be identified. However, in some rare cases, GDP-4-keto-6-deoxy-D-mannose is also a precursor of GDP-Rha, as for example, in *Xanthomonas campestris* and *Pseudomonas aeruginosa* (59, 60). It has also been previously described that in *Synechocystis*, the sequence of *slr0583* is similar to that of *fucS*, and while the biological function of the corresponding protein remains unknown. Mohamed et al. (24) noted that *slr0583* is also similar to an *rmd* gene in *Xanthomonas campestris* that is essential for the biosynthesis of GDP-Rha. Therefore, one cannot discard the possibility that *Synechocystis* could produce GDP-Rha via a pathway closely related to the pathway of GDP-Fuc.

Interestingly, the transcript levels of *sll1212* (the putative *gmd/rfbD* immediately upstream of *fucS*; Fig. 1B) did not vary significantly in all strains, although this gene could be considered the prime *gmd* candidate as observed in many other organisms (23). In contrast, the transcript levels of the other *gmd* candidate (*slr1072*) were upregulated in all strains, suggesting that *slr1072* is important for the biosynthesis of GDP-Fuc, dTDP-Rha, or other closely related pathways, such as GDP-Rha. If *slr1072* does encode a *Gmd*, its striking upregulation in $\Delta rfbC1\Delta rfbC2$ could be explained by an activation of a GDP-Rha pathway due to the inability to produce dTDP-Rha.

In conclusion, deoxyhexose biosynthetic pathways in *Synechocystis* are more complex and/or have more players than initially anticipated. Here, we demonstrated that *fucS* (*sll1213*) deletion impairs both fucose and rhamnose biosynthesis and that the putative *rfbC* (*slr0985* and *slr1933*) deletion(s) do not impair rhamnose biosynthesis despite affecting the EPS export. Additionally, the increased expression of the putative *gmd* (*slr1072*) in $\Delta fucS$ and $\Delta rfbC$ strains suggests that its gene product might have an important role in the deoxyhexoses' pathways. This study amounts to the evidence that many proteins of *Synechocystis* have incorrectly assigned functions, as previously pointed out by Mills et al. (36), and highlights how little is actually known regarding carbohydrate metabolism in cyanobacteria. These findings could pave the way to identify key players, namely, for the production of D- or L-rhamnose in *Synechocystis*, as well as their regulatory mechanisms.

MATERIALS AND METHODS

Bacterial strains and standard growth conditions

The cyanobacterium *Synechocystis* sp. PCC 6803 wild type (sub-strain GT-Kazusa; glucose tolerant, with S-layer and non-motile) and strains were cultivated in BG11 medium (61) at 30°C under a 12 h light (25 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$)/12 h dark regimen with orbital shaking (150 rpm). For solid medium, BG11 medium was supplemented with 1.5% (w/v) Noble agar (Difco), 0.3% (w/v) sodium thiosulfate, and 10 mM TES [N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid]-potassium hydroxide buffer (pH 8.2). For the selection and maintenance of strains, BG11 medium was supplemented with kanamycin (Km; Merck Millipore) up to 400 $\mu\text{g mL}^{-1}$ and/or chloramphenicol (Cm; Merck Millipore) up to 75 $\mu\text{g mL}^{-1}$. The *Escherichia coli* TOP10 (Thermo Fisher Scientific) was cultivated at 37°C in lysogeny broth medium (62) supplemented with ampicillin (100 $\mu\text{g mL}^{-1}$; Amp; Merck Millipore), Km (50 $\mu\text{g mL}^{-1}$), or Cm (25 $\mu\text{g mL}^{-1}$).

Cyanobacterial DNA extraction and recovery

Cyanobacterial genomic DNA was extracted by the phenol-chloroform method described previously (63). Agarose gel electrophoresis was performed according to standard protocols (64), and the DNA fragments were isolated from gels using the Monarch DNA Gel Extraction Kit (New England Biolabs) and from enzymatic assay or PCR mixtures using the Monarch PCR & DNA Cleanup Kit (New England Biolabs).

Plasmid construction for *Synechocystis* transformation

The *Synechocystis* sp. PCC 6803 chromosomal regions flanking *rfbC1* (*slr0985*) and *rfbC2* (*slr1933*) were amplified by PCR using specific oligonucleotide primers (Table S1). An overlapping region containing an *StuI* restriction site was included in primers *slr0985.5I* and *slr0985.3I*, and an *XmaI* restriction site was included in primers *slr1933.5R* and *slr1933.3F* for cloning purposes. For each gene, the purified PCR fragments were fused by “overlap PCR.” The resulting products were purified and cloned into the vector pGEM-T Easy (Promega), creating plasmids pGDslr0985 and pGDslr1933. A selection cassette containing the *nptII* gene (resistance to neomycin and kanamycin) was excised from plasmid pKm.1 (65) with the restriction enzyme *SmaI* or *XmaI* (Thermo Fisher Scientific), and *cat* (resistance to chloramphenicol) was amplified from pSEVA351 (66) by PCR using primers containing an *XmaI* restriction site. Subsequently, the selection cassettes were cloned into the *XmaI* restriction site of the plasmids to form pGDslr0985.Km and pGDslr1933.Cm.

Generation of *Synechocystis* $\Delta rfbC1$ and $\Delta rfbC1\Delta rfbC2$

$\Delta fucS$ ($\Delta slr1213$) was kindly provided by P. Oliveira (47). To generate the other strains, *Synechocystis* sp. PCC 6803 cultures were grown until the optical density at 730 nm (OD_{730}) reached ~ 0.8 , and cells were harvested by centrifugation and suspended in BG11 medium to an OD_{730} of ~ 2.5 . Five hundred microliters of cells was incubated with $20 \mu\text{g mL}^{-1}$ plasmid DNA for 5 h before spreading them onto Immobilon-NC membranes ($0.45 \mu\text{m}$ pore size; Merck Millipore) resting on solid BG11 plates and kept at 26°C under 16 h light/8 h dark for 24 h. Then, membranes were transferred to selective plates containing $10 \mu\text{g mL}^{-1}$ Km or $12.5 \mu\text{g mL}^{-1}$ Cm. After 1 week, transformants were transferred to plates containing $20 \mu\text{g mL}^{-1}$ Km or $25 \mu\text{g mL}^{-1}$ Cm. For complete segregation, colonies were grown in increasing antibiotic concentrations.

Strain confirmation by PCR and Southern blot

Segregation was confirmed by PCR using the primer pairs listed in Table S1 and depicted in Fig. S1. Each PCR reaction contained 2–10 ng of genomic DNA, $0.25 \mu\text{M}$ of each primer, $200 \mu\text{M}$ of dNTPs mix (Promega), $1\times$ Green GoTaq Flexi buffer, 1.5 mM of MgCl_2 , and 0.5 U of GoTaq G2 Flexi DNA polymerase (Promega). For the PCRs, an initial denaturation of 95°C for 5 min was performed, as well as a final extension at 72°C for 7 min. Strain's segregation was further confirmed by Southern blot. To confirm the deletion of *rfbC1* (*slr0985*), approximately 2–3 μg of genomic DNA of *Synechocystis* sp. PCC 6803 wild type, $\Delta rfbC1$, and $\Delta rfbC1\Delta rfbC2$ was digested with *DraI* (Thermo Fisher Scientific). For the deletion of *rfbC2* (*slr1933*), 2–3 μg of genomic DNA of wild type and $\Delta rfbC1\Delta rfbC2$ was digested with *NcoI* (Thermo Fisher Scientific). The DNA fragments were separated on a 0.8% (w/v) agarose gel and transferred by vacuum (5 inHg , Bio-Rad, vacuum regulator) onto an Amersham Hybond-N+ (Cytiva) with a Model 785 vacuum blotter (Bio-Rad). For the transfer, the DNA was depurinated and denatured with 0.25 M HCl (20 min), followed by 0.5 M NaOH 1.5 M NaCl (20 min). The gel was soaked in neutralizing buffer (1 M Tris-HCl pH 7.5, 1.5 M NaCl) for 20 min and subsequently in $20\times$ SSC (3 M NaCl , $0.3 \text{ M sodium citrate}$ pH 7.0) for 30 min before being equilibrated in $6\times$ SSC for 5 min. The membrane was removed from the vacuum blotter equilibrated in $6\times$ SSC for 5 min and cross-linked at 80 mJ cm^{-2} . For hybridization, probes covering the flanking regions 5' of *rfbC1* and 3' of *rfbC2* were amplified by PCR using primer pairs *slr0985_SB_Fwd* and *slr0985_SB_Rev* and *slr1933.3F* and *slr1933.3R*, respectively (Table S1). Probes were then labeled using the DIG DNA Labeling Kit (Roche) according to the manufacturer's instructions. Hybridizations were performed overnight at 60°C in a solution containing 2% (w/v) blocking reagent (Roche), 0.1% (w/v) N-lauroylsarcosine, $3\times$ SSC, and 0.02% (w/v) SDS. Subsequently, the membrane was immersed in low-stringency buffer ($2\times$ SSC, 0.1% [w/v] SDS) at room temperature for 5 min (twice) and in high-stringency buffer ($0.5\times$ SSC, 0.1% [w/v] SDS) at 60°C for 15 min (twice). The membrane was placed in

washing buffer (0.1 M maleic acid buffer, pH 7.5 with 0.3% [v/v] Tween-20) for 2 min and then incubated in 1% blocking reagent for 30 min, followed by 1% blocking reagent (w/v) with anti-DIG antibody at 1:10,000 for 30 min before being washed with 2× washing buffer for 15 min. Finally, the membrane was incubated with detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) for 5 min, followed by incubation with CDP-Star (Roche) for 5 min and revealed using a ChemiDoc Imager (Bio-Rad).

Growth assessment

Synechocystis cultures were inoculated to an OD₇₃₀ of 0.5 in 150 mL of BG11 medium in 250 mL Erlenmeyer flasks and grown for 21 days under standard growth conditions. Growth measurements were performed every 7 days by monitoring the OD₇₃₀ (67) using a spectrophotometer (Shimadzu Uvmini-1240, Shimadzu Corporation) and determining the chlorophyll *a* content as described previously (68). All experiments were performed with three technical and three biological replicates.

Determination of total carbohydrate, RPS, and CPS contents

Total carbohydrate and RPS contents were determined as described previously by Mota et al. (69) and CPS as described by Santos et al. (70) using the phenol-sulfuric acid method (71). All contents were expressed as micrograms of carbohydrates per microgram of chlorophyll *a*. All experiments were performed with three technical and three biological replicates.

Sedimentation index

Cell culture sedimentation was quantified by measuring the OD₇₃₀ of the cultures at 0, 24, and 48 h. For that, 4 mL of homogeneous culture at OD₇₃₀ of 0.8 was transferred to plastic cuvettes (Fisher Scientific) and left to sediment for 48 h at room temperature. Sedimentation index was calculated using the equation $[(OD_{730i} - OD_{730t}) \times (OD_{730i})^{-1}] \times 100$, where OD_{730i} is the measurement of the initial OD₇₃₀ (at 0 h), and OD_{730t} is the OD₇₃₀ at other timepoints.

Optical microscopy

Cells were observed directly using an Axio Lab.A1 light microscope, and micrographs were acquired with an AxioCam Erc 5s camera using ZEN 2.6 software (ZEISS).

Transmission electron microscopy

Cells were fixed directly in culture medium with final concentrations of 2.5% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) (overnight), washed three times in double-strength sodium cacodylate buffer, followed by post-fixation with 2% (v/v) osmium tetroxide and 1.5% (w/v) potassium ferrocyanide in 0.1 M sodium cacodylate buffer (pH 7.4) for 3 h, and washed three times with MilliQ-grade water. Samples were then suspended and incubated in 2% (v/v) tannic acid for 1 h and washed three times with MilliQ-grade water. Pellets were suspended in 2% (v/v) uranyl acetate, left to incubate overnight at 4 °C, and washed three times in MilliQ-grade water. Warm HistoGel (Fisher Scientific) was added to the cell pellets and left to cool at 4 °C until solid. The sample was gradually dehydrated with solutions of increasing concentration of ethanol in MilliQ-grade water and a final step with propylene oxide. Embedding was performed gradually with an increasing concentration of EPON resin in propylene oxide until pure resin was added and left to incubate for 48 h at 60 °C until completely solid. Ultrathin sections were then mounted on 300 mesh copper grids, and samples were stained with 2% (v/v) uranyl acetate and 2% (w/v) lead citrate (Reynolds' method [72]) for 15 min each. Samples were examined under a JEM-2100-HT TEM (JEOL, Ltd.) operating at 80 kV and equipped with a fast-readout "OneView" 4k × 4k CCD camera that operates at 25 fps (300 fps with 512 × 512 px).

Rhamnose binding assay

Cell-surface rhamnose was quantified using a bacteriophage receptor-binding protein (gp17) fused to GFP (gp17-GFP) provided by M. Loessner (73). Cultures were inoculated in 10 mL BG11 medium at OD₇₃₀ of 0.2 and incubated at 30 °C under a 12 h light (25 μmol photons m⁻² s⁻¹)/12 h dark regimen with orbital shaking (150 rpm) until an OD₇₃₀ = 1.5. Cultures were centrifuged for 10 min at 4,470 g, and cell pellets were suspended in fresh BG11 medium to a final concentration of OD₇₃₀ = 5. One hundred microliters of culture was transferred to a 96-well microplate (Greiner Bio-One), and cells were washed with phosphate-buffered saline (PBS) (10 min at 3,200 g). Then, cells were incubated in PBS complemented with gp17-GFP at 17 μg mL⁻¹ for 20 min in the dark. After that, cells were washed three times with PBS to remove unbound protein. To prevent cell clumping, cells were resuspended in 20 mM Tris-HCl (pH 7.8) with 4% (w/v) SDS. OD₇₃₀ and GFP fluorescence were measured using a CLARIOstar Plus plate reader (BMG Labtech). Fluorescence data were normalized by OD₇₃₀. All experiments were performed with three technical and three biological replicates. Images were collected with an Olympus BX53 (Olympus Corporation) fluorescence microscope with ISO set to 400. Autofluorescence micrographs were captured using the filter for tetramethylrhodamine and an exposure of 714.2 ms, while the micrographs of the gp17-GFP fluorescence were obtained using the filter for fluorescein and an exposure of 957.4 ms. Images were then processed using Fiji (version 2.15.0, 74).

Neutral sugar and uronic acid analysis

To determine the monosaccharidic composition of the RPS, cultures at OD₇₃₀ = 3 were processed as described by Flores and Tamagnini (75). To determine the monosaccharidic composition of the biomass, cell cultures at OD₇₃₀ = 1.5 were centrifuged and washed with BG11. Cell pellets were left to dry at 55 °C for 48 h. Both freeze-dried RPS and biomass (1 to 2 mg) were processed and analyzed as detailed by Aveiro et al. (76). In short, all samples were subjected to a prehydrolysis with 0.2 mL of 72% (v/v) H₂SO₄ for 3 h at room temperature, followed by 2.5 h hydrolysis with 1 M H₂SO₄ at 100 °C. Neutral sugars were determined by converting the hydrolyzed sugars in alditol acetates using 2-deoxyglucose as the internal standard. Then, gas chromatography with a flame ionization detector (GC-FID; Perkin-Elmer Clarus 400) equipped with a DB-225 capillary column (Agilent J&W GC columns) was used to identify the alditol acetates. The uronic acid content was determined by the m-phenylphenol colorimetric method, and d-galacturonic acid solutions (0–80 μg mL⁻¹) were used to construct a calibration curve. The analyses were performed in triplicate.

RNA extraction, cDNA synthesis, and transcription analysis by RT-qPCR

For RNA extraction, cultures were inoculated to OD₇₃₀ = 0.5 in a final volume of 150 mL of BG11 medium in 250 mL Erlenmeyer flasks and grown for 21 days (see subsection “Bacterial strains and standard growth conditions”). Samples were collected at 0, 7, and 21 days, and cell pellets were treated as previously detailed by Ferreira et al. (77). The RNA concentration, purity, and integrity were checked as stated by Ferreira et al. (77), and for cDNA synthesis, 1 μg of total RNA was transcribed with the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad) in a final volume of 20 μL following the manufacturer’s instructions. Fivefold standard dilutions of the cDNAs were made (1/5, 1/25, 1/125, and 1/625) and stored at –20 °C.

RT-qPCRs were performed on hard-shell 384-well PCR plates (thin wall, skirted, clear/ white) covered with Microseal B adhesive seal (Bio-Rad). The reactions (10 μL) were manually assembled and contained 0.125 μM of each primer (Table S2), 5 μL of iTaq Universal SYBR Green Supermix (Bio-Rad), and 1 μL of template cDNA (dilution 1/5). The PCR protocol used was: 3 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 56 °C, and 30 s at 72 °C. In the end, a melting curve analysis of the amplicons (10 s cycles between 65 and 95 °C with a 0.5 °C increment per cycle) was carried out.

Standard dilutions of the cDNA were used to check the relative efficiency and quality of primers, and negative controls (no cDNA template) were included. RT-qPCRs were performed with three biological and three technical replicates of each cDNA sample in the CFX384 Touch Real-Time PCR Detection System (Bio-Rad). The data obtained were analyzed using Bio-Rad CFX Maestro 1.1 software (Bio-Rad), implementing an efficiency-corrected delta-delta Cq method ($\Delta\Delta Cq$). The genes *sll1395* and *sll1212* were validated as reference genes for data normalization using the reference gene selection tool available in the Bio-Rad CFX Maestro software (version 2.3, Bio-Rad). Standard *t*-test was performed using the same software, and tests were considered significant if $P < 0.05$.

Statistical analysis

Data were statistically analyzed with GraphPad Prism (version 8.0.1, GraphPad Software) using analysis of variance, followed by Dunnett's multiple-comparisons test.

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ADDITIONAL FILES

The following material is available [online](#).

Supplemental Material

Supplemental material (AEM00702-25-s0001.pdf). Tables S1 and S2; Figures S1 and S2.

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Supplementary material

**Rhamnose biosynthesis is not impaired by the deletion of putative *rfbC* genes,
slr0985 and *slr1933*, in *Synechocystis* sp. PCC 6803**

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Table S1 List of primers used for strain generation and confirmation.

Primer Name	Code	Primer Sequence 5' → 3'	Amplicon (bp)	Source
slr0985.5O ^{a,b}	1F	CGCCTGCGAAATCGTAGTAGCCAGTT ATC	709	This work
slr0985.5I ^a		GATAATTCGGTAATTGAGGCCTATGG GCTGACGGATAACGGTTCTGAGT		
slr0985.3O ^{a,b}	1R	GCTCATGGTAAACCGTATCAAATTGG GTC	1157	
slr0985.3I ^a		TTATCCGTCAGCCCATAGGCCTCAAT TACCGAATTATCCCAGCGAGACC		
slr0985.5F ^b	2F	TCGCCATTTCAGGCTGCGCAACGGAG ATTGGTCAGAGGATCGTCTCATTCC	535	
slr0985.5R ^b	2R	TAGGCGAGGACCCGGGTCAGATAGC AAGGTCTGACAGAGC		
slr0985_SB_Fwd ^c	3F	CCTGATGTTGTCCGCGCCCTA	333	
slr0985_SB_Rev ^c	3R	CCAACTAGGACGATATCCCAGTA		
slr1933.3F ^{a,c}	6F	TTTAATCTCCCGGGGTTTCGCCTCTTTC CTGAATTACCCAAAAGTTTGC	574	
slr1933.3R ^{a,c}	6R	CACTCATTAGGCACCCCAGGCCCA ATTTTGC GTTGGTTCCTGG		
slr1933.5F ^a		TCGCCATTTCAGGCTGCGCAACCCATG GCTGGAAATAACCATAGACCGTTTCG	680	
slr1933.5R ^a		GGCGAACCCCGGGAGATTAAATTCCT CTTTAGGGTTACGACACC		
slr1933.FwdO ^b	4F	CTTTGAGCCGAACCTTTCTC	1836 (WT) 2080 (MT)	
slr1933.RevO ^b	4R	CATACCGTTGAACAATGGAC		
slr1933.FwdI ^b	5F	CTTTGAGAGCTACAACGACAAG	347	
slr1933.RevI ^b	5R	CATAGGACGGTGCATAGTAATC		
pSEVA351.Cm.Fwd ^d		GCGAAACCCGGGCGTTGATCGGCACG TAAGAG	805	
pSEVA351.Cm.Rev ^d		TCCACACCCGGGACGATTTAAGTCAAA TTACGCC		

^aPrimers used for plasmids construction.^bPrimers used for the confirmation of the knockout strains' segregation by PCR.^cPrimers used for Southern blot probes.^dPrimers used for the amplification of the *cat* cassette.

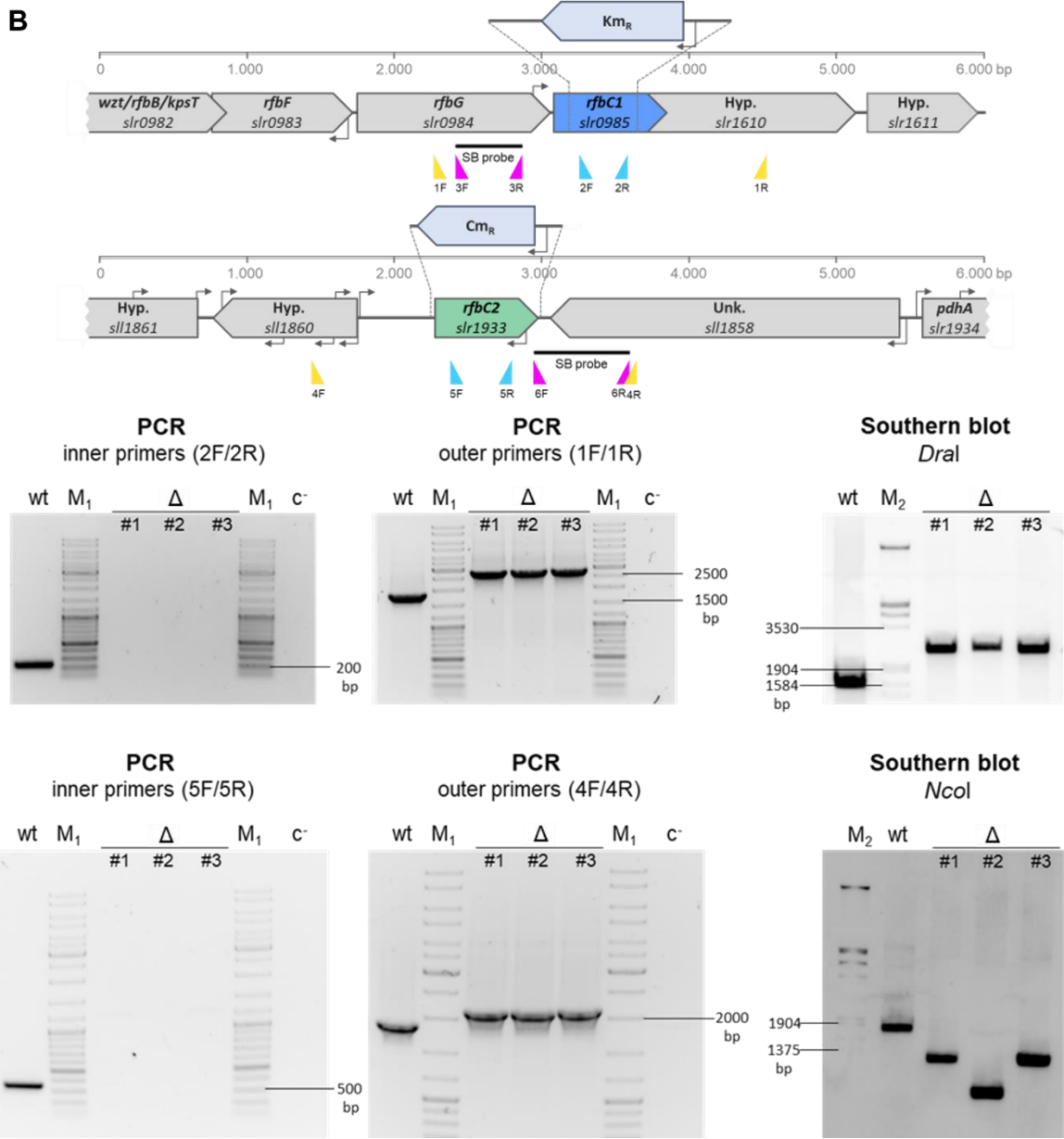
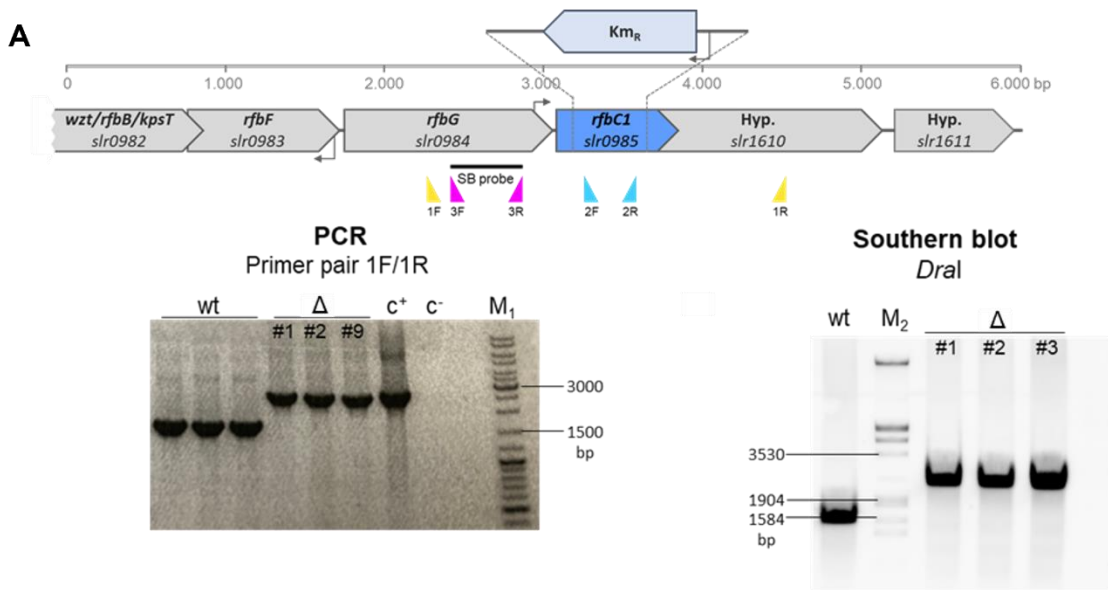


Fig S1 RfbC encoding genes in *Synechocystis* sp. PCC 6803, and confirmation of the segregation of the $\Delta rfbC1$ and $\Delta rfbC1\Delta rfbC2$ strains. **(A)** Schematic representation of the genomic context, position of the primers used in the PCR reactions, and regions replaced by the selection cassette (conferring resistance to kanamycin). Agarose gel electrophoresis showing the PCR amplicons obtained with a primer pair targeting the *rfbC1* region (slr0985.5O/slr0985.3O, expected sizes 1828 bp for the wild type and 2778 bp for the knockout strain). Southern blot hybridization with genomic DNA from *Synechocystis* wild type and $\Delta rfbC1$ clones digested with *DraI* and hybridized with a probe covering the 5' flanking region of *rfbC1* gene (obtained by PCR with the primer pair slr0985_SB_Fwd/slr0985_SB_Rev). Expected sizes of restriction fragments are 1636 bp for wild type and 2586 bp for the knockout strain clones. **(B)** Schematic representation of the genomic context, position of the primers used in the PCR reactions, and regions replaced by the selection cassettes (conferring resistance to kanamycin or chloramphenicol). Agarose gel electrophoresis showing the PCR amplicons obtained with a primer pair targeting *rfbC1* (inner primers: slr0985.F/slr0985.R, expected size 232 bp for the wild type) and the gene region (outer primers: slr0985.5O/slr0985.3O, expected sizes 1828 bp for the wild type and 2778 bp for the knockout strain). Southern blot hybridization with genomic DNA from *Synechocystis* wild type and $\Delta rfbC1\Delta rfbC2$ clones digested with *DraI* and hybridized with a probe covering the 5' flanking region of *rfbC1* gene (obtained by PCR with the primer pair slr0985_SB_Fwd / slr0985_SB_Rev). Expected sizes of restriction fragments are 1636 bp for wild type and 2586 bp for the knockout strain clones. Agarose gel electrophoresis showing the PCR amplicons obtained with a primer pair targeting *rfbC2* (inner primers: slr1933.FwdI/slr1933.RevI, expected size 347 bp for the wild type) and the gene region (outer primers: slr1933.FwdO/slr1933.RevO, expected sizes 1836 bp for the wild type and 2080 bp for the knockout strain). Southern blot hybridization with genomic DNA from *Synechocystis* wild type and $\Delta rfbC1\Delta rfbC2$ clones digested with *NcoI* and hybridized with a probe covering the 3' flanking region of *rfbC2* gene (obtained by PCR with the primer pair slr1933.3F/slr1933.3R). Expected sizes of restriction fragments are 1819 bp for wild type and 1256 or 795 bp for the knockout strain clones, depending on the orientation of the resistance cassette.

wt - wild type, Δ - knockout strain, # clone number, c⁺ - positive control, c⁻ - negative control, M1 - GeneRuler DNA Ladder mix (Thermo Scientific), M2 – DNA Weight Marker III DIG-Labeled (Roche).

Table S2 List of primers used in the RT-qPCR analysis.

Primer Name	Primer Sequence 5' → 3'	Amplicon (bp)	Source
slI1395_RT_fwd	GAAATCGGTGCCTATGTG	196	
slI1395_RT_rev	CATGGACTCCATAAACCC		
slr0982_RT_fwd	ATTGATGAGCCCCTAACG	217	
slr0982_RT_rev	ATCCCACCGAGTAAACAC		
slr0985_RT_fwd	CTGAGGAGTTGAAGGAAG	181	
slr0985_RT_rev	GAATCAACCCGCAAATCC		
slr1933_RT_fwd	GGTTGTGTGCTCAATGAC	130	
slr1933_RT_rev	CATAGGACGGTGCATAGT		
slr1072_RT_fwd_ii	CAGGTGGTTTCACAGGTCAA	218	This work
slr1072_RT_rev_ii	TCATCAGCGGCGGAATTG		
slI1213_RT_fwd_ii	CTGGAATCCTACCGCTTGC	215	
slI1213_RT_rev_ii	GCCGCATCAGTGGAGTAAA		
slI1212_RT_fwd_ii	CGGCATCCAAGTAAGGTTC	181	
slI1212_RT_rev_ii	GCCGTTACAGGCGAATAAGTC		
slr0983_RT_fwd_ii	GCTACGGTCACAGCAGTTG	196	
slr0983_RT_rev_ii	ATTCAGTTAGGGGCTCACC		
slr1610_RT_fwd_ii	GCCCAGACCCAATTTGAT	189	
slr1610_RT_rev_ii	TTCCCTTACTCGCTCACT		

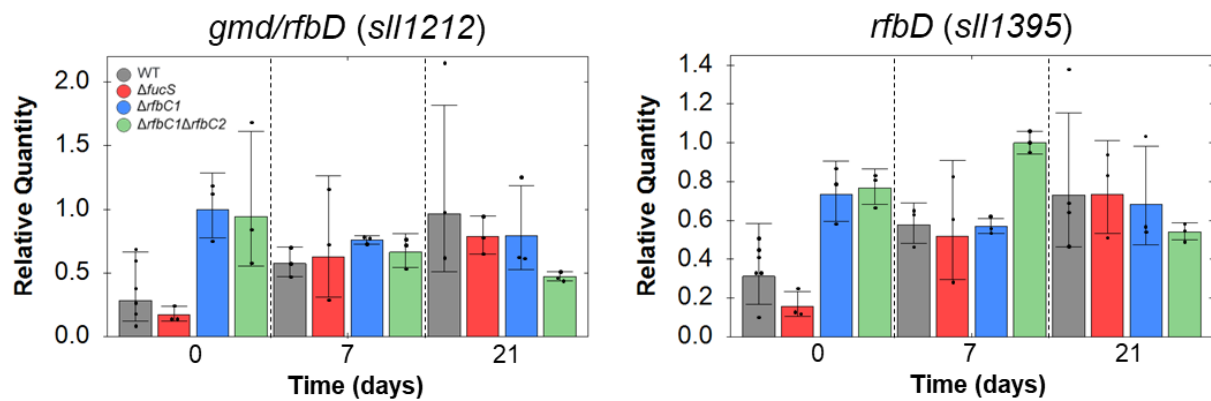


Fig S2 RT-qPCR analysis of *slI1212* and *slI1395* relative expression in *Synechocystis* sp. PCC 6803 wild type (WT) and $\Delta fucS$, $\Delta rfbC1$ and $\Delta rfbC1\Delta rfbC2$ strains. RNA was extracted from cells of the different strains collected at three time points - 0, 7, and 21 days. The bars represent the expression of *gmd/rfbD (slI1212)* and *rfbD (slI1395)* from *Synechocystis* sp. PCC 6803 wild type, $\Delta fucS$, $\Delta rfbC1$ and $\Delta rfbC1\Delta rfbC2$ at each time point. The data were obtained from three biological replicates with technical triplicates, error bars represent the standard deviations, and individual measurements are shown.

Chapter III

**Comparison of polymers' isolation methods,
further characterization of $\Delta fucS$, and
preliminary characterization of $\Delta rfbC2$**

Comparison of polymers' isolation methods, further characterization of $\Delta fucS$, and preliminary characterization of $\Delta rfbC2$

1. Comparison of two methods for the isolation of released polysaccharides (RPS)

1.1. Introduction

Cyanobacterial extracellular polymers struggle to compete with biopolymers already established in the market mainly due to scalability and productivity issues, and/or processing costs (Agarwal et al., 2022; Gomes Gradíssimo et al., 2020; Laroche, 2022; Pierre et al., 2019). As such, for the isolated polymers to be commercially viable, it is important to optimize the extraction method to maximize yield without sacrificing the desired properties. For that reason, the polymers previously isolated in Chapter II (Pissarra et al., 2025), from the *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) wild type and the $\Delta fucS$ and $\Delta rfbC1$ knockout strains via ethanol precipitation, were compared against polymers isolated by another method (freeze-dried culture medium) aiming at increasing the final yield without the use of solvents. The resulting polymers were compared regarding yield and total sugar and peptide contents.

1.2. Materials and methods

1.2.1. Polymer isolation

Two methods were used to isolate polymers from *Synechocystis* wild type and $\Delta fucS$ and $\Delta rfbC$ strains: (Method 1) by ethanol precipitation of the cell-free and dialyzed culture media and freeze drying of the precipitates, as described previously in Chapter II (Pissarra et al., 2025); or (Method 2) by freeze drying the cell-free and dialyzed culture media without precipitation, as described by Pereira et al. (2019).

1.2.2. Total sugar and peptides quantification

Total proteins/peptides were quantified by the Lowry method (Lowry et al., 1951), and total sugars were quantified by gas chromatography with a flame ionization detector (GC-FID) as detailed in Chapter II (Pissarra et al., 2025).

1.3. Results and discussion

1.3.1. Characterization of polymers isolated with or without ethanol precipitation

To test if the isolated polymers obtained from the wild type and the $\Delta fucS$ and $\Delta rfbC1$ knockout strains would be different depending on the isolation method, two different methodologies were tested. The first method, ethanol precipitation (Method 1), previously used in Chapter II (Pissarra et al., 2025) resulted in small amounts of light yellow polymers with similar appearance between strains. Whereas the second method, freeze-dried culture media (Method 2), produced larger quantities but the polymers were generally darker, with the color intensity varying depending on the strain (Figure 1).

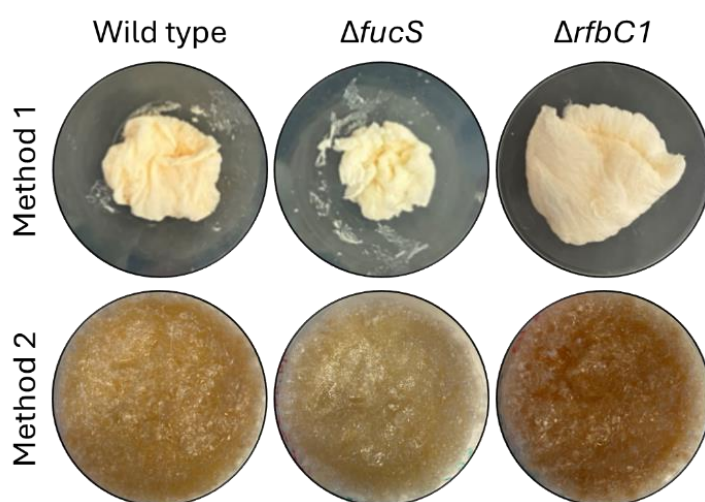


Figure 1. Comparison of polymers isolated from *Synechocystis* sp. PCC 6803 wild type and $\Delta fucS$ and $\Delta rfbC1$ strains by two different methods: ethanol precipitation (Method 1), and freeze-dried culture media (Method 2).

Preliminary results regarding the protein content of the polymers obtained by Method 1 showed minor differences between strains (Table 1). While Method 2 resulted in polymers with more variability (Table 1). The polymers isolated by Method 1 (ethanol precipitation) had similar total sugar contents and had more sugar than the polymers isolated by Method 2, this was particularly noticeable for the polymers from the knockout strains (Table 1). In general, total sugar and peptide contents correspond only to ~60-70% of the polymer weight but it is likely that the sugar content is underestimated due to the quantification method. Since no other compositional tests were performed, it can be expected that the remainder of the weight percentage can be attributed to other constituents. This can include pigments, usually responsible for the yellow/orange color of the polymers.

Table 1. Comparison between polymers of *Synechocystis* sp. PCC 6803 wild type and $\Delta fucS$ and $\Delta rfbC1$ knockout strains obtained by ethanol precipitation (Method 1) and freeze-dried culture media (Method 2).

Strain	Method 1				Method 2			
	Total peptide % (w/w)	SD	Total sugar % (w/w)	SD	Total peptide % (w/w)	SD	Total sugar % (w/w)	SD
Wild type	47.3	1.0	23.9	1.7	36.9	0.4	20.5	1.0
$\Delta fucS$	45.8	2.1	23.6	2.5	50.4	1.7	13.5	0.7
$\Delta rfbC1$	41.5	2.3	25.3	2.0	43.0	1.8	13.6	0.4

While freeze-drying directly the cell-free culture medium (Method 2) resulted in greater yield by weight, the isolated polymers showed a darker color (indicating higher amounts of contaminants such as carotenoids), variation in protein content, and less total sugar content. Therefore, the ethanol precipitation step seems to be important to increase the purity of the polymers, which can influence the desired properties and be particularly relevant for certain medical applications. However, if the color observed is indeed due to the presence of carotenoids, and since it is known that these pigments can have anti-inflammatory and antioxidant properties (Gonçalves et al., 2024; Morone et al., 2024; Paz-Montelongo et al., 2024; Rodrigues et al., 2024), Method 2 can be useful for example if the polymers are intended for cosmetic applications.

2. Fucose and rhamnose on the anchorage of the S-layer

2.1. Introduction

Synechocystis lacking *sll1213* ($\Delta fucS$) has been shown to be unable to maintain an S-layer (Mohamed et al., 2005) despite the S-layer protein (Sll1951) having been detected in the culture media (Trautner & Vermaas, 2013). In the previous chapter, the absence of an S-layer in the $\Delta fucS$ strain was confirmed, but it was also possible to observe an S-layer-like structure detaching from the cells (Chapter II; Pissarra et al., 2025). Considering that this strain is also unable to synthesize fucose and rhamnose, it was hypothesized that fucose and/or rhamnose is essential for the anchorage of the S-layer. In here, we tested if the supplementation of the growth medium with fucose or fucose plus rhamnose leads to changes in the phenotype previously observed in $\Delta fucS$.

2.2. Materials and methods

2.2.1. Bacterial strains and standard growth conditions

The cyanobacterium *Synechocystis* sp. PCC 6803 wild type (sub-strain GT-Kazusa; glucose tolerant, with S-layer and non-motile) and strain were cultivated in BG11 medium (Stanier et al., 1971) in the standard conditions stated before (Chapter II; Pissarra et al., 2025). For the maintenance of strains, BG11 medium was supplemented with 100 $\mu\text{g mL}^{-1}$ of kanamycin (Km; Merck Millipore). For the deoxyhexose incorporation test, BG11 medium was supplemented with L-fucose (3 mM) or both L-fucose and L-rhamnose (3 mM).

2.2.2. Transmission electron microscopy

For transmission electron microscopy (TEM) analysis, cyanobacterium culture was centrifuged at 2500 $\times$ g for 10 min, and the collected cells were fixed in 2.5% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) (overnight). After fixation, cells were washed three times in sodium cacodylate buffer followed by postfixation in 2% (v/v) osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.2) (overnight at 4°C) and washed three times in water. Pellets were suspended in 1% (v/v) uranyl acetate (overnight at 4°C) and washed three times in water. Warm HistoGel (Fisher Scientific, USA) was added to the cell pellets and left to cool at 4°C until solid. Samples were gradually dehydrated with solutions of increasing concentration of ethanol (50% - 100%; v/v), and a final step with 100% propylene oxide repeated three times. Samples were embedded in a series of solutions of EMBed-812 resin (Electron Microscopy Sciences) in propylene oxide of increasing concentrations (25% - 100%; v/v) and left in 100% resin for 48 h to fully polymerize. Ultrathin sections were mounted on 300 mesh nickel grids, and samples were stained by the Reynolds' method (Reynolds, 1963) for 5 min each. Grids were examined using a JEM-1400Plus electron microscope (JEOL, USA) operating at 80 kV and images were digitally recorded using a CCD digital camera Orious 1100W (Hamamatsu Photonics, Japan).

2.3. Results and discussion

2.3.1. Deoxyhexoses supplementation does not rescue the S-layer phenotype observed for $\Delta fucS$

To elucidate if the strain lacking *slr1213* ($\Delta fucS$) can incorporate exogenous L-fucose and/or L-rhamnose, allowing the formation of a structured S-layer, the knockout strain was cultivated in media supplemented with L-fucose or with L-fucose and L-rhamnose. Afterwards, the presence/absence of the S-layer was visualized using TEM. It was possible

to observe that the $\Delta fucS$ knockout strain growing with fucose only (data not shown) or with fucose and rhamnose (Figure 2) was still unable to form a structured S-layer. This result suggests that *Synechocystis* is incapable of incorporating exogenous L-fucose and L-rhamnose into the synthesis of nucleotide sugars.

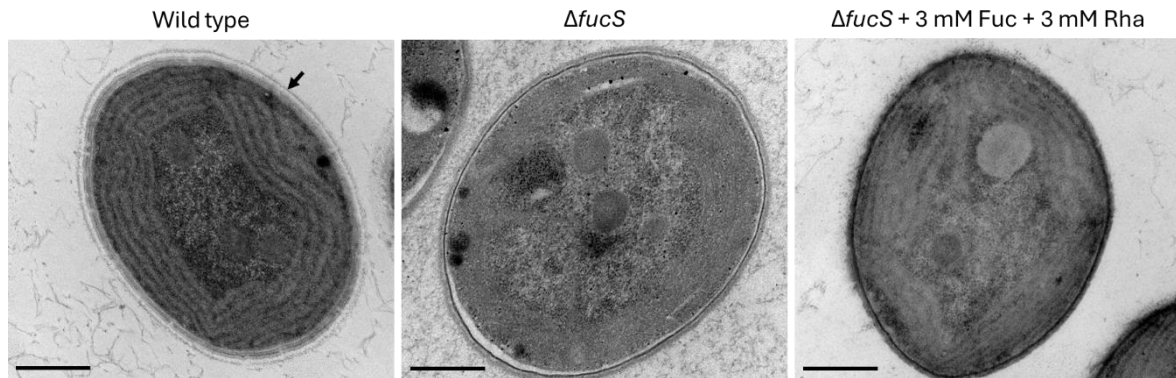


Figure 2. Ultrastructure of *Synechocystis* sp. PCC 6803 wild type, and $\Delta fucS$ (*slr1213*) strain Grown in BG11 or BG11 supplemented with fucose and rhamnose ($\Delta fucS$ + 3 mM Fuc + 3 mM Rha). S-layer is highlighted (arrow). Scale bars: 400 nm.

3. Preliminary characterization of *Synechocystis* $\Delta rfbC2$ (*slr1933*) strain

3.1. Introduction

As explored in the previous chapter, it is expected that *Synechocystis* produces deoxythymidine diphosphate-L-rhamnose (dTDP-Rha) (Kanehisa et al., 2023; Mills et al., 2020; Moore et al., 2024). Despite being extensively characterized in several bacteria (Giraud & Naismith, 2000; Mäki & Renkonen, 2004), most of what is known regarding this pathway in *Synechocystis* is inferred based on the similarities between the sequences of identified genes (Mills et al., 2020). The biosynthetic pathway of dTDP-Rha consists of a reaction catalyzed by four enzymes (RfbA, RfbB, RfbC and RfbD) that act in sequence to convert the precursor glucose-1-phosphate into dTDP-Rha (Giraud & Naismith, 2000; Mäki & Renkonen, 2004). In *Synechocystis*, genes encoding for each of these proteins have been identified: RfbA is likely encoded by *slr0207*, RfbB by *slr0836*, RfbC by *slr0985* and/or *slr1933* and RfbD by *slr1395* (Kanehisa et al., 2023; Mills et al., 2020; Moore et al., 2024). However, we demonstrated that *slr0985* (*rfbC1*) and *slr1933* (*rfbC2*) are not essential for the production of rhamnose since the single $\Delta rfbC1$ knockout strain as well as the double knockout strain ($\Delta rfbC1\Delta rfbC2$) were still capable of synthesizing rhamnose (Chapter II; Pissarra et al., 2025). This result raised questions regarding the true biosynthetic pathway for rhamnose in *Synechocystis*, and the actual role of the previously assumed RfbC

enzymes. Here, the other generated single knockout strain lacking *slr1933* ($\Delta rfbC2$) was characterized.

3.2. Materials and methods

3.2.1. Bacterial strains and standard growth conditions

The cyanobacterium *Synechocystis* sp. PCC 6803 wild type (sub-strain GT-Kazusa; glucose tolerant, with S-layer and non-motile) and strain were cultivated in BG11 medium (Stanier et al., 1971) in the standard conditions stated before (Chapter II; Pissarra et al., 2025). For the selection and maintenance of strains, BG11 medium was supplemented with kanamycin (Km; Merck Millipore) up to 2000 $\mu\text{g mL}^{-1}$. The *Escherichia coli* TOP10 (Thermo Fisher Scientific) was cultivated at 37°C in lysogeny broth medium (Bertani, 1951) supplemented with ampicillin (100 $\mu\text{g mL}^{-1}$; Amp; Merck Millipore), Km (50 $\mu\text{g mL}^{-1}$).

3.2.2. Cyanobacterial DNA extraction and recovery

Cyanobacterial genomic DNA was extracted by the phenol-chloroform method described previously (Tamagnini et al., 1997). Agarose gel electrophoresis was performed according to standard protocols (Sambrook & Russel, 2001), and the DNA fragments were isolated from gels using the Monarch DNA Gel Extraction Kit (New England Biolabs) and from enzymatic assay or PCR mixtures using the Monarch PCR & DNA Cleanup Kit (New England Biolabs).

3.2.3. Plasmid construction for *Synechocystis* transformation

The *Synechocystis* chromosomal regions flanking *rfbC2* (*slr1933*) were amplified by PCR using specific oligonucleotide primers (Table 2). An overlapping region containing an *XmaI* restriction site was included in primers *slr1933.5R* and *slr1933.3F* for cloning purposes. For each gene, the purified PCR fragments were fused by “overlap PCR.” The resulting product was purified and cloned into the vector pGEM-T Easy (Promega), creating plasmid pGDslr1933. A selection cassette containing the *nptII* gene (resistance to neomycin and kanamycin) was excised from plasmid pKm.1 (Pinto et al., 2015) with the restriction enzyme *XmaI* (Thermo Fisher Scientific). Subsequently, the selection cassette was cloned into the *XmaI* restriction site of the plasmid to form pGDslr1933.Km.

Table 2. List of primers used for strain generation and confirmation.

Primer Name	Code	Primer Sequence 5' → 3'	Amplicon (bp)	Source
slr1933.3F ^{a,c}	1F	TTTAATCTCCCGGGGTTTCGCCTCTTT CCTGAATTACCCAAAAGTTTGC	574	Pissarra et al., 2025
slr1933.3R ^{a,c}	1R	CACTCATTAGGCACCCCAGGCCAC AATTTTGC GTTGGTTCTCTGG		
slr1933.5F ^a	2F	TCGCCATTTCAGGCTGCGCAACCCAT GGCTGGAAATAACCATAGACCGTTTCG	680	
slr1933.5R ^a	2R	GGCGAACCCCGGGAGATTAAATTCC TCTTTAGGGTTACGACACC		
slr1933.FwdO ^b	3F	CTTTGAGCCGAACCTTTCTC	1836 (WT) 2659 (MT)	
slr1933.RevO ^b	3R	CATACCGTTGAACAATGGAC		
slr1933.FwdI ^b	4F	CTTTGAGAGCTACAACGACAAG	347	
slr1933.RevI ^b	4R	CATAGGACGGTGCATAGTAATC		

^aPrimers used for plasmids construction.^bPrimers used for the confirmation of the knockout strain's segregation by PCR.^cPrimers used for Southern blot probe.

3.2.4. Generation of $\Delta rfbC2$ and confirmation by PCR and Southern blot

The knockout strain lacking *slr1933* ($\Delta rfbC2$) was generated as detailed in Chapter II (Pissarra et al., 2025) using *Synechocystis* culture incubated with pGDslr1933.Km plasmid DNA. Resulting colonies were grown in increasing kanamycin concentration for complete segregation. Segregation was confirmed by PCR and Southern blot as detailed in Chapter II (Pissarra et al., 2025) using the same primers and probe used for the confirmation of the deletion of *rfbC2* in the knockout strain $\Delta rfbC1\Delta rfbC2$ (Table 1).

3.2.5. Transmission electron microscopy

Transmission electron microscopy was performed as detailed in subsection 2.2.2 of Chapter III.

3.2.6. Growth assessment and determination of carbohydrate contents

Synechocystis cultures were grown and monitored for 21 days with growth (OD_{730} and chlorophyll *a* content) and total carbohydrates, released polysaccharides (RPS), and capsular polysaccharides (CPS) measured every 7 days as previously stated (Chapter II; Pissarra et al., 2025).

3.3. Results and discussion

3.3.1. Generation and segregation of $\Delta rfbC2$

Aiming at better understanding the impact of the absence of Slr1933 (RfbC2) in the model cyanobacterium *Synechocystis* sp. PCC 6803, a single knockout strain lacking *slr1933* ($\Delta rfbC2$) was generated, and segregation of clone #1 was confirmed by PCR and Southern blot (Figure 3).

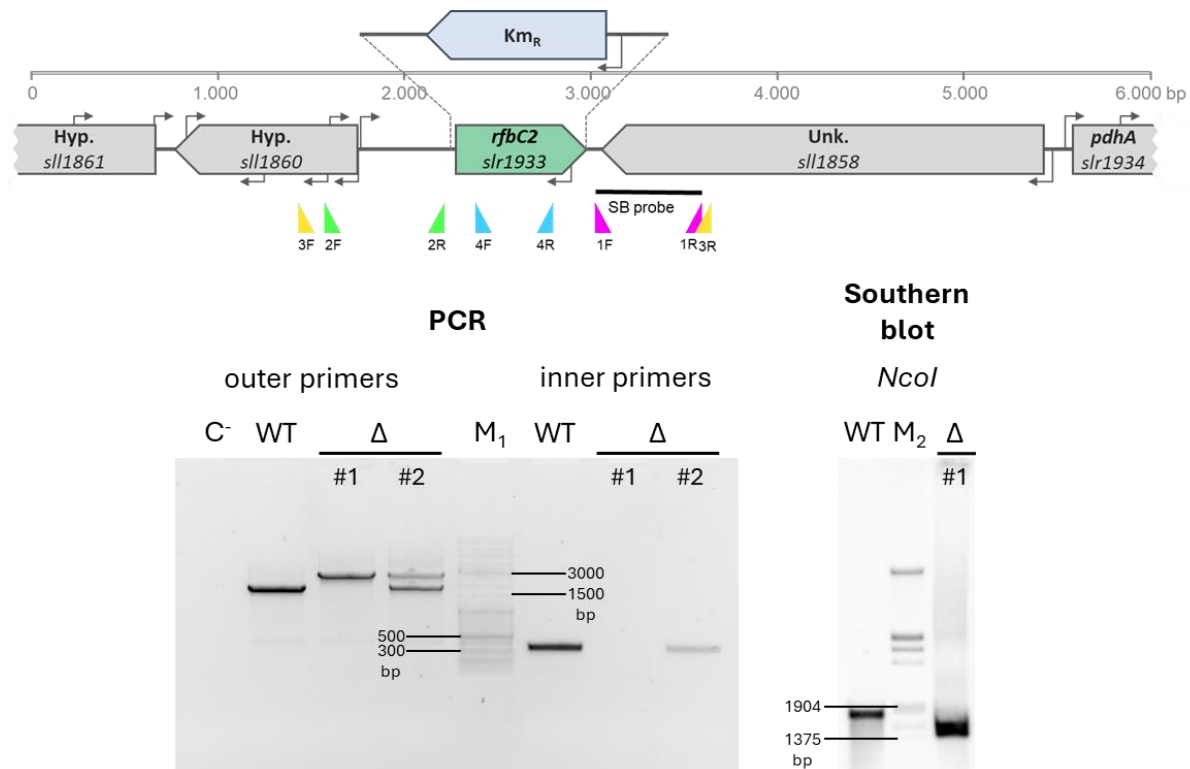


Figure 3. RfbC2 encoding gene in *Synechocystis* sp. PCC 6803, and confirmation of the segregation of the $\Delta rfbC2$ strain. Schematic representation of the genomic context, position of the primers used in the PCR reactions, and regions replaced by the selection cassette (conferring resistance to kanamycin). Agarose gel electrophoresis showing the PCR amplicons obtained with a primer pair targeting *rfbC2* (inner primers: 4F/4R, expected size 347 bp for the wild type) and the gene region (outer primers: 3F/3R, expected sizes 1836 bp for the wild type and 2659 bp for the knockout strain). Southern blot hybridization with genomic DNA from *Synechocystis* wild type and $\Delta rfbC2$ clones digested with *Nco*I and hybridized with a probe covering the 3' flanking region of *rfbC2* gene (obtained by PCR with the primer pair 1F/1R). Expected sizes of restriction fragments are 1819 bp for wild type and 1456 bp for the knockout strain clones, depending on the orientation of the resistance cassette. WT - wild type, Δ - knockout strain $\Delta rfbC2$, # clone number, C⁻ - negative control, M₁ - GeneRuler DNA Ladder mix (Thermo Scientific), M₂ – DNA Weight Marker III DIG-Labeled (Roche).

3.3.2. S-layer, growth and carbohydrate production of $\Delta rfbC2$

Considering the noticeable alterations of the S-layers previously observed for the $\Delta slr0985$ ($\Delta rfbC1$) and $\Delta slr0985\Delta 1933$ ($\Delta rfbC1\Delta rfbC2$) strains, which were slightly less electron dense and more uneven than the wild type (Chapter II; Pissarra et al., 2025), TEM was also used here to understand how the deletion of *slr1933* (*rfbC2*) alone affects *Synechocystis*' cellular surface. It is possible to observe that the $\Delta rfbC2$ strain produces an S-layer (Figure 4) quite similar to the one observed in the wild type, without the alterations seen in $\Delta rfbC1$ and $\Delta rfbC1\Delta rfbC2$. Additionally, while not quantified, this knockout strain seems to produce more storage granules (Figure 4).

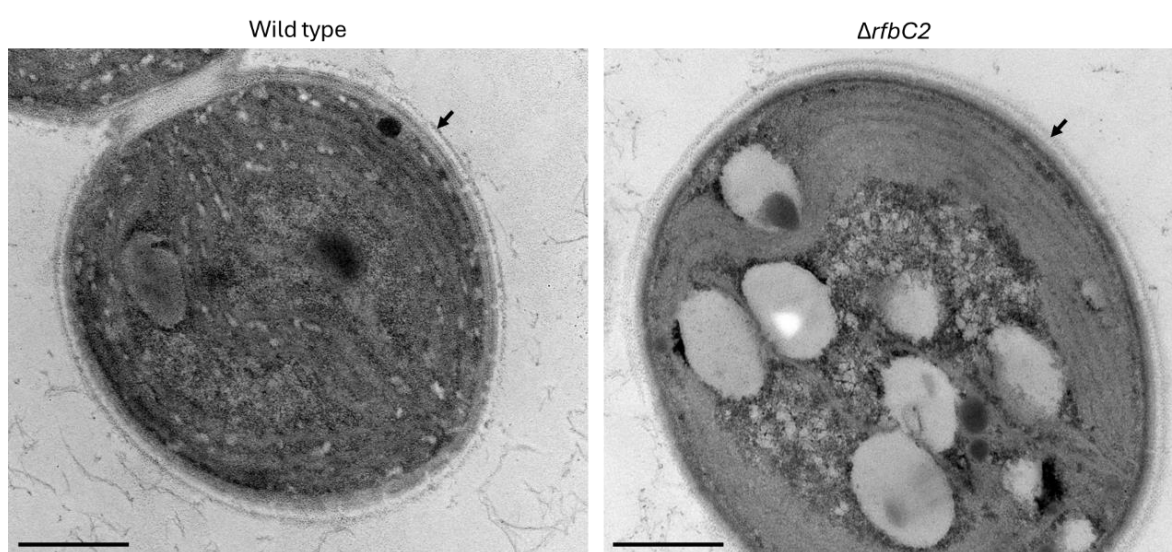


Figure 4. Ultrastructure of *Synechocystis* sp. PCC 6803 wild type and $\Delta rfbC2$ (*slr1933*) strain with the S-layer highlighted (arrow). Scale bars: 400 nm.

Preliminary results show that the deletion of *rfbC2* did not affect the growth (OD_{730} and chlorophyll *a*, Figure 5A), as previously observed for the deletion of *rfbC1*, and in contrast with the growth impairment observed for the double mutant strain, $\Delta rfbC1\Delta rfbC2$ (Chapter II; Pissarra et al., 2025). As such, it seems that the deletion of both *rfbC1* and *rfbC2* has a synergistic impact on growth.

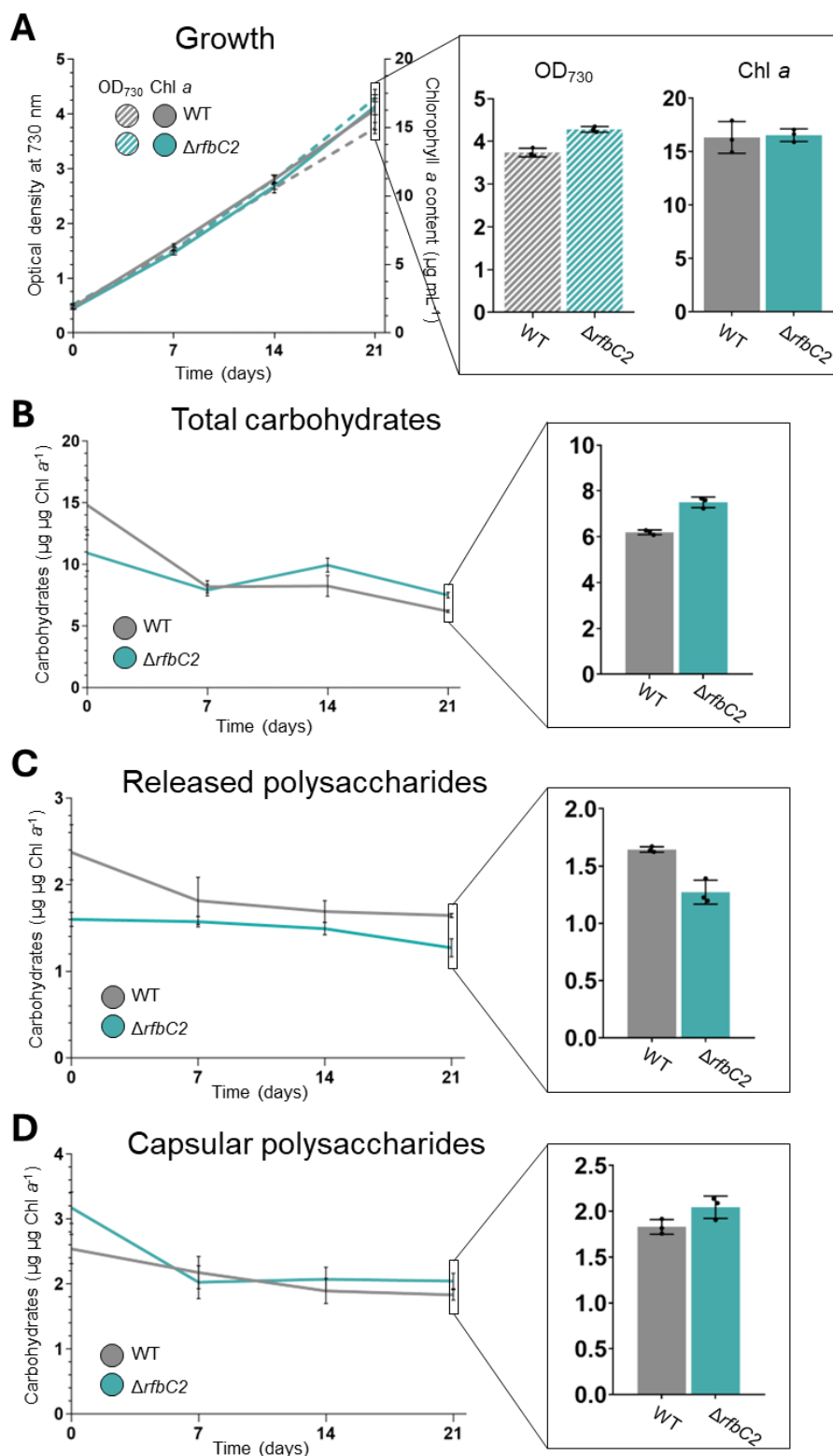


Figure 5. Growth, total carbohydrates, released polysaccharides, and capsular polysaccharides of *Synechocystis* sp. PCC 6803 wild type (WT) and ΔrfbC2 strain. **(A)** Growth (optical density at 730 nm) and micrograms of chlorophyll a per milliliter of culture and production of **(B)** total carbohydrates, **(C)** released polysaccharides, and **(D)** capsular polysaccharides expressed as micrograms of carbohydrates per microgram of chlorophyll a. Data represent means $\pm$ SD ($n = 3$), and individual measurements are shown.

Concerning carbohydrates quantification, preliminary results show that $\Delta rfbC2$ has more total carbohydrates at 21 days compared to the wild type (Figure 5B), similar to what was observed previously for the double knockout $\Delta rfbC1\Delta rfbC2$ (Chapter II; Pissarra et al., 2025). This suggests that the absence of *rfbC2* can lead to the accumulation of carbohydrates both in $\Delta rfbC2$ and $\Delta rfbC1\Delta rfbC2$, something that was not observed for $\Delta rfbC1$. Additionally, $\Delta rfbC2$ seems to produce less released polysaccharides (RPS) compared to the wild type at 21 days (Figure 5C), consistent with what was observed for both $\Delta rfbC1$ and $\Delta rfbC1\Delta rfbC2$ (Chapter II; Pissarra et al., 2025). However, no differences were observed regarding capsular polysaccharides (CPS) (Figure 5D), contrary to what was observed for $\Delta rfbC1$ and $\Delta rfbC1\Delta rfbC2$ (Chapter II; Pissarra et al., 2025), implying that *rfbC2* does not play a key role in CPS formation. Moreover, since this strain produces more total carbohydrates but less RPS, this likely results in an internal accumulation which agrees with the perceived increase of storage granules observed in TEM micrographs (Figure 4).

Chapter IV

Final remarks

Final remarks

This work lays an important foundation to fully understand the deoxyhexoses' biosynthetic pathways in *Synechocystis* sp. PCC 6803 by establishing the role of putative key enzymes on the composition and amount of extracellular polymeric substances (EPS). Furthermore, it sheds some light regarding how these enzymes may affect features that could be useful for industrial/commercial applications such as sedimentation and production of polymers. Overall, this study also serves to highlight how little is known regarding nucleotide sugar and EPS biosynthesis in *Synechocystis* and cyanobacteria in general.

1. Uncovering the nucleotide deoxyhexoses' pathways

Despite *Synechocystis* being the most studied cyanobacterium, most of what is known regarding pathways of nucleotide sugars in this organism is inferred from pathways studied in other bacteria (Han et al., 2017; Mills et al., 2020; Santos-Merino et al., 2019). Many of these pathways have been extensively studied in other organisms (such as humans, plants, fungi, and bacteria) and several have been shown to be reasonably conserved (Bisso et al., 1999; Figueroa et al., 2021; Ren et al., 2010; van der Beek et al., 2019; Yang et al., 2021). However, identification of putative key enzymes often relies on weak identity scores (Mills et al., 2020) since most of these sugar-modifying enzymes are highly strain specific (Pereira et al., 2013). This not only makes it difficult to identify all the proteins but also results in incorrect and misleading annotations. Nevertheless, *Synechocystis* is expected to synthesize guanosine diphosphate-L-fucose (GDP-Fuc) and deoxythymidine diphosphate-L-rhamnose (dTDP-Rha), since several genes putatively encoding key enzymes of each pathway have been identified (Kanehisa et al., 2023; Mills et al., 2020; Moore et al., 2024).

Here, it was demonstrated that *Synechocystis* deficient in *sll1213* ($\Delta fucS$) lacks fucose altogether (Chapter II; Pissarra et al., 2025), and not only in the EPS as previously demonstrated by Fisher et al. (2013), strengthening the idea that this gene encodes the GDP-L-fucose synthase (FucS/WcaG) essential for the *de novo* biosynthetic pathway of GDP-Fuc. Additionally, some organisms can incorporate exogenous fucose into the nucleotide sugar metabolism through the use of an alternative *salvage* pathway (Liu et al., 2011; Mäki & Renkonen, 2004; Niittymäki et al., 2004), with very few cases described in bacteria such as *Bacteroides fragilis* that is capable of incorporating fucose through the use of a multifunctional L-fucokinase/GDP-fucose pyrophosphorylase (Lin et al., 2025). However, the results previously shown in Chapter III support the idea that *Synechocystis* does not have this *salvage* pathway for GDP-Fuc. Most surprisingly, it has also been shown that the $\Delta fucS$ strain is also incapable of synthesizing rhamnose (Chapter II; Pissarra et al.,

2025), suggesting that the biosynthesis of rhamnose in *Synechocystis* is dependent of FucS or has fucose as a precursor. This result is very intriguing since the pathways for GDP-Fuc and dTDP-Rha should be completely independent and there is no known rhamnose pathway that uses fucose as a precursor. If anything, an increase in rhamnose could be expected since some strains, such as the cyanobacterium *Nostoc* sp. PCC 7120, have the capacity to substitute fucosylated metabolites/products for rhamnosylated ones in the absence of fucose (Mochimaru et al., 2008). Furthermore, deletion of both putative *rfbC* genes (*slr0985* and *slr1933*) – presumed to encode for dTDP-4-dehydrorhamnose 3,5-epimerase (RfbC), essential for the biosynthesis of dTDP-Rha – does not impair biosynthesis of rhamnose in *Synechocystis* (Chapter II; Pissarra et al., 2025). This result suggests that either there are other RfbC encoding genes yet to be identified, or that *Synechocystis* produces rhamnose resorting to an alternative pathway such as the GDP-D-rhamnose (GDP-Rha) one. While there are no other *Synechocystis* genes with similar sequences to *rfbC1* and *rfbC2*, it is common for these types of genes to have low identity scores, which makes their identification more difficult. Additionally, there are several other apparently redundant genes presumed to encode nucleotide sugar epimerases (such as *slr1078*, *slr1067* and *slr0244*, all assumed to encode epimerases of the UDP-D-galactose or UDP-N-acetyl-D-glucosamine pathways) that could hypothetically encode for the epimerase of the dTDP-Rha pathway (RfbC). Additionally, independent deletions of *slr0985* ($\Delta rfbC1$) and *slr1933* ($\Delta rfbC2$) result in distinct characteristics with $\Delta rfbC1$ displaying an altered S-layer, decrease in released polysaccharides (RPS) and increase in capsular polysaccharides (CPS) (Chapter II; Pissarra et al., 2025), while $\Delta rfbC2$ displays an accumulation of total carbohydrates despite a reduction in RPS (Chapter III) (Figure 1). These results suggest that each gene encodes for enzymes belonging to different EPS-related biological processes. For instance, *rfbC1*, due to its location [in the same transcriptional unit as *slr0983* (*rfbF*) and *slr0984* (*rfbG*), in the *slr0977* cluster], could encode for a CDP-4-dehydro-6-deoxyglucose reductase (AscC/RfbH), transforming the end product of RfbG (CDP-4-dehydro-6-deoxy-D-glucose) into CDP-4-dehydro-3,6-dideoxy-D-glucose.

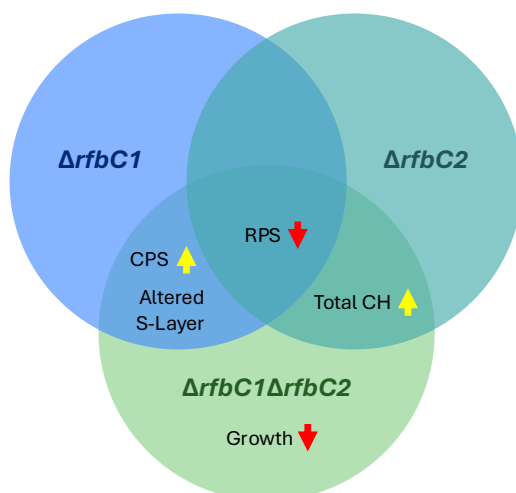


Figure 1. Venn diagram illustrating which characteristics of $\Delta rfbC1$ and $\Delta rfbC2$ strains could contribute to the observed traits of $\Delta rfbC1\Delta rfbC2$.

The results presented, however, raise more questions than answers regarding the pathway for biosynthesis of rhamnose in *Synechocystis* and how it is related to the fucose pathway. It has been explored before in Chapter II (Pissarra et al., 2025) that the deletion of *fucS* could lead to an accumulation of the precursor sugar (GDP-4-keto-6-deoxy-D-mannose). In one hand, if *Synechocystis* produces dTDP-Rha, this accumulation could lead to a feedback inhibition of the glucose-1-phosphate thymidyltransferase (RfbA). However, this is rather unlikely since GDP-sugars (GDP-Rha) have been shown to not inhibit RfbA in other organisms (Zheng et al., 2023). On the other hand, production of GDP-Rha also seems unlikely since the accumulation of the common precursor (GDP-4-keto-6-deoxy-D-mannose) would likely result in an outflow towards the GDP-Rha pathway, triggering an increase of rhamnose. With this in mind, at least two other hypotheses can be raised: (i) *Synechocystis* may have an unidentified enzyme that could catalyze the conversion of GDP-Fuc into GDP-Rha instead of using the precursor GDP-4-keto-6-deoxy-D-mannose. If that is the case, a good candidate would be Slr0583, since it has been previously described as being similar to FucS (in *Synechocystis*) and to GDP-6-deoxy-D-mannose reductase (Rmd; essential for GDP-Rha biosynthesis in *Xanthomonas campestris*) (Mohamed et al., 2005). (ii) Alternatively, an arguably more far-fetched hypothesis would be for Slr1213 (FucS) to act as both a FucS and dTDP-glucose 4,6-dehydratase (RfbB), since Slr0836 (putative RfbB of the dTDP-Rha pathway) is the enzyme most similar to FucS in *Synechocystis*. However, FucS acts as both an epimerase and reductase in the GDP-Fuc pathway, while RfbB is a dehydratase that acts before the epimerization step of the dTDP-Rha pathway. For Slr1213 to have such a multitude of catalytic functions in two distinct nucleotide sugar pathways would be exceptionally unique and therefore unlikely. Nevertheless, all hypotheses (Figure 2) should be properly investigated.

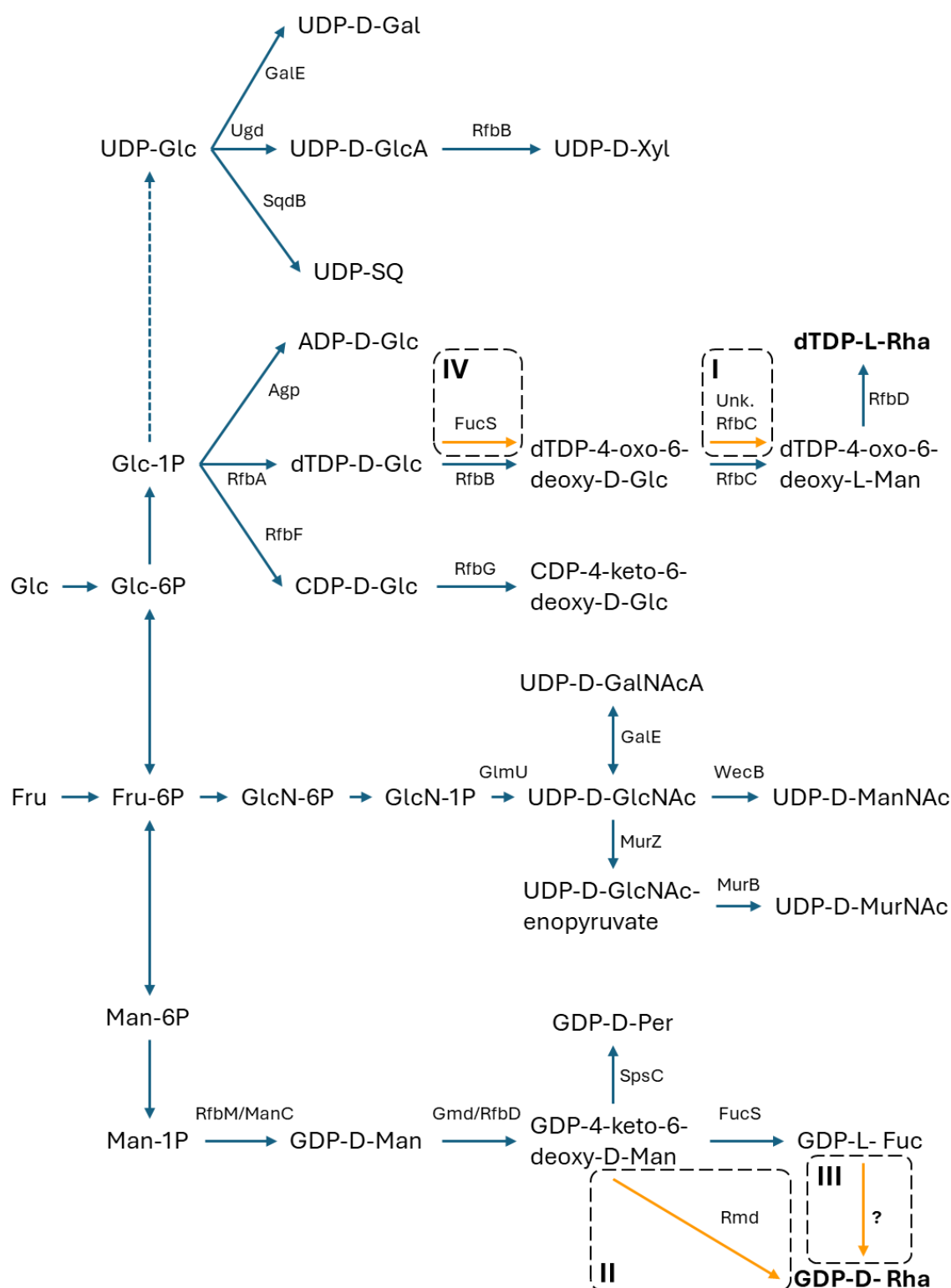


Figure 2. Expected and hypothesized nucleotide sugar pathways in *Synechocystis* sp. PCC 6803. Hypotheses raised here for alternative pathways for the synthesis of rhamnose are highlighted (dashed boxes, orange arrows): (I) existence of an unknown RfbC protein in the dTDP-L-Rha pathway; (II) biosynthesis of GDP-D-Rha from GDP-4-keto-6-deoxy-D-Man catalyzed by an Rmd protein; (III) biosynthesis of GDP-D-Rha from GDP-L-Fuc catalyzed by an unknown protein; and (IV) SII1213 may act as both FucS and RfbB on the GDP-L-Fuc and dTDP-L-Rha pathways.

2. Attributes beneficial for commercial applications

In several organisms, fucose and rhamnose have been shown to be important for the integrity of the outer membrane and capsule, and have been associated with several bioactive properties (Bischer et al., 2020; Carvalho et al., 2018; Mäki & Renkonen, 2004; Mohamed et al., 2005; Reiter, 2002). In particular, bacterium-, plant-, and synthetically-derived rhamnosides have been linked to antitumoral, antibacterial and even anti-inflammatory activities (Chen et al., 2018; Lee et al., 2014; Mei et al., 2019; Sylla et al., 2019), and bacterial EPS rich in fucose or rhamnose have been found to have antioxidant, antibacterial and biofilm-inhibiting properties (Baptista et al., 2023; Ramachandran et al., 2020; Xu et al., 2020). These deoxyhexoses confer hydrophobic properties that can contribute to cell adhesion and polymers emulsifying properties (Chamizo et al., 2019, 2020b; Pereira et al., 2019; Rossi & De Philippis, 2015). As such, it is essential to study the associated pathways in order to optimize production and enhance the desired properties for the envisioned industrial/commercial applications.

As previously shown here (Chapter II; Pissarra et al., 2025) and by others, deletion of *fucS* ($\Delta fucS$) resulted in cell aggregation, fast sedimentation and absent S-layer (Fisher et al., 2013; Mohamed et al., 2005; Trautner & Vermaas, 2013). Additionally, it had also been shown that the O-antigen portions of the lipopolysaccharides (LPS) of $\Delta fucS$ are truncated (Fisher et al., 2013; Matinha-Cardoso et al., 2022). Considering that this strain is unable to produce fucose or rhamnose (Chapter II; Pissarra et al., 2025), the lack of deoxyhexose moieties likely explains the truncated LPS, which in turn justifies the absence of the S-layer since this structure is typically anchored to the O-antigen region of the LPS in Gram-negative bacteria (Bharat et al., 2021). Moreover, $\Delta rfbC1$ and $\Delta rfbC1\Delta rfbC2$ have also shown fast sedimentation, and an S-layer that was less electron dense and with an uneven surface (Chapter II; Pissarra et al., 2025). While alterations to the S-layer are not necessarily useful for commercial applications, they are probably linked to the observed cell aggregation and/or fast sedimentation since higher sedimentation rates have been associated with alterations to the cellular surface, usually due to changes regarding hydrophobicity (Zedler et al., 2023). Such characteristics are extremely desirable in industrial settings since they allow for an efficient and cost-effective separation of biomass from the culture medium.

It has also been previously discussed that $\Delta fucS$ produces EPS lacking fucose and rhamnose and that the deletion of *rfbC1* and *rfbC2* did not impair the biosynthesis of rhamnose (Chapter II; Pissarra et al., 2025). While this was unexpected, RfbC1 and RfbC2 are still expected to be involved in the nucleotide sugar metabolism since $\Delta rfbC1$, $\Delta rfbC2$ and $\Delta rfbC1\Delta rfbC2$ have been demonstrated to affect total carbohydrates and production of

EPS (Chapter II; Pissarra et al., 2025; Chapter III). With this in mind, and while not all knockout strains had their polymers characterized, it is reasonable to consider that the produced polymers may have distinct features that can potentially be commercially exploited. Notably, the lack of deoxyhexoses in the polymer of $\Delta fucS$ can likely contribute to a reduction in hydrophobicity, and therefore increased hydrophilicity, that could result in a polymer with more affinity to metal ions, nutrients and water (Chamizo et al., 2019, 2020b; Kauser et al., 2024; Okajima et al., 2008; Pereira et al., 2009). This in turn could be particularly useful for wastewater and soils remediation/amelioration. Furthermore, when considering the second method of polymer isolation described in Chapter III, $\Delta rfbC1$ produced a polymer much darker in color compared to the polymers produced by other strains and isolated by different methods. This is suggestive of a high concentration of carotenoids that, if confirmed, could give anti-inflammatory and antioxidant properties to the polymer, as previously discussed (Chapter III). Moreover, $\Delta rfbC2$ produces more total carbohydrates (despite producing a normal amount of CPS and less RPS) and seems to have more carbon storage granules (Chapter III). While not confirmed, these types of carbohydrate granules are commonly the accumulation of glycogen or polyhydroxybutyrate (PHB) (Gao et al., 2023; Koch & Forchhammer, 2021; Luan et al., 2019), the latter being highly relevant in industrial settings, in particular for the production of biodegradable bioplastics (Markl et al., 2018; Meixner et al., 2018).

3. Future perspectives

Though this work provided an important new perspective on the putative pathways of nucleotide deoxyhexoses and how they may affect EPS biosynthesis, it also opened several new possibilities that should be properly addressed. Additionally, obtained polymers should be further characterized. For those reasons, several research routes are proposed:

(i) Experimentally validate the remaining putative enzymes of the dTDP-Rha pathway (RfbA, RfbB and RfbD) by generating knockout and knockdown (using CRISPRi) strains of the respective best gene candidates: *slr0207*, *slr0836*, and *slr1395*.

(ii) Elucidate the role of putative Gmd (Slr1072), whose encoding gene (*slr1072*) was experimentally shown to be upregulated in $\Delta fucS$, $\Delta rfbC1$ and $\Delta rfbC1\Delta rfbC2$.

(iii) Investigate the existence of a GDP-Rha pathway by targeting the best Rmd candidate: Slr0583.

(iv) Elucidate the role of other putative nucleotide sugar epimerases, not exclusively but including putative GalE encoded by *slr1078*, *slr1067* and/or *slr0244*.

(v) Quantify the carotenoid content of the polymers and test for any associated antioxidant and/or anti-inflammatory bioactivities.

(vi) Determine the hydrophobicity of polymers with and without deoxyhexoses and produce polymers with various degrees of hydrophobicity by upregulating and downregulating *fucS*.

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