1 **Impairment of a cyanobacterial glycosyltransferase that modifies a pilin results** 2 **in biofilm development**

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11 **Running title:** Biofilm inhibition requires PilA glycosylation

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15 **Originality-Significance Statement**

16 The molecular mechanisms that underlie biofilm development in cyanobacteria are just
17 emerging. Using the cyanobacterium *S. elongatus* as a model, we demonstrate that
18 glycosylation of the pilus subunit is crucial for the biofilm self-suppression mechanism,
19 however, it is dispensable for DNA competence.

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21

22 **Summary**

23 A biofilm inhibiting mechanism operates in the cyanobacterium *Synechococcus*
 24 *elongatus*. Here, we demonstrate that the glycosyltransferase homolog, Ogt,
 25 participates in the inhibitory process – inactivation of *ogt* results in robust biofilm
 26 formation. Furthermore, a mutational approach shows requirement of the
 27 glycosyltransferase activity for biofilm inhibition. This enzyme is necessary for
 28 glycosylation of the pilus subunit and for adequate pilus formation. In contrast to wild-
 29 type culture in which most cells exhibit several pili, only 25% of the mutant cells are
 30 piliated, half of which possess a single pilus. In spite of this poor piliation, natural DNA
 31 competence was similar to that of wild-type, therefore, we propose that the
 32 unglycosylated pili facilitate DNA transformation. Additionally, conditioned medium
 33 from wild-type culture, which contains a biofilm inhibiting substance(s), only partially
 34 blocks biofilm development by the *ogt*-mutant. Thus, we suggest that inactivation of
 35 *ogt* affects multiple processes including production or secretion of the inhibitor as well
 36 as the ability to sense or respond to it.

37

38 Introduction

39 Post-translational modification is a pivotal mechanism that regulates protein function
 40 in all cell types, including the surface appendages of bacterial cells (Proft and Baker,
 41 2009; Nothaft and Szymanski, 2010; Giltner et al., 2012). Surface modifications are
 42 likely to be important for cyanobacteria, which are photosynthetic prokaryotes that are
 43 highly prevalent in the environment, have an important role in global ecology (Garcia-
 44 Pichel et al., 2003; Falkowski et al., 2008; Braakman, 2019), and are found in microbial
 45 assemblages known as biofilms (Gorbushina, 2007; Stal et al., 2010; Mieszkin et al.,
 46 2013; Salta et al., 2013; Romeu et al., 2019; Kuhl et al., 2020). Among post-
 47 translational modifications, glycosylation is known to affect protein folding, localization
 48 and trafficking, protein solubility, antigenicity, biological activity and protein half-life
 49 (Blaser et al., 1986; Hounsell et al., 1996; Marceau and Nassif, 1999; Shental-Bechor
 50 and Levy, 2008; Vagin et al., 2009; Cummings, 2019).

51 Subunits of cell appendages required for motility are known to be glycosylated in
 52 diverse bacterial cells (Marceau and Nassif, 1999; Arora et al., 2005; Logan, 2006;
 53 Tytgat and Lebeer, 2014). For example, the flagellins FlaA and FlaB that comprise the
 54 flagellum of *Campylobacter jejuni* are heavily N-link glycosylated (Logan et al., 2002;
 55 Schirm et al., 2005). Glycosylation of *Campylobacter* flagellin is essential for flagellar
 56 assembly and consequent motility. Mutants defective in biosynthetic genes for the
 57 sugar moiety pseudominic acid in several strains of *Campylobacter* are non-motile and
 58 accumulate intracellular flagellin of reduced molecular mass due to lack of
 59 glycosylation (Hitchen et al., 2010). Another common glycosylation is addition of O-
 60 linked sugar onto pilus subunits; e.g., type IV pilins are O-glycosylated in some strains
 61 of *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae* and *Neisseria meningitidis*,
 62 (DiGiandomenico et al., 2002; Power et al., 2003; Aas et al., 2007).

63 Studies of the molecular mechanisms underlying cyanobacterial biofilm development
 64 are currently emerging (Enomoto et al., 2014; Enomoto et al., 2015; Agostoni et al.,

2016; Lacey and Binder, 2016; Enomoto et al., 2018). Our previous studies revealed a mechanism that represses biofilm formation in *Synechococcus elongatus* PCC 7942 (hereafter *S. elongatus*) (Schatz et al., 2013; Parnasa et al., 2016; Nagar et al., 2017; Sendersky et al., 2017; Parnasa et al., 2019). Inactivation of the gene Synpcc7942_2071 [recently designated as *pilB* (Yegorov et al., 2021)], which encodes an ATPase homologue of type II protein secretion (T2S) and/or type IV pilus assembly systems (T4P), impairs the inhibitory process, resulting in a mutant (PilB::Tn5) that develops robust biofilms (Schatz et al., 2013; Parnasa et al., 2016).

Factors that affect biofilm formation were recently shown to also affect multicellularity in *Synechocystis*. Motile strains of *Synechocystis* sp. PCC 6803 form floating aggregates termed flocs (Conradi et al., 2019) and impairment of PilB or the RNA chaperone homologue Hfq prohibited *Synechocystis* flocculation (Conradi et al., 2019). In contrast, inactivation of these components in *S. elongatus* elicited biofilm formation (Schatz et al., 2013; Yegorov et al., 2021), thus, these components impart different regulation of multicellularity in those cyanobacteria.

To identify additional components of the biofilm self-suppression mechanism of *S. elongatus*, a high throughput screen using a barcoded transposon library was employed (manuscript in preparation). This screen suggested involvement of several genes, including Synpcc7942_0051, in biofilm inhibition. Here, we demonstrate that the glycosyltransferase encoded by this gene is involved in glycosylation of the pilus subunit, PilA and discuss possible involvement of this enzyme in the biofilm inhibitory mechanism.

Results

Inactivation of Synpcc7942_0051 results in biofilm formation

A genetic screen using a barcoded transposon library suggested that inactivation of gene Synpcc7942_0051 causes biofilm development (manuscript in preparation).

Briefly, this *S. elongatus* library of mutants was grown, a biofilm was allowed to form, and DNA extracted from the biofilm was analyzed by high throughput sequencing. Data analysis revealed mutants highly enriched in the biofilm, including a strain impaired in a gene encoding a glycosyltransferase homolog, O-linked β -N-acetylglucosamine (O-GlcNAc) transferase (Ogt). This enzyme is characterized by several tetratricopeptide (TPR) repeats at the N-terminus followed by two glycosyltransferase 41 domains [Fig. 1A, also see (Sokol and Olszewski, 2015)]. These domains are conserved in Ogt enzymes that catalyze attachment of O-GlcNAc to serine or threonine residues, a modification that is reversible due to an antagonistic activity by β -N-acetylglucosaminidases that remove O-GlcNAc (Golks and Guerini, 2008; Zeidan and Hart, 2010). However, the number of TPR repeats and the domain organization varies between enzymes [Fig. S1; (Lubas and Hanover, 2000; Iyer and Hart, 2003)].

Enrichment of a mutant in the sessile subpopulation of the library implies that the particular strain is capable of biofilm development; however, it is possible that the mutant merely intercalated into and proliferated within a biofilm formed by other mutants in the library. Therefore, we insertionally inactivated Synpcc7942_0051 and tested biofilm formation by the mutant, Ogt::Mu, when grown in pure culture. The Ogt::Mu strain forms robust biofilms under static conditions (Fig. 1 B-D). Substantial biofilm development in flasks was revealed by crystal violet staining in Ogt::Mu similarly to PilB::Tn5 (Fig. 1 B&C). This assay suggested only trace cell adhesion or sugar-matrix deposition in wild-type (WT) culture (Fig. 1 B&C). Additionally, biofilms were quantified by assessment of the percentage of chlorophyll in suspension; about 95% of the total chlorophyll in cultures of this mutant is found in biofilm-cells, compared with 100% of the chlorophyll in WT cultures in planktonic cells (Fig. 1D). Total chlorophyll accumulated in the culture was not significantly different between WT and Ogt::Mu (Fig. S2).

Ogt::Mu did not reproducibly develop biofilms under the continuous bubbling conditions in which PilB::Tn5 biofilms are typically assayed (Schatz et al., 2013; Parnasa et al., 2016; Nagar et al., 2017; Sendersky et al., 2017; Parnasa et al., 2019). However, Ogt::Mu biofilms formed in a flow-cell system with continuous fresh BG-11 medium (Fig. 1E). In this setting, WT cells are observed in the groove of the flow-cell device but are not attached to the substratum (Fig. 1E, lower panel). Further characterization of the mutant was performed under static conditions.

Previously, we reported that conditioned medium from WT culture (hereafter CM) prohibited biofilm development by PilB::Tn5 under continuous bubbling, indicating that this mutant is capable of sensing and responding to biofilm-inhibitor(s) produced and secreted by the WT strain (Schatz et al., 2013; Parnasa et al., 2016). Here we tested the effect of CM on PilB::Tn5 and Ogt::Mu biofilm development under static conditions. In the absence of constant bubbling that maintained the cells suspended, PilB::Tn5 cells, which are non-piliated (Nagar et al., 2017), precipitated by gravity to the very bottom of the tube when grown in CM (Fig. 2, upper left tube). This pellet, however, was clearly distinct from the biofilm formed on the tube walls under fresh growth medium (Fig. 2, compare two upper tubes). In contrast to PilB::Tn5, the Ogt-mutant formed biofilms in CM, although not as robustly as under fresh medium and some cell pelleting was also observed in CM (Fig. 2, lower tubes). This observation suggests that Ogt::Mu is indifferent, to some extent, to the biofilm inhibitor present in CM from a WT culture.

Lysine 445 essential for catalysis is required for biofilm self-suppression

Biofilm development by Ogt::Mu indicates that Ogt takes part in the biofilm self-suppression mechanism that operates in *S. elongatus*. Next, we tested whether the predicted glycosyltransferase activity of Ogt is required for this process. We mutated the conserved lysine 445 in the C-terminal glycosyltransferase domain (Fig. 1A), which was previously demonstrated to be essential for catalytic activity (Clarke et al., 2008;

Sokol and Olszewski, 2015). A gene encoding a lysine to alanine substitution (K445A) with its native regulatory region was introduced into the Ogt-mutant (Ogt::Mu/K445A, see Table S1). This strain formed biofilms similarly to Ogt::Mu, in contrast to the planktonic growth of strain Ogt::Mu/Ogt in which the native gene was inserted into the mutant (Fig. 3A). Because introduction *in trans* of *ogt* into Ogt::Mu restored planktonic growth, the biofilm-forming phenotype of Ogt::Mu is not due to effect of the antibiotic cassette inserted in *ogt* on downstream genes. Together, these data support requirement of Ogt glycosyltransferase activity for biofilm self-suppression.

Absence of restoration of planktonic growth in Ogt::Mu/K445A, could in principal result from lower stability or solubility of the mutated protein. Thus, we validated the presence of the mutant Ogt. A gene encoding C-terminal triple FLAG-tagged Ogt (hereafter Ogt::FLAG) was introduced with its native regulatory region into Ogt::Mu (Table S1). Western blot analysis using anti-FLAG antibodies indicated similar amount of tagged mutated and WT Ogt proteins, which were present mostly in the soluble cell fraction (Fig. 3B, Ogt::Mu/K445A::FLAG and Ogt::Mu/Ogt::FLAG, respectively). Moreover, the tagged-WT protein complemented the biofilm-forming phenotype of Ogt::Mu; in strain Ogt::Mu/Ogt::FLAG 100% of the chlorophyll is in planktonic cells (Fig. 3A). In contrast, strain Ogt::Mu/K445A::FLAG formed biofilms similarly to Ogt::Mu (Fig. 3A). Together, biofilm assays and Western analysis substantiate the requirement of the glycosyltransferase activity for the biofilm self-suppression process.

Is Ogt involved in PilA glycosylation?

Our previous studies revealed involvement of the T4P assembly complex in the biofilm self-suppression mechanism operating in *S. elongatus*. Additionally, subunits of the pilus undergo glycosylation in diverse bacteria (Faridmoayer et al., 2007; Giltner et al., 2012; Harding et al., 2015; Elhenawy et al., 2016; Goncalves et al., 2018), and physical adjacency of genes encoding the pilus subunit and their cognate glycosyltransferases were reported e.g. in *Pseudomonas syringae* pv. tabaci 6605 (Nguyen et al., 2012)

and in *Ralstonia solanacearum* (Elhenawy et al., 2016). Synteny analysis of cyanobacterial genomes revealed physical adjacency of *ogt* and pilin genes in (38%) of the genomes (see Fig. S3 for synteny in selected genomes). Given these facts, we tested whether Ogt is involved in PilA glycosylation in *S. elongatus*. Analysis of CM by gel-electrophoresis followed by silver staining indicated a major band of ~14 kDa in WT (Fig. 4A). This band, which is largely missing in PilB::Tn5 (Fig. 4A), corresponds to PilA (Nagar et al., 2017). In Ogt::Mu, however, a band of lower intensity and smaller MW compared to WT was detected (Fig. 4A). A similar shift as well as lack of glycosylation of the pilus subunit has been previously reported in diverse bacteria (Faridmoayer et al., 2007 ; Harding et al., 2015; Elhenawy et al., 2016) including cyanobacteria (Goncalves et al., 2018). The complemented strain Ogt::Mu/Ogt is characterized by a band similar in intensity and MW to that of WT. In contrast, a band akin to the one observed in Ogt::Mu was detected in Ogt::Mu/K445A (Fig. 4A). Taken together, these observations support involvement of Ogt in PilA glycosylation.

In vitro analysis has demonstrated that Ogt uses UDP-GlcNAc as a sugar donor (Sokol and Olszewski, 2015); therefore, we tested whether this glycosyltransferase is involved in addition of GlcNAc to PilA. Succinylated wheat Germ Agglutinin (succinyl-WGA), a lectin that detects GlcNAc, revealed a band of ~14 kDa in WT, supporting presence of GlcNAc residue(s) in PilA in this strain (Fig. 4B). This band is absent from Ogt::Mu; however, it was recovered in the *ogt* mutant bearing a native *ogt* gene (Fig 4B Ogt::Mu and Ogt::Mu/Ogt, respectively) but not with the active-site mutant allele (Fig. 4B, Ogt::Mu/K445A). In conclusion, the difference in MW (Fig. 4A) and GlcNAc detection (Fig. 4B) indicate involvement of Ogt in PilA glycosylation.

Inactivation of ogt impairs pilus formation

Several studies demonstrated that subunits of cell appendages required for motility including T4P are glycosylated, and given the requirement of Ogt for PilA glycosylation we examined cell piliation in Ogt::Mu. TEM analyses of negatively stained whole cells

revealed the presence of pili in WT [Fig. 5, (Nagar et al., 2017)]. The majority of the cells (96%) are pilated with most cells exhibiting several pili (see Table within Fig. 5). In contrast, only 25% of *Ogt::Mu* cells are pilated, half of which exhibit only a single pilus (Fig. 5). Introduction of *ogt* into the mutant largely restored pilus assembly (Fig. 5; 87% of the observed cells were pilated). Furthermore, in strain *Ogt::Mu/K445A*, which does not glycosylate PilA (Fig. 4), 21% of the cells were pilated, similar to *Ogt::Mu* (Fig. 5). Together, TEM analyses of the different strains support requirement of *Ogt* activity for proper pilus formation.

Type 4 pili are involved in DNA transformation (Ellison et al., 2018; Craig et al., 2019; Piepenbrink, 2019). As we previously reported (Nagar et al., 2017; Yegorov et al., 2021), non-piliated PilB::Tn5 completely lacks ability to take up external DNA (Fig. 6) in agreement with a recent study (Taton et al., 2020). Given the lower number of pilated cells and of pili per cell observed in *Ogt::Mu* compared to WT, we tested natural transformation competence of the mutant. Interestingly, despite the lower abundance of pili in *Ogt::Mu*, DNA competency of this strain is similar to WT (Fig. 6). Together, data suggest that the unglycosylated pili facilitate DNA transformation.

Interestingly, inactivation of *ogt* affected fitness similarly to inactivation of genes encoding components of T4P and the DNA competence machinery (Table S2). The fitness data were collected using a randomly barcoded transposon-library that was subject to a large variety of growth conditions (Wetmore et al., 2015; Price et al., 2018). The abundance of a particular strain at the end of a growth experiment compared to its beginning served for fitness calculation. Genes are defined to have strong cofitness if they have similar fitness patterns (cofitness > 0.75). Very high cofitness values (0.89-0.99) were obtained for *ogt* and mutants in various *pil* genes encoding subunits of T4P assembly complex. Additionally, newly identified components of the T4P machinery that are essential for biofilm suppression [EbsA and Hfq, (Yegorov et al., 2021)] show high cofitness with *Ogt* (0.96 and 0.95, respectively, Table S2). These cofitness data

support the involvement of Ogt in cellular processes related to the T4P assembly. Moreover, recently identified proteins required for natural transformation of *S. elongatus* [RntA and RntB, (Taton et al., 2020)] also show strong cofitness with Ogt (Table S2). Because inactivation of *ogt* did not affect DNA competence, an additional cellular function common with the Rnt proteins may be suggested.

Discussion

The glycosyltransferase Ogt is involved in biofilm self-suppression

The glycosyltransferase homologue Ogt is involved in the biofilm self-suppression mechanism operating in *S. elongatus* as manifested in biofilm development by the Ogt::Mu (Fig. 1). Sokol et al. (Sokol and Olszewski, 2015) reported that impairment of this glycosyltransferase results in cell settlement and formation of small cell aggregates, in contrast to development of robust biofilms reported here for the mutant. In both studies, cultures were analyzed under static conditions; however, other growth conditions (including temperature and light quality and intensity) varied between the studies. Additionally, we previously reported that freshly prepared iron stock is important for promotion of biofilm development, and we use it in our studies (Sendersky et al., 2017). Therefore, the conditions in the Sokol et al study may not have favored the biofilm development program beyond cell settling.

Previous studies indicated that impairment of pilus assembly is accompanied by low abundance of numerous proteins in the exo-proteome (Nagar et al., 2017; Yegorov et al., 2021). Furthermore, biofilm formation in non-piliated PilB::Tn5 was completely blocked by CM from a WT culture ((Schatz et al., 2013) and Fig. 2). Together, these observations support the hypothesis that the T4P complex in *S. elongatus* is engaged in protein secretion and is possibly involved in deposition of biofilm inhibitor(s) to the extracellular milieu. Distinct responses to CM, however, were observed for PilB::Tn5 and Ogt::Mu: Cell pelleting, apparently by gravity, was observed in PilB::Tn5 culture

indicating complete inhibition of biofilm development, in contrast to the partial inhibition of biofilm formation in Ogt::Mu cultures that was manifested by some cell pelleting along with formation of biofilms on the tube walls (Fig. 2). These data may be explained by a pleiotropic effect of inactivation of *ogt* if Ogt affects the T4P apparatus as well as additional process(es) related to biofilm inhibition: e.g., the ability to properly respond to biofilm inhibitor(s) present in WT-CM. Pleiotropic effects resulting from abrogation of *ogt* in *S. elongatus* were reported by Sokol *et al.*, (Sokol and Olszewski, 2015), including wider lumen of the photosynthetic membranes and high inorganic phosphate levels compared to WT. Our analysis revealed chlorophyll levels in Ogt::Mu similar to WT (Fig. S2), suggesting that biomass accumulation is not affected by impairment of the glycosyltransferase activity.

Ogt is required for PilA glycosylation and adequate pilus formation

Several observations support involvement of *S. elongatus* Ogt in PilA glycosylation: PilA of Ogt::Mu is of lower molecular weight compared to WT (Fig. 4A) in agreement with lack of glycosylation in the mutant. Moreover, GlcNAc detection validated involvement of Ogt for PilA glycosylation (Fig. 4B). PilA glycosylation is commonly found in heterotrophic bacteria (Nothaft and Szymanski, 2010; Giltner *et al.*, 2012). Recent studies in *Synechocystis* indicated that mutants defective in genes *sll0141*, *sll0180* and *slr0369*, encoding homologues of TolC-related secretion systems, are impaired in PilA glycosylation. The particular role, however, of these proteins in the process of PilA glycosylation is unknown (Goncalves *et al.*, 2018). An additional study of this cyanobacterium revealed that inactivation of *sll0899* altered PilA glycosylation and impaired motility (Kim *et al.*, 2009).

Impairment of PilA glycosylation (Fig. 4) abolished piliation in ~75% of the Ogt::Mu cells, whereas the rest were characterized by a single or few pili (Fig. 5). These observations show that despite the defective glycosylation, PilA secretion and assembly occurs albeit at low efficiency. Absence of motility of the lab strain of *S.*

elongatus on agar plates prohibits assessment of the consequence of *ogt* inactivation on function of the assembled pili in motility.

The impact of PilA glycosylation on pilus assembly varies between different cyanobacteria. For example, deletion of *slr0180* in *Synechocystis*, which results in lower MW PilA indicative of altered glycosylation, did not affect pilus formation as revealed by TEM (Goncalves et al., 2018). The deletion of *ogtA* in *Nostoc punctiforme* prevented accumulation of PilA, suggesting that PilA-glycosylation is required for its stability, and abolished cell motility. Additionally, transcription of *ogtA* of *N. punctiforme* is induced upon induction of development of motile filaments in this cyanobacterium, known as hormogonia. Together, these observations support requirement of OgtA for normal pilus function (Khayatan et al., 2015).

Ogt is dispensable for DNA competence

Type IV pili are commonly involved in DNA uptake in diverse bacteria (Ellison et al., 2018; Craig et al., 2019; Piepenbrink, 2019) including cyanobacteria (Bhaya et al., 2000; Yoshihara et al., 2001; Schuergers and Wilde, 2015; Chen et al., 2020; Taton et al., 2020). For example, inactivation of *pilB*, completely eliminated pili and rendered the cells non-competent for DNA uptake (Nagar et al., 2017). A recent genome-wide screen of the same barcoded transposon library that identified *ogt* revealed the components required for natural transformation competence in *S. elongatus*, including various homologs of T4P assembly system (Taton et al., 2020). According to this screen, gene *ogt* is not required for DNA uptake, in agreement with the similar DNA competence of Ogt::Mu and WT reported here (Fig. 6). Though not completely abrogating pilus formation, inactivation of *ogt* severely reduced the number of pilated cells (Fig. 5). Therefore, a full suite of pili is not necessary to support maximal transformation competency. It is also possible that, as proposed for other organisms (Muschiol et al., 2015; Neuhaus et al., 2020), only particular pili serve in DNA uptake by *S. elongatus*, and *ogt* inactivation may not affect assembly of competence pili.

Experimental Procedures

Strains, culture conditions and biofilm quantification: Culture starters of *S. elongatus* PCC 7942 and all derived strains were bubbled with air enriched with CO₂ as described previously (Sendersky et al., 2017). Starters were diluted with BG-11 to OD₇₅₀ of 0.5 (30 ml culture in 100 ml flasks). Construction of mutants and details of molecular manipulations are provided in Table S1. Biofilms formed under static conditions at 28°C with incandescent light illumination (6 $\mu\text{mole photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Biofilms formed in standing cultures under the conditions indicated above and were quantified after 6 d growth as described (Parnasa et al., 2019), either by percentage of chlorophyll in suspension (Sendersky et al., 2017) or by crystal violet staining. For examination of biofilm formation in a flow cell system, initial cultures were diluted with BG-11 medium to OD₇₅₀ of 1.0, and incubated for 48 h under 28°C and 6 $\mu\text{mole photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Subsequently, a constant flow of BG-11 at 5 ml/h was provided by a peristaltic pump. Biofilms were observed following 7 d from experiment initiation using Leica SP8 confocal microscope (excitation: 588nm; emission: 603-710 nm).

Analysis of conditioned medium: Medium from a 50 ml culture was collected after centrifugation (5000 g, 10 min, room temperature). The supernatant was removed and passed through 0.22 mm filter, desiccated, and resuspended in 1.1 ml TE buffer supplemented with a protease inhibitor cocktail (Sigma, P8465-5ML). Analysis of conditioned medium by SDS-PAGE and silver staining was described previously (Schatz et al., 2013). Detection of *N*-Acetylglucosamine was performed as described (Cao et al., 2013) with the following modifications: 5% bovine serum albumin (BSA) in TBST was used as a blocking solution, 5 $\mu\text{g/ml}$ biotinylated succinylated wheat germ agglutinin (Succinyl-WGA, VE-B-1025S, Vector Labs), which allows specific detection of GlcNAc was used rather than WGA, and horseradish peroxidase streptavidin (VE-SA-5014, Vector Labs) was adjusted to 3 $\mu\text{g/ml}$.

Transmission electron microscopy: One day old cultures that had not yet initiated biofilm formation were sampled (10µl) and applied onto ultra-thin carbon coated grids. Following 5 minutes liquid was removed by blotting and cells were negatively stained twice with 2% fresh aquatic uranyl acetate (10 µl) for 1 min each step. Stain was removed by blotting, grids were briefly washed with double distilled water (10 µl) and left to dry over-night at room temperature. Images were acquired with a Technai G2 Fei transmission electron microscope, operating at 120 KV with a 1KX1K camera.

DNA competence analysis: Assessment of DNA competence was performed essentially as described previously (Golden et al., 1987). Exponentially growing cells were centrifuged (5,000 x g for 8 min at room temperature), washed once with 10mM NaCl, and resuspended to an optical density at 750 nm (OD₇₅₀) of 4.0. A shuttle vector (1,000 ng) was added to 600 µl of cells, which were gently agitated overnight at 28°C in the dark. Transformants were selected by plating on selective solid growth medium (50 µg/ml spectinomycin) supplemented with NaHCO₃ (5 mM) and sodium thiosulfate (0.3%, wt/vol). The shuttle vector replicates autonomously, thus allowing the assessment of DNA uptake without a possible impact of the efficiency of DNA integration into the chromosome.

Bioinformatics and confitness analysis: TPR repeats and glycosyltransferase domains were detected by TPRpred (Karpenahalli et al., 2007) and PFAM (Finn et al., 2014), respectively. Synteny analysis was performed using SyntaX (Oberto, 2013). This tool is limited to analysis of 100 genomes at a time; therefore, analyses of 201 cyanobacterial genomes was performed in batches as follows: 50 genomes (grouped in SyntaX under bacteria/cyanobacteria), and 100 and 51 (grouped in SyntaX under bacteria:unclassified/cyanobacteria:unclassified). Cofitness data were recruited using the “Fitness Browser” web site (Wetmore et al., 2015; Price et al., 2018).

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Figure Legends

Figure 1: Inactivation of Synpcc7942_0051 results in biofilm formation. A.

Genomic region of Synpcc7942_0051 (*ogt*). Tetratricopeptide (TPR) repeat region and conserved glycosyltransferase (GT41) domains are shown. The insertion point of a chloramphenicol resistance cassette (Cm^R) is indicated. Asterisk indicates the position of the conserved lysine, K445. Small arrows indicate primers used to PCR amplify a DNA fragment for complementation (See Table S1). Genes Synpcc7942_0049/0048 encode the pilus subunit PilA. Synpcc7942_0050 and Synpcc7942_0052 are annotated 'hypothetical'. **B.** Quantitation of biofilms using crystal violet staining. **C.** Biofilms formed at culture flask bottom as revealed by crystal violet staining. **D.** Percentage of chlorophyll in suspension as a proxy for biofilm development. Data in **B** and **D** represent average and standard error from 3 independent biological repetitions (with 3 technical repeats in each). **E.** Confocal microscopy imaging of biofilms formed in a flow cell. Red color represents auto-fluorescence (excitation: 588 nm; emission: 603-710 nm).

Figure 2: Biofilm development by PilB::Tn5 and Ogt::Mu under fresh medium (FM) and conditioned medium (CM) from WT culture. Cultures were imaged following 6 d of growth.

Figure 3: The glycosyltransferase activity of Ogt::Mu is required for biofilm self-suppression. A.

Percentage of chlorophyll in suspension as a proxy for biofilm development. Data represent average and standard error from 3 independent biological repetitions (with 3 technical repeats in each). Strains analyzed: Ogt::Mu, Ogt-mutant harboring mutated and native Ogt (Ogt::Mu/ K445A and Ogt::Mu/Ogt, respectively) and corresponding strains with tagged mutated or native Ogt (Ogt::Mu/K445A::FLAG and Ogt::Mu/Ogt::FLAG, respectively). **B.** Western blot analysis of whole cell lysate (L), soluble (S) and pelletable (P) fractions using anti-

FLAG antibodies. Arrow indicates the FLAG-tagged Ogt protein (~70 kDa). Each lane corresponds to 2 µg chlorophyll.

Figure 4: Ogt is involved in PilA glycosylation. Gel-electrophoresis of conditioned medium followed by silver staining **(A)** or GlcNAc detection **(B)**. Strains analyzed: *S. elongatus* (WT), *pilB*- and *ogt*-inactivated strains (PilB::Tn5 and Ogt::Mu, respectively) and Ogt::Mu into which native or mutated Ogt was introduced (Ogt::Mu/Ogt and Ogt::Mu/K445A, respectively). Each lane represents 0.5 ml of conditioned medium.

Figure 5: Ogt is required for adequate pilus assembly. TEM images of whole negatively stained cells and quantitative analysis of pili. Strains analyzed: *S. elongatus* (WT), *ogt*-inactivated strain (Ogt::Mu) and Ogt::Mu into which native or mutated *ogt* was introduced (Ogt::Mu/Ogt and Ogt::Mu/K445A, respectively). White arrow indicates the single pilus of an Ogt::Mu cell.

Figure 6: Inactivation of *ogt* does not affect natural DNA competence. DNA uptake by WT, PilB::Tn5 and Ogt::Mu. A shuttle vector capable of replication in *S. elongatus* was used, so that the transformation assay represents ability to take up the DNA without requirement for integration into the chromosome. WT and Ogt::Mu plates represent plating of 100 µL of 100⁻² diluted transformation mixture whereas in the case of PilB::Tn5 200 µL of non-diluted mixture was plated. Data represent averages and standard error of 3 technical repeats.

Legends to Supplementary Figures and Tables

Figure S1: Domain organization of various Ogt enzymes. TPR repeats (purple) and glycosyltransferase 41 domains (red) of cyanobacteria (*Synechococcus elongatus* PCC 7942, synpcc7942_0051; *Microcystis aeruginosa* PCC 7806, BH695_0839 and *Nostoc punctiforme* PCC 73102, Npun_F0677), gram positive bacteria (*Thermobaculum terrenum* strain ATCC BAA-798, Tter_2822 and *Listeria*

monocytogenes strain EGD-e, lmo0688) and the gram-negative bacterium *Xanthomonas campestris* pv. *campestris* str. ATCC 33913, XCC0866.

Figure S2: Inactivation of *ogt* does not affect biomass accumulation as measured by total chlorophyll. Data represent average and standard error from 3 independent biological repetitions (with 3 technical repeats in each).

Figure S3: Genomic analysis of different cyanobacteria indicates physical adjacency of *ogt* and *pilA* genes. Selected genomic maps of the 201 cyanobacterial genomes analyzed by SynTax are presented.

Table S1: Summary of cloning and mutational information

* Insertional inactivation of Mu transposon, which confers Chloramphenicol resistance cassette (Cm^R) was performed using the indicated vector from the unigene set (UGS) library (Holtman et al., 2005). Gene disruption in *S. elongatus* was obtained by transformation and replacement of the native gene by homologous recombination.

PCR products were cloned into the *Swal* in NSII vector.

All cloning products were validated by PCR analyses and sequencing. Complete chromosome segregation of cyanobacterial strains were confirmed by PCR.

Lowercase letters in primers indicate the regions that encode the triple FLAG-tag.

Table S2: Cofitness data of *ogt*-mutant. Data were recruited from the Fitness Browser website (<https://fit.genomics.lbl.gov/cgi-bin/myFrontPage.cgi>) (Wetmore et al., 2015; Price et al., 2018).

CM

FM

PilB::Tn5

Ogt::Mu

B

AMW,
kDa

WT

PilB::Tn5

Ogt::Mu

Ogt::Mu/Ogt

Ogt::Mu/K445A

25—

15—

10—

← PilA

BMW,
kDa

WT

PilB::Tn5

Ogt::Mu

Ogt::Mu/Ogt

Ogt::Mu/K445A

25—

15—

10—

← PilA

WT

Ogt::Mu

Ogt::Mu/Ogt

Ogt::Mu/K445A

Strains	Number of cells				% of piliated cells
	Total	a single pilus	pili	non-piliated	
WT	45	4	39	2	96
Ogt::Mu	44	6	5	33	25
Ogt::Mu/Ogt	54	3	44	7	87
Ogt::Mu/K445A	53	7	4	42	21

Strain	Colonies per ug DNA	Standard error
WT	103556	8988
PilB::Tn5	0	0
Ogt::Mu	103333	6429