environmental microbiology reports

Environmental Microbiology Reports (2022) **00**(00), 00–00 doi:10.1111/1758-2229.13050

Brief Report

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

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Summary

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

Introduction

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

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

Studies of the molecular mechanisms underlying cyanobacterial biofilm development 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;

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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 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). Furthermore, we revealed that the wild-type (WT) strain secretes small compound(s) (<3 kDa) that govern expression of genes enabling biofilm development (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 regulations 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 (Simkovsky *et al*., 2022). 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 (Simkovsky *et al*., 2022). Briefly, this *S. elongatus* library of mutants was grown, a biofilm was allowed to form, and DNA extracted from the biofilm was analysed by high-throughput sequencing. Data analysis revealed mutants highly abundant in the biofilm. 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 constructed individual mutants and examined their ability to develop biofilms in a pure culture (Simkovsky *et al*., 2022). One of the mutants enriched in the biofilm is impaired in a gene encoding a glycosyltransferase homologue, 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 catalyse 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)]. Using *S. elongatus* Ogt purified protein, Sokol and Olszewski (2015) demonstrated UDP-GlcNAc hydrolysis thus providing evidence for the glycosyltransferase activity of this protein.

We insertionally inactivated Synpcc7942_0051, the gene encoding Ogt of *S. elongatus*, 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. 1B–D). Substantial biofilm development in flasks was revealed by crystal violet staining in Ogt::Mu similarly to PilB::Tn5 (Fig. 1B and C). This assay suggested only trace cell adhesion or sugar-matrix deposition in WT culture (Fig. 1B and 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, precipitated by gravity to the very bottom of the tube when grown in CM in contrast to WT cells that

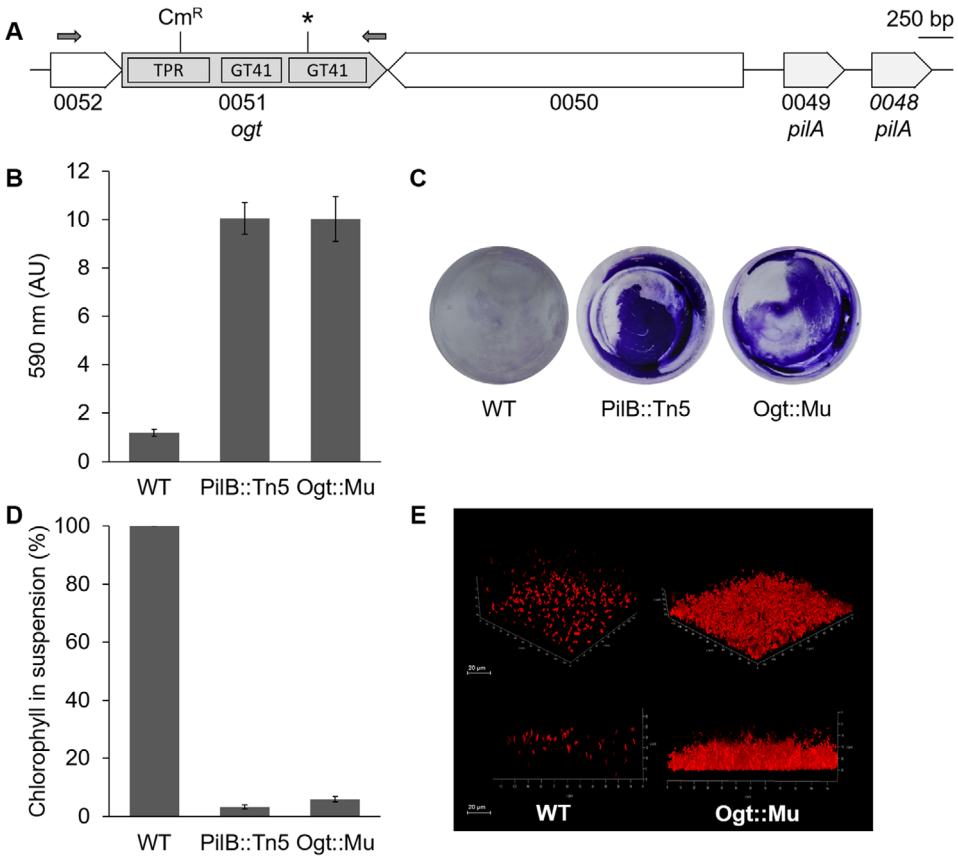


Fig. 1. Inactivation of Synpcc7942_0051 results in biofilm formation.A. Genomic region of Synpcc7942_0051 (*ogt*). Tetratricopeptide (TPR) repeat region and conserved glycosyltransferase (GT41) domains are shown. The insertion point of a chloramphenicol resistance cassette (Cm^R) is indicated. Asterisk 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 three independent biological repetitions (with three technical repeats in each).E. Confocal microscopy imaging of biofilms formed in a flow cell. Red colour represents auto-fluorescence (excitation: 588 nm; emission: 603–710 nm).

remain suspended (Fig. S3). Cells of this mutant are similar in size to WT cells thus, pelleting is most likely due to absence of pili from this mutant (Nagar *et al*., 2017). The cell pellet, however, was clearly distinct from the biofilm formed on the tube walls under fresh growth medium (Fig. S3, 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. S3). 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. 2A). 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.

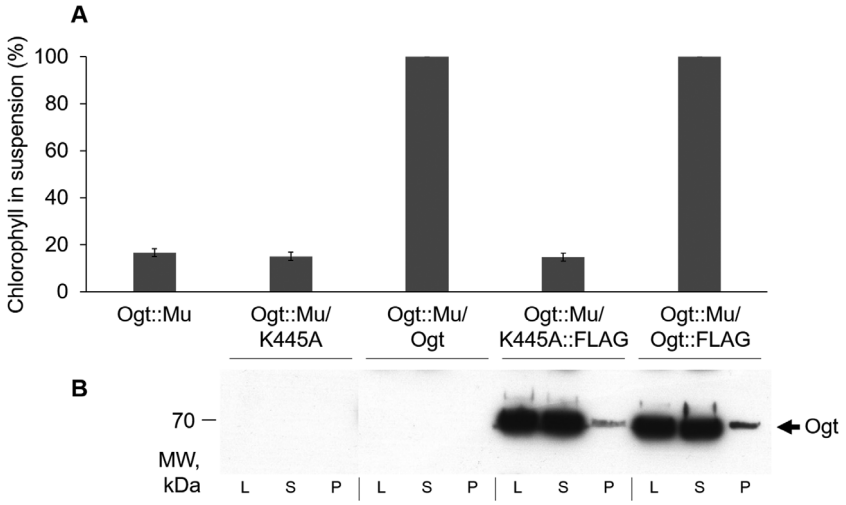


Fig. 2. The glycosyltransferase activity of Ogt is required for biofilm self-suppression.A. Percentage of chlorophyll in suspension as a proxy for biofilm development. Data represent average and standard error from three independent biological repetitions (with three technical repeats in each). Strains analysed: Ogt::Mu, Ogt-mutant harbouring 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.

Absence of restoration of planktonic growth in Ogt::Mu/K445A could in principle 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. 2B, 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. 2A). In contrast, strain Ogt::Mu/K445A::FLAG formed biofilms similarly to Ogt::Mu (Fig. 2A). 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 201 cyanobacterial genomes revealed physical adjacency of *ogt* and pilin genes in 77 (38%) of the genomes (see Fig. S4 for synteny in selected genomes). Given these facts, we tested whether Ogt is involved in PilA glycosylation in *S. elongatus*. Analysis by transmission electron microscopy revealed detached pili in cultures of this cyanobacterium (Nagar *et al*., 2017). This observation indicates that CM analysis most likely represents functional pili filaments released into the medium and not only individual PilA subunits obtained following pilus disassembly. Analysis of CM by gel-electrophoresis followed by silver staining indicated a major band of ~14 kDa in WT (Fig. 3A). This band, which is largely missing in PilB::Tn5 (Fig. 3A), 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. 3A). 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. 3A). Analyses of excised bands from WT and Ogt::Mu preparations by mass spectrometry (MS) validates the presence of PilA1 in these gel regions in addition to other proteins (Supplemental data file S1). 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);

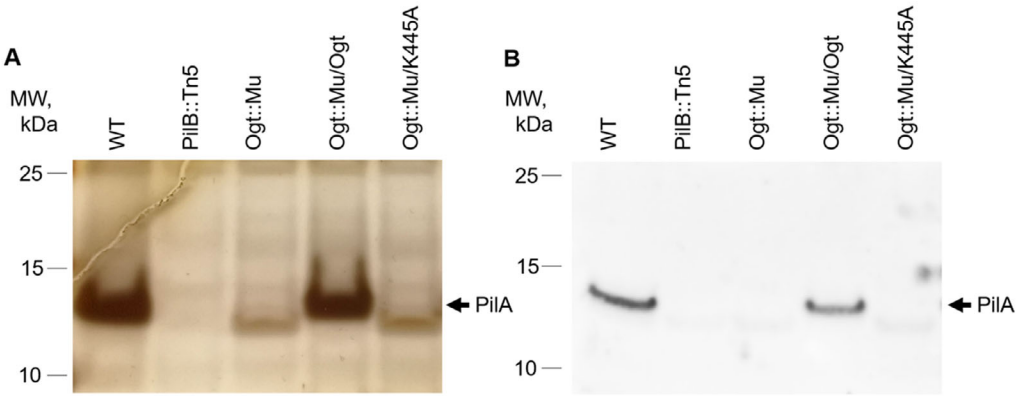


Fig. 3. Ogt is involved in PilA glycosylation. Gel electrophoresis of conditioned medium followed by silver staining (A) or GlcNAc detection (B). Strains analysed: *S. elongatus* (WT), *pilB*- and *ogt*-inactivated strains (PilB::Tn5 and Ogt::Mu respectively) and Ogt::Mu into which native or mutated Ogt was introduced (Ogt::Mu/Ogt and Ogt::Mu/K445A respectively). Each lane represents 0.5 ml of conditioned medium.

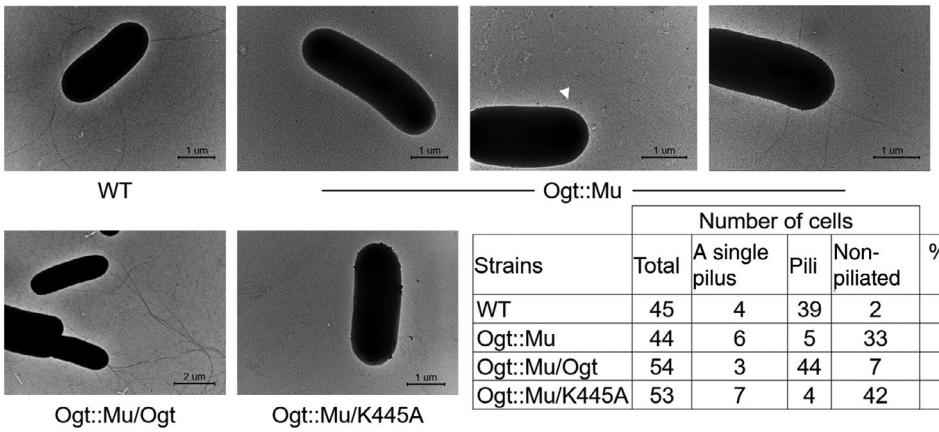
therefore, we tested whether this glycosyltransferase is involved in addition of GlcNAc to PilA. Succinylated wheat Germ Agglutinin (succinyl-WGA), a lectin that detects GlcNAc, revealed a band of ~14 kDa in WT, supporting presence of GlcNAc residue(s) in PilA in this strain (Fig. 3B). A faint band is apparent in OgtB::Mu preparation; however, a very weak signal is also detected in PilB::Tn5, which lacks pili (Nagar *et al*., 2017; Yegorov *et al*., 2021); thus, this minor signal does not represent PilA glycosylation (Fig. 3B). Furthermore, MS analyses of excised bands indicate that PilA1 (encoded by synpcc7942_0049/0048) is the most dominant protein comprising ~95% of the estimated amount of protein in the mutant gel region, whereas in the WT strain PilA1 is only ~9% in the corresponding band (Fig. S5 and Supplemental data file S1). Thus, absence of GlcNAc signal in Ogt::Mu in spite of excessive presence of the pilin protein in the corresponding gel region compared to WT supports a role for Ogt in PilA glycosylation. Moreover, the band corresponding to the molecular weight typical of PilA of WT was recovered in the *ogt* mutant bearing a native *ogt* gene but not with the active-site mutant allele (Fig. 4B, Ogt::Mu/Ogt and Ogt::Mu/K445A respectively). In conclusion, the difference in MW (Fig. 3A) and GlcNAc detection (Fig. 3B) indicates 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. 4; (Nagar *et al*., 2017)]. The majority of the cells (96%) are piliated with most cells exhibiting several pili (see Table within Fig. 4). In contrast, only 25% of Ogt::Mu cells are piliated, half of which exhibit only a single pilus (Fig. 4). Introduction of *ogt* into the mutant largely restored pilus assembly (Fig. 4; 87% of the observed cells were piliated). Furthermore, in strain Ogt::Mu/K445A, which does not glycosylate PilA (Fig. 3), 21% of the cells were piliated, similar to Ogt::Mu (Fig. 4). Together, TEM analyses of the different strains support requirement of Ogt activity for proper pilus formation.

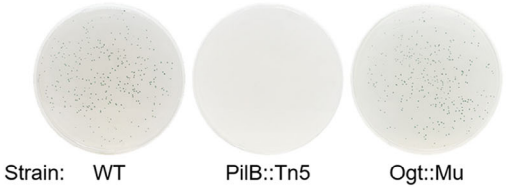
Type 4 pili are involved in DNA transformation (Ellison *et al*., 2018; Craig *et al*., 2019; Piepenbrink, 2019). As we previously reported (Nagar *et al*., 2017; Yegorov *et al*., 2021), non-piliated PilB::Tn5 completely lacks ability to take up external DNA (Fig. 5) in agreement with a recent study (Taton *et al*., 2020). Given the lower number of piliated 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. 5). 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 1). The fitness data were collected using a randomly barcoded transposon library that was subject to a large variety of stress 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



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

Fig. 4. Ogt is required for adequate pilus assembly. TEM images of whole negatively stained cells and quantitative analysis of pili. Strains analysed: *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 arrowhead indicates the single pilus of an Ogt::Mu cell.



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

Fig. 5. 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 three technical repeats.

Table 1. Cofitness data of ogt mutant.

Gene	Name	Description	Cofitness
1935	*pilD*	Type 4 prepilin peptidase	0.97
1436		Hypothetical protein	0.96
0862	*ebsA*	Hypothetical protein	0.96
2069	*pilC*	Fimbrial assembly protein PilC-like	0.96
2486	*rntA*	Hypothetical protein	0.96
2071	*pilB1*	ATPase	0.95
2479	*pilA2*	Pilin-like protein	0.95
2451	*pilO*	Putative type IV pilus protein PilO	0.95
2484		Hypothetical protein	0.95
1139		Hypothetical protein	0.95
2452	*pilN*	Tfp pilus protein PilN-like	0.95
0168		Hypothetical protein	0.95
2453	*pilM*	Type IV pilus assembly protein PilM	0.95
1926	*hfq*	Hypothetical protein	0.95
2450	*pilQ*	General secretion pathway protein D	0.95
2485	*rntB*	Hypothetical protein	0.95
1510	*sigF1*	RNA polymerase sigma factor SigF	0.94
1110		Response regulator receiver (CheY-like)	0.93
1525	*typA*	GTP-binding protein TypA	0.89
0049	*pilA*	Pilin polypeptide PilA-like	0.89

Column 'Gene' describes the last four digits of full gene name (synpcc7942_XXXX). Genes Synpcc7942_2484 and Synpcc7942_2485 encode PilA candidates (Taton *et al*., 2020). 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).

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 1). 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 1). 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 and Olszewski (2015) reported that impairment of this glycosyltransferase results in cell settlement and formation of small cell aggregates. Such small aggregates may represent an initial stage of biofilm formation referred to as microcolonies; however, they may as well be a consequence of cell surface changes that result in clumping of few cells without relation to elicitation of an intricate developmental program that leads to formation of multilayered large cell consortia, referred to as biofilms. Thus, our study provides novel information by demonstrating that inactivation of *ogt* triggers a complex process that culminates in robust biofilms thereby assigning a crucial role for Ogt activity in the biofilm suppression mechanism that operates in *S. elongatus*. In both studies, cultures were analysed 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 favoured 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. S3]. 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. S3). The nature of this heterogeneous phenotype is unknown; however, biofilm development by a subpopulation in the culture in the presence of the inhibitor indicates that Ogt is required for proper response to biofilm inhibitor(s) present in WT-CM. Additionally, substantially reduced piliation in Ogt::Mu (Fig. 4) and cofitness data (Table 1) indicate the requirement of Ogt for proper activity of the T4P complex and suggest that this enzyme is also engaged in inhibitor secretion, as previously proposed for PilB::Tn5 (Nagar *et al*., 2017). A few studies demonstrated involvement of T4P complex in protein secretion (Sauvonnet *et al*., 2000; Kirn *et al*., 2003; Hager *et al*., 2006; Han *et al*., 2007) thus, the biofilm inhibitor may be directly secreted. Indirect involvement is also possible, e.g. deposition to the outer membrane of an enzyme required for inhibitor synthesis. Together, these data support a pleiotropic effect of inactivation of *ogt*. Pleiotropic effects resulting from abrogation of *ogt* in *S. elongatus* were reported by 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. 3A) in agreement with lack of glycosylation in the mutant. Moreover, GlcNAc detection validated involvement of Ogt for PilA glycosylation (Fig. 3B). Secretion signals were not identified in Ogt, in support of cytoplasmic localization of this enzyme and accordingly, the cellular site of the glycosylation process. 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 (Goncalves *et al*., 2018). Additionally, inactivation of *Synechocystis kpsM* that encodes a putative protein required for release of extracellular matrix affected PilA glycosylation (Santos *et al*., 2021). An additional study of this cyanobacterium revealed that inactivation of sll0899 altered PilA glycosylation and impaired motility (Kim *et al*., 2009).

The particular role, however, of these proteins in the process of PilA glycosylation is unknown.

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. It is worth noting that a wild isolate of *S. elongatus* exhibits phototactic motility on agar plates, an observation that supports the suggestion that laboratory strains of this cyanobacterium lost this kind of motility due to a domestication process (Yang *et al*., 2018).

The impact of PilA glycosylation on pilus assembly varies between different cyanobacteria. For example, deletion of sll0180 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 triggered 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 homologues 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. 5). Though not completely abrogating pilus formation, inactivation of *ogt* severely reduced the number of piliated cells (Fig. 4). Therefore, a full suite of pili is not necessary to support maximal transformation competency. *S. elongatus* possesses several *pilA* candidate genes: synpcc7942_0049/0048, Synpcc7942_2479, Synpcc7942_2482, Synpcc7942_2484, Synpcc7942_2485, Synpcc7942_2590 and Synpcc7942_2591. It is possible that, as proposed for other organisms (Muschiol *et al*., 2015; Neuhaus *et al*., 2020; Oeser *et al*., 2021), only particular pili serve in DNA uptake by *S. elongatus*, and *ogt* inactivation may not affect the 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 CO_2 as described previously (Sendersky *et al*., 2017). Starters were diluted with BG-11 to OD_{750} 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 μmol photons m^{-2} s^{-1}). Biofilms formed in standing cultures under the conditions indicated above and were quantified after 6 days 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 OD_{750} of 1.0, and incubated for 48 h under 28°C and 6 μmol photons m^{-2} s^{-1}. Subsequently, a constant flow of BG-11 at 5 ml h^{-1} was provided by a peristaltic pump. Biofilms were observed following 7 days from experiment initiation using Leica SP8 confocal microscope (excitation: 588 nm; 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 re-suspended 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 in TBST was used as a blocking solution, 5 μg ml^{-1} 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 μg ml^{-1}.

Transmission electron microscopy. One day old cultures that had not yet initiated biofilm formation were sampled (10 μl) and applied onto ultra-thin carbon-coated grids. Following 5 min 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 overnight at room temperature. Images were acquired with a Tecnai 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 (5000*g* for 8 min at room temperature), washed once with 10 mM NaCl, and re-suspended to an optical density at 750 nm (OD_{750}) of 4.0. A shuttle vector (1000 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^{-1} spectinomycin) supplemented with $NaHCO_3$ (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 on the efficiency of DNA integration into the chromosome.

Bioinformatics and cofitness analysis. TPR repeats and glycosyltransferase domains were detected by TPRpred (Karpenahalli *et al*., 2007) and PFAM (Finn *et al*., 2014) respectively. Search for a secretion signal was done using Phobius, Signal IP – 5.0, TatFind and SecretomeP. Synteny analysis was performed using SyntTaX (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 SyntTaX under bacteria/cyanobacteria), and 100 and 51 (grouped in SyntTaX under bacteria:unclassified/cyanobacteria:unclassified). Cofitness data were recruited using the 'Fitness Browser' website (Wetmore *et al*., 2015; Price *et al*., 2018).

Acknowledgements

Studies in the laboratories of Rakefet Schwarz and Susan Golden were supported by the program of the National Science Foundation and the U.S.-Israel Binational Science Foundation (NSF-BSF 2012823). This study was also supported by grants from the Israel Science Foundation (ISF 1406/14 and 2494/19) to Rakefet Schwarz.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. 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.

Fig. 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).

Fig. S3. 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.

Fig. S4. Genomic analysis of different cyanobacteria indicates physical adjacency of *ogt* and *pilA* genes. Selected genomic maps of the 201 cyanobacterial genomes analysed by SynTax are presented.

Fig. S5. Gel-electrophoresis of conditioned medium followed by silver staining of wild-type (WT) and Ogt::Mu culture. Rectangles indicate bands excised for mass spectrometry analyses.

Table S1. Summary of cloning and mutational information.

File S1. Mass spectrometry analysis of excised 'PilA bands'. Peptide spectrum matches (PSM) displays the total number of identified peptide sequences (including those redundantly identified). 'Area' corresponds to the amount of peptides.