FdTonB is involved in the photoregulation of cellular morphology during complementary chromatic adaptation in *Fremyella diplosiphon*

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We have characterized a *Fremyella diplosiphon* TonB protein (FdTonB) and investigated its function during complementary chromatic adaptation. Sequence similarity analysis of FdTonB (571 aa) led to identification of several conserved domains characteristic of TonB proteins, including an N-terminal transmembrane domain, a central proline-rich spacer and a C-terminal TonB-related domain (TBRD). We identified a novel glycine-rich domain containing (Gly-X)_n repeats. To assess FdTonB function, we constructed a Δ tonB mutant through homologous recombination based upon truncation of the central proline-rich spacer, glycine-rich domain and TBRD. Our Δ tonB mutant exhibited an aberrant cellular morphology under green light, with expanded cell width compared to the parental wild-type (WT) strain. The cellular morphology of the Δ tonB mutant recovered upon WT tonB expression. Interestingly, tonB expression was found to be independent of RcaE. As Δ tonB and WT strains respond in the same way when grown under iron-replete versus iron-limited conditions, our results suggest that FdTonB is not involved in the classic TonB function of mediating cellular adaptation to iron limitation, but exhibits a novel function related to the photoregulation of cellular morphology in *F. diplosiphon*.

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INTRODUCTION

Cyanobacteria use light as a primary source of energy for growth and development in their natural environment. They capture light energy using light-harvesting complexes called phycobilisomes (PBSs). To enhance their photosynthetic capacity under varying light qualities and quantities, some cyanobacterial species alter the protein or pigment content of their PBS structures. Alterations of PBS composition can be identified readily by observing the cell colour phenotype. The classic example of PBS acclimation to variations in light colour results in cells that change between brick red and bright blue-green in colour. This change in colour phenotype, which is a key feature of a light-dependent acclimation process referred to as com-

plementary chromatic adaptation (CCA; Bogorad, 1975), occurs primarily in response to green light (GL) and red light (RL). The changes that occur during CCA have been well characterized in the freshwater cyanobacterium *Fremyella diplosiphon*, which is also designated *Calothrix* sp. PCC 7601 (Kehoe & Gutu, 2006).

In addition to the light-dependent pigment changes during the process of CCA, physiological and cellular changes also occur in *F. diplosiphon* in response to changes in light quality (Bennett & Bogorad, 1973; Campbell, 1996). Filaments are longer and individual cells are larger and more brick-like in shape under GL, whereas shorter filaments and smaller, rounded cells are observed under RL (Bennett & Bogorad, 1973; Bordowitz & Montgomery, 2008). Recent results indicate that RcaE is the photoreceptor that is responsible for regulating light-dependent changes in cell shape and filament length in *F. diplosiphon* (Bordowitz & Montgomery, 2008). The identity of specific effectors operating downstream of RcaE in this cellular morphology pathway is still under investigation.

In a microarray study that examined the impact of light quality on gene expression in *F. diplosiphon*, 17 novel genes were found to be differentially expressed under GL and RL (Stowe-Evans *et al.*, 2004). *F. diplosiphon* DNA clone

Abbreviations: AP, allophycocyanin; CCA, complementary chromatic adaptation; chl*a*, chlorophyll *a*; DIC, differential interference contrast microscopy; GL, green light; GRP, glycine-rich protein; PBP, phycobiliprotein; PBS, phycobilisome; PC, phycocyanin; PE, phycoerythrin; RL, red light; TBRD, TonB-related domain; WT, wild-type.

The GenBank Third Party Annotation (TPA) database accession number for the annotated nucleotide sequence reported in this paper is BK006919.

Supplementary tables of primers and plasmids, and a supplementary figure, are available with the online version of this paper.

3091A7, containing two predicted ORFs, exhibited nearly 1.59-fold upregulation under GL (Stowe-Evans *et al.*, 2004). When the expression of the individual ORFs was determined, ORF 3091A7-A exhibited GL-dependent upregulation, whereas the second ORF, 3091A7-B, was induced under RL (Stowe-Evans *et al.*, 2004). Using Pfam analysis (Finn *et al.*, 2006), the polypeptide encoded by ORF 3091A7-A was determined to exhibit sequence similarity to periplasmic protein TonB (Montgomery, 2008). Based on this similarity, 3091A7-A was designated FdTonB (Montgomery, 2008).

According to Entrez Gene (Maglott *et al.*, 2007), genes encoding TonB-family proteins are specified as members of the cell envelope/membrane biogenesis group. Upregulation of *FdtonB* gene expression under GL and the occurrence of elongated cells under the same light condition may indicate a role for FdTonB in light-dependent regulation of cell envelope and cell wall components during CCA (Montgomery, 2008). Indirect evidence for the involvement of TonB in the regulation of cell wall biosynthesis and cell shape exists in bacterial systems; e.g. a TonB-dependent transporter directly interacts with TonB and, in association with additional proteins, regulates cell wall biosynthetic complexes and bacterial cell shape in *Caulobacter crescentus* (Divakaruni *et al.*, 2005). We therefore hypothesized that FdTonB may play a significant, novel role in the photoregulation of cellular morphology during CCA in *F. diplosiphon*.

In Gram-negative bacteria, TonB is known to be involved in regulation and active transport of iron complexes and vitamin B12 across the outer membrane (Postle & Kadner, 2003; Postle & Larsen, 2007; Schwyn & Neilands, 1987). Iron is essential for all living organisms, but most importantly for photosynthetic micro-organisms, as it is an essential metal in photosynthetic electron transfer for photosystems I and II (Keren *et al.*, 2004). Iron transportation is required for a number of metabolic processes in cyanobacterial cells, including photosynthesis, respiration and nitrogen fixation. Under iron-limited conditions, several bacteria and cyanobacteria secrete iron chelators known as siderophores to complex iron from the environment (Neilands, 1995; Nicolaisen *et al.*, 2008). Using TonB-dependent outer-membrane receptors, iron-bound siderophores transport through the outer membranes into the cytoplasm, mediated by an ABC transporter present in the cytoplasmic membrane (Schalk *et al.*, 2004). Although the presence of sequences with similarity to TonB-dependent transporters has been recently reported for a number of cyanobacterial genomes (Mirus *et al.*, 2009), the functions of TonB-dependent receptors have not been studied extensively in cyanobacteria. Several proteins with homology to siderophore receptors have been characterized in the freshwater cyanobacterium *Synechocystis* sp. PCC 6803 (Katoh *et al.*, 2001) and in *Anabaena* sp. PCC 7120 (Nicolaisen *et al.*, 2008). Transportation of ferric complexes into the periplasm occurs via a proton-motive force and requires an integral

inner-membrane complex consisting of proteins TonB, ExbB and ExbD (Moeck & Coulton, 1998). *F. diplosiphon* ORF 3091A7-B was predicted to encode FdExbB (Stowe-Evans *et al.*, 2004), but it is oriented in the opposite direction to the gene encoding FdTonB. Furthermore, the expression of *FdtonB* is induced by GL, whereas expression of *FdexbB* is induced by RL (Stowe-Evans *et al.*, 2004). Based on these results, we predicted that in *F. diplosiphon*, while FdTonB may have an Fe-dependent function, it is also likely to exhibit a novel function.

In this study, we investigated the function of FdTonB in *F. diplosiphon*. We demonstrate that FdTonB is required for the photoregulation of cellular morphology during CCA. Unlike other *tonB* mutants that exhibit pleiotropic phenotypes, a $\Delta tonB$ mutant in *F. diplosiphon* exhibits a specific light-dependent phenotype. Our $\Delta tonB$ mutant has significantly wider cells than the parental strain only under GL growth conditions. These GL-grown $\Delta tonB$ cells appear to release greater levels of phycobiliproteins, indicating that the cell wall and/or cell envelope structure is compromised in the absence of FdTonB. In addition, FdTonB does not appear to play a significant role in adaptation of *F. diplosiphon* cells to changes in iron availability. To our knowledge, this is the first report of the characterization of the function of a cyanobacterial TonB protein. We also provide novel insight into the environmental regulation of a TonB-family protein, about which little is currently known for any TonB protein in any biological system. Our results suggest that FdTonB is a novel factor involved in the photoregulation of cellular morphology during CCA in *F. diplosiphon*.

METHODS

Cyanobacterial strains and culture conditions. *Fremyella diplosiphon* strain SF33 (Cobley *et al.*, 1993), which we use as a wild-type (WT) pigmentation strain, and the $\Delta tonB$ mutant were grown as described previously (Bordowitz & Montgomery, 2008). For iron-limited medium (BG11-_{Fe}), ammonium citrate replaced ferric ammonium citrate in BG11 and the water used for the medium was run through Chelex-100 resin (Bio-Rad) to eliminate traces of iron (Sherman & Sherman, 1983). BG11 or BG11-_{Fe} medium used for each experiment was always supplemented with 20 mM HEPES. Glassware used in making the BG11-_{Fe} medium was rinsed extensively with distilled water prior to use. Cells to be used for growth assays in iron-limited medium were washed three times with BG11-_{Fe} medium prior to final resuspension. Initially, cells were adjusted to an OD₇₅₀ of ~0.1 and grown in 25 ml BG11 as a control and in BG11-_{Fe}, under either red light (RL) or green light (GL) conditions. RL and GL sources were those previously described (Bordowitz & Montgomery, 2008). The OD₇₅₀ of the cultures was observed at 3 day intervals for up to 15 days. Growth analysis of three individual cultures in each light condition was carried out with subsequent dilution of the culture in BG11 or BG11-_{Fe} as indicated. Cyanobacterial cultures grown on plates were incubated under continuous white light, provided by Cool White Plus fluorescent bulbs (Philips; model F32T8/TL741/ALTO). Light intensity was measured using a Li-Cor light meter (model LI-250) with a connected Li-Cor quantum sensor (model LI-190SA). *Escherichia coli* cultures were grown at 37 °C in Luria-Bertani (LB) broth with the appropriate antibiotic whenever

necessary. For growth on solid medium, LB was solidified with 1.5 % (w/v) Bacto-agar.

Bioinformatic analyses. To identify conserved domains in FdTonB we analysed the sequence using the National Center for Biotechnology Information (NCBI) Conserved Domains Database (Marchler-Bauer *et al.*, 2009). Putative transmembrane regions were identified using the DAS Transmembrane Prediction server (Cserző *et al.*, 1997). Sequence similarity analysis to detect FdTonB homologues was conducted using the NCBI BLAST program (Altschul *et al.*, 1990). Multiple sequence alignments were conducted using CLUSTAL W (Thompson *et al.*, 1994).

RT-PCR amplification analyses. Purified RNA was isolated from WT and FdBk14 (*ΔrcaE* mutant) cells grown in GL and RL as previously described (Seib & Kehoe, 2002). Purified RNA was then treated with a TURBO DNA-free kit (Ambion) to remove contaminating genomic DNA according to the manufacturer's instructions. Reverse transcription was conducted with 1 µg RNA using the Reverse Transcription System (Promega). RT-PCR was performed using 1 µl cDNA product, GoTaq Green master mix (Promega) with primer sets designed for *tonB*, i.e. FdtonBRTF and FdtonBRTR (see Supplementary Table S1, available with the online version of this paper, for all primer sequences and descriptions), as well as the 23S ribosomal control gene, i.e. *ribo23sintF* and *ribo23sintR*. The gene-specific annealing temperatures were 55 °C for *tonB* and 51 °C for the 23S ribosomal gene. Gene-specific extension times were 30 s for *tonB* and 40 s for the 23S ribosomal gene. The PCR programme was as follows: denaturation at 94 °C for 1 min, then 30 (gene-specific) or 21 (ribosomal) cycles of 94 °C for 30 s, annealing at the indicated temperature for 30 s, and extension at 68 °C for the indicated time, followed by an additional cycle of 68 °C for 1 min, ending with a hold at 4 °C. The PCR products were analysed by agarose gel electrophoresis and gels imaged using Quantity One 1-D Software (Bio-Rad).

Mutant construction. A plasmid carrying the truncated *tonB* gene was originally prepared from allelic-exchange vector pJCF276 (see Supplementary Table S2 for plasmid descriptions; Cobley *et al.*, 1999). The pJCF276 vector was digested with *NcoI* for linearization and concomitant inactivation of the *Cm^R* gene, which allowed double selection of positive clones after ligation. The primers 5'-*tonB*_delHR and 3'-*tonB*_delHR, each with an introduced *NcoI* restriction site, were used to amplify a 3.2 kb fragment of the *F. diplosiphon* genome, including *tonB* with its native promoter and a downstream ORF from genomic DNA. The resulting PCR product was digested with *NcoI* and ligated to the *NcoI*-linearized, alkaline-phosphatase-treated pJCF276 vector using a TaKaRa DNA Ligation kit version 2.1. The predicted functional part of the *tonB* gene (1203 bp) was deleted by digestion with *BbsI* and *XcmI*. The verified plasmid, pJCF276_*tonB*_del, was then transformed into *E. coli* DH5α containing plasmid pJCF173, which carries genes encoding methylases that work to prevent plasmid digestion in the *F. diplosiphon* host (Cobley *et al.*, 1999).

Conjugation and selection of transconjugants. We used a triparental mating procedure adapted from Elhai & Wolk (1988) and Cobley *et al.* (2002) with modifications. *F. diplosiphon* was the recipient strain, *E. coli* 803 (RP4) was used for mobilization, and *E. coli* strain DH5α containing pJCF173 and the cargo plasmid (carrying a truncated *tonB* gene, pJCF276_*tonB*_del) was used for DNA transfer. Overnight-grown *E. coli* strains and a culture of *F. diplosiphon* at an OD₇₅₀ of ~0.1 were utilized for the mating process. Initial transconjugants were selected by screening for resistance to 10 µg neomycin ml⁻¹ with growth on nitrocellulose membrane layered on medium D plates as previously described (Cobley *et al.*, 1993, 2002). Colonies selected from the plates were grown in BG11

medium with 10 µg neomycin ml⁻¹ for the first selection. Medium D and BG11 medium were compared for positive conjugant selection and BG11 medium appeared to be best for transconjugant selection. For the selection of double recombinant transconjugants, neomycin-resistant cells were spread on BG11 plates containing 5 % sucrose at pH 6.75 to select plasmid-excised transconjugants (Cobley *et al.*, 2002). Selected colonies from sucrose-containing plates were used for PCR screening with the 5'-*tonB*_delHR and 3'-*tonB*_delHR primers. We confirmed the integration of the truncated gene into the correct location of the cyanobacterial genome. To this end, we amplified a genomic fragment containing *tonB* using the following primers: 5'-flank *tonB* and 3'-flank *tonB*. These primers anneal 127 bp away from the 5' end and 287 bp away from the 3' end of the *tonB* region contained within the plasmid used for homologous recombination. Transconjugants were maintained by subculturing on BG11 agar plates. After several cycles of subculturing, *ΔtonB* mutant strains segregating a WT copy of the genome were isolated and confirmed via PCR with the 5'-*tonB*_delHR and 3'-*tonB*_delHR primers (as described before). These strains were hence designated *ΔtonB*: WT.

Cell density measurements and spectral scans. Densities of cell cultures grown under RL and GL conditions were measured as OD₇₅₀. Whole-cell spectral scans were obtained in the range of 400–750 nm after the cells were adjusted to an OD₈₀₀ of ~0.1 using a SpectraMax M2 microplate reader (Molecular Devices).

Pigment quantification assays. Chlorophyll *a* and phycobiliproteins were extracted, analysed and quantified as described previously (Bordowitz & Montgomery, 2008).

Microscopy-based analysis of cellular morphology. Slide preparation and imaging parameters were essentially those described previously (Bordowitz & Montgomery, 2008). Differential interference contrast (DIC) and autofluorescence images were acquired for each strain assessed, and cell size (length and width) was determined from confocal laser scanning microscope images using the LSM FCS Zeiss 510 Meta AIM imaging software (Carl Zeiss MicroImaging). For the cell measurement experiments, WT and mutant cells were initially adjusted to an OD₇₅₀ of ~0.1 and grown in 15 ml BG11 or BG11_{-Fe} under either RL (~15 µmol m⁻² s⁻¹) or GL (~5 µmol m⁻² s⁻¹) conditions. Cells were observed after growth for 3 and 6 days to compare the cellular morphology in response to growth in iron-replete or iron-limited conditions over time. At each time point, samples were also collected for whole-cell spectral scans. Medians and standard error for the cell size were determined from cells imaged and measured in three independent experiments. The cell size of *ΔtonB*: WT strains was also measured to compare with the WT and *ΔtonB* mutant cells.

Statistical analysis. Data accumulated from individual experiments for the cell size measurement were not normally distributed. Therefore, statistical analysis was completed using the Mann-Whitney U Test (<http://elegans.swmed.edu/~leon/stats/utest.html>). Two-tailed, unpaired *t*-tests were carried out on the results accumulated for pigment analysis using GraphPad Software (<http://www.graphpad.com/quickcalcs/index.cfm>).

RESULTS

Bioinformatic analysis of FdTonB

F. diplosiphon gene 3091A7-A, whose expression is upregulated 1.63-fold in GL conditions according to Northern blot analysis (Stowe-Evans *et al.*, 2004), encodes a protein that contains a putative TonB domain and was

denoted a putative TonB protein, i.e. FdTonB (Montgomery, 2008). The predicted length of FdTonB is 571 aa. We conducted sequence similarity analysis of the predicted FdTonB protein and found numerous motifs and domains characteristic of TonB-family proteins, including a predicted transmembrane domain, a proline-rich spacer, and a C-terminal TonB domain (see Fig. 1a).

Using the DAS Transmembrane Prediction server (Cserző *et al.*, 1997), we identified the domain encompassing amino acids 21–41 as a putative transmembrane domain. A conserved SXXXH motif (amino acids 29–33), which delimits the minimum required energy-transduction element of TonB proteins, is found within the predicted transmembrane α -helix (Fig. 1a; Larsen & Postle, 2001). As in most TonB-family proteins, the domain between the predicted transmembrane domain and the TonB domain is proline-enriched. This region contains ~11% Pro residues in the FdTonB protein as compared to ~17% in the *E. coli* TonB (Evans *et al.*, 1986; Postle & Good, 1983). The C-terminal TonB-related domain (TBRD) spans amino acids 335–411 as identified by the NCBI Conserved Domains Database (Fig. 1b; Marchler-Bauer *et al.*, 2009). The TBRD includes a conserved RY motif, i.e. R335 and Y336, and a conserved SSG motif (amino acids 369–371) (Fig. 1b; Chu *et al.*, 2007). We also identified a novel, central glycine-rich domain spanning amino acids 220–307 (Fig. 1c). This domain contains a stretch of (Gly-X)_n repeats that have been previously recognized in glycine-rich proteins (GRPs; Sachetto-Martins *et al.*, 2000).

FdTonB, at a length of 571 residues, was found to be larger than most TonB-related proteins, which average 225–300 residues (Chu *et al.*, 2007). Other predicted cyanobacterial TonB-family members that were identified by BLAST

analysis show significant similarity to FdTonB (Fig. 2). These proteins also are larger than average, including related TonB-family proteins in *Nostoc* sp. PCC 7120 (All5036; 470 aa in length; Mirus *et al.*, 2009), *Nostoc punctiforme* PCC 73102 (480 aa), *Anabaena variabilis* ATCC 29413 (507 aa) and *Nodularia spumigena* CCY9414 (526 aa). All of these proteins contained the conserved SXXXH motif of the transmembrane domain and the conserved Y and SSG motif in the TBRD (Fig. 2). Markedly, the TonB-family proteins from *A. variabilis* and *N. punctiforme* also contain the glycine-rich domain identified in FdTonB (Fig. 2). Prior analysis of *F. diplosiphon* protein lysates with monoclonal antibodies recognizing TonB resulted in the identification of an immunoreactive signal for bands much higher than the known sizes of *E. coli* and related TonB proteins, which were 33–40 kDa in size (Larsen *et al.*, 1996). This result correlates well with the much larger FdTonB protein, i.e. predicted size ~60 kDa, according to the ExPASy server (Gasteiger *et al.*, 2005), which we have identified as a putative *F. diplosiphon* TonB homologue.

Regulation of *tonB* expression

As *FdtonB* is upregulated under GL conditions (Stowe-Evans *et al.*, 2004), we initiated an investigation to determine whether *FdtonB* expression is regulated by RcaE. We conducted RT-PCR analyses to determine relative levels of *tonB* expression for WT as compared with the *rcaE* null mutant FdBk14. Our analyses demonstrated that the level of *tonB* expression did not change significantly in FdBk14 in comparison to WT (Fig. 3). The observed changes are due specifically to changes in gene expression and not to genomic DNA contamination as we

Fig. 1. Physical map of FdTonB, indicating conserved domains and motifs. (a) Diagram of the predicted N-terminal putative transmembrane domain (black box) containing the conserved SXXXH motif (underlined), proline-rich domain, novel glycine-rich spacer and C-terminal TonB-related domain (TBRD). (b) Sequence comparison of the TBRD from *F. diplosiphon* (top) and the conserved TonB domain [bottom; COG0810 (Marchler-Bauer *et al.*, 2009)], with conserved residues shaded in grey. Conserved Y (asterisk) and SSG (boxed) motifs are indicated. (c) Sequence containing the novel, central glycine-rich domain, with conserved glycine motifs underlined.

Fig. 4. PCR amplification and agarose gel electrophoresis analyses of *tonB*-containing genomic regions in WT or Δ *tonB* mutants. (a) Δ *tonB* mutant and WT and (b) Δ *tonB*:WT and WT were assessed for the presence of a truncated product (~2 kb) in the mutant or a full-length product (~3.2 kb) in the WT.

putative transconjugants isolated from sucrose plates grown under continuous white illumination (data not shown). We utilized PCR amplification to confirm the presence of a truncated *tonB* gene in these putative Δ *tonB* transconjugants (Fig. 4a). Positive transconjugants (i.e. Δ *tonB* mutants) exhibited an amplified, truncated *tonB* gene product of ~2 kb, compared to the WT *tonB* product of ~3.2 kb (Fig. 4a). Approximately 90 % of the colonies were positive for carrying a mutated *tonB* gene.

Characterization of the Δ *tonB* mutant

To assess the CCA pigmentation response in the Δ *tonB* mutant strain, we obtained whole-cell spectral scans that demonstrated accumulation of the phycobiliprotein phycoerythrin (PE) under GL and phycocyanin (PC) under RL, which is essentially identical to the response observed for the WT strain (Fig. 5). Chlorophyll *a* (*chl**a*) and phycobiliproteins were extracted from the Δ *tonB* mutant, their concentrations quantified, and levels compared with those determined for the WT strain grown under both GL and RL conditions (Fig. 6). The amounts of *chl**a* extracted from WT and the Δ *tonB* mutant strain were similar (Fig. 6a). Allophycocyanin (AP) and PC concentrations relative to *chl**a* differed significantly ($P < 0.0001$) in the mutant strain as compared to those of the WT strain under both light conditions (Fig. 6b, c). However, only under GL conditions was the PE content for the Δ *tonB* mutant strain significantly higher ($P < 0.001$) than that of the WT strain (Fig. 6d). Notably, the differences in levels between WT and Δ *tonB* for all phycobiliproteins were much greater under GL than RL conditions.

Although the overall levels of phycobiliproteins extracted from cells of the Δ *tonB* strain relative to *chl**a* were significantly higher than those obtained from the WT strain, particularly under GL, assessment of the phycobiliprotein ratios yielded distinct results (Table 1). WT phycobiliprotein ratios under both GL and RL conditions were higher than those determined for the Δ *tonB* mutant

Fig. 5. Whole-cell absorption spectral scans of *F. diplosiphon* WT and Δ *tonB* cultures grown under green light (GL) and red light (RL). WT (a) and Δ *tonB* (b). The peaks at 565 nm and 620 nm indicate accumulation of PE and PC under GL and RL, respectively.

(Table 1). However, it should be noted that the general trend of high PE/PC ratios under GL and low PE/PC ratios under RL was observed for both WT and Δ *tonB* strains.

Microscopy-based analyses of TonB-dependent cellular morphology

To determine whether the cell shape of *F. diplosiphon* is influenced by FdTonB activity, we grew the Δ *tonB* mutant strain under GL and RL conditions and qualitatively compared its cellular morphology with that of the WT strain grown in similar conditions. The WT and Δ *tonB* strains showed similar growth responses, as reflected by the OD₇₅₀ after 3 and 6 days of growth under each light condition (data not shown). However, confocal micrographs clearly show the cell shape for the Δ *tonB* mutant to be different from that of the WT strain (Fig. 7). As demonstrated by our microscopy-based analyses and as previously reported, the WT strain has a more elongated shape under GL and a smaller, rounded cell shape under RL (Fig. 7a, b, g, h; Bordowitz & Montgomery, 2008). The Δ *tonB* mutant is also smaller under RL than GL conditions (Fig. 7c, d, i, j). Markedly under GL, the Δ *tonB* mutant showed a distinctive morphological phenotype that was very different from the cylindrical WT cells (Fig. 7c, i).

Fig. 6. Pigment content of *F. diplosiphon* WT and $\Delta tonB$ strains. (a) Chla ($\mu\text{g ml}^{-1}$), (b) allophycocyanin (AP; $\mu\text{g per } \mu\text{g chla}$), (c) phycocyanin (PC; $\mu\text{g per } \mu\text{g chla}$), and (d) phycoerythrin (PE; $\mu\text{g per } \mu\text{g chla}$). WT and $\Delta tonB$ mutant strains were grown under GL and RL. Bars represent the means $\pm$ SD for at least three independent experiments.

Table 1. Phycobiliprotein ratios in WT and $\Delta tonB$ strains

Values represent means $\pm$ SD from at least three independent experiments.

Strain	Light	PE/AP	PC/AP	PE/PC
WT	GL	3.35 (0.19)	0.66 (0.02)	5.10 (0.25)
	RL	0.36 (0.19)	1.96 (1.70)	0.21 (0.09)
$\Delta tonB$	GL	2.53 (0.19)	0.54 (0.06)	4.68 (0.26)
	RL	0.18 (0.11)	1.09 (0.68)	0.13 (0.10)

Under these conditions, $\Delta tonB$ cells appeared expanded in width compared to cells of the parental WT strain. Although always wider than WT cells grown under similar conditions, the $\Delta tonB$ mutant cell shape varied in the degree of increase in cell width under GL conditions (Supplementary Fig. S1). This variation appeared to be correlated with the age of the culture (data not shown). Notably, when we assessed the cellular morphology for $\Delta tonB$: WT, in which a WT copy of the *tonB* gene (~ 3.2 kb amplified band) as well as a *tonB* truncated gene (~ 2 kb) was detected on an agarose gel (Fig. 4b), these cells were found to exhibit a recovered, WT-like morphology (Fig. 7e, f, k, l).

We also quantified the observed differences in cellular morphology on the basis of cell length and width under GL and RL. After 3 and 6 days of growth under RL, both WT and $\Delta tonB$ strains showed very similar cell length, with GL-grown cells being longer than RL-grown cells (Fig. 8a, c). The width of GL-grown $\Delta tonB$ cells was found to be highly significantly different ($P < 0.001$) from the WT strain consistently throughout our experiments, i.e. GL-grown $\Delta tonB$ cells are much wider than WT cells after both 3 days and 6 days (Fig. 8b, d).

We also quantified the observed differences in cellular morphology on the basis of cell length and width for $\Delta tonB$: WT under GL and RL. After 3 and 6 days of growth under RL, $\Delta tonB$: WT cells exhibited similar a cell length pattern to WT (Fig. 8a, c). Markedly, under GL conditions, $\Delta tonB$: WT cells displayed cell widths the same as WT (Fig. 8b, d), both of which are significantly less wide than $\Delta tonB$.

Response of *F. diplosiphon* strains to growth in iron-limited medium

TonB is known to be involved in iron transport across the outer membrane in many Gram-negative bacteria. To investigate whether FdTonB plays a similar role in *F. diplosiphon*, we grew $\Delta tonB$ and WT strains in iron-limited BG11_{-Fe} medium under GL and RL conditions and compared them to strains grown in complete BG11 medium under identical conditions. The rate of growth of the cultures was observed at 3 day intervals on the basis of the OD₇₅₀. No significant differences in the growth rates were observed for the $\Delta tonB$ mutant strain when compared with the WT strain in either medium under GL or RL conditions (Fig. 9a, b). The GL-grown WT strain in iron-limited medium displayed morphological similarities to the $\Delta tonB$ mutant strain grown in iron-replete BG11 medium under GL (compare Figs 9c and 7c) and sometimes formed vacuolated cells (data not shown). Markedly, the $\Delta tonB$ mutant formed vacuoles in the cells when grown under GL conditions in BG11_{-Fe} medium (Fig. 9e). GL-grown cultures (WT or $\Delta tonB$ mutant strain) in BG11_{-Fe} medium showed curly filaments (data not shown). Under RL conditions, vacuolated cells were observed for both WT and $\Delta tonB$ cells grown in BG11_{-Fe} medium (Fig. 9d, h, f, j)

Fig. 7. Cellular morphologies of *F. diplosiphon* WT and ΔtonB mutant strains grown under GL and RL. (a–f) Representative slices from a Z-series of DIC images for WT (a, b), ΔtonB (c, d) and $\Delta\text{tonB:WT}$ (e, f). (g–l) Maximum-projection phycobiliprotein autofluorescence images from a Z-series of images of GL- and RL-grown filaments of WT (g, h), ΔtonB (i, j), and $\Delta\text{tonB:WT}$ (k, l) strains captured using a 40 $\times$ oil-immersion lens objective with 2 $\times$ zoom setting. Bars represent 10 μm .

DISCUSSION

TonB is a periplasmic protein that connects the outer and cytoplasmic membrane (NCBI AAT41882.1) in Gram-negative bacteria. The functions of TonB proteins have

been extensively characterized in bacteria but very little is known about their function in cyanobacteria. To our knowledge, this functional characterization of FdTonB in *F. diplosiphon* provides the first report on the role of TonB in a cyanobacterium.

Fig. 8. Light-dependent differences in cell lengths and widths for *F. diplosiphon* WT, ΔtonB and $\Delta\text{tonB:WT}$ strains grown for 3 days (a, b) and 6 days (c, d) under GL and RL. Bars represent the median $\pm$ SE of 50 cells measured from each of at least three independent experiments.

Using bioinformatic sequence-similarity analysis for FdTonB, we identified characteristics of several motifs and domains of TonB-family proteins. The N-terminal portion of FdTonB has a predicted transmembrane domain and its C-terminal portion has a TonB-related domain. The conserved domains of the N- and C-termini are connected by a proline-rich linker (Fig. 1a). Previous studies in *E. coli* have shown that the N-terminal domain of TonB is anchored to the cytoplasmic membrane and makes contacts with proteins ExbB and ExbD to form a complex, which transduces the energy of the cytoplasmic membrane proton-motive force across the periplasmic space to outer-membrane transporters. The C-terminal domain of TonB proteins appears to interact directly with receptors in the outer membrane (Chu *et al.*, 2007; Postle & Larsen, 2007).

The conserved tyrosine (Y) in TonB-related domains has been implicated as a contact point with the 'TonB box' of interacting outer-membrane transporters (Pawelek *et al.*, 2006). The SSG motif is conserved in many TonB proteins and is in close proximity, though not in direct contact, with the outer-membrane receptor, and thus has been proposed to play a role in receptor recognition (Chu *et al.*, 2007). The functional significance of the Pro-rich domain, apart from serving as a periplasmic spacer, is still under debate (Chu *et al.*, 2007). However, it has been demonstrated definitively that the Pro-rich domain is not essential for function of the *E. coli* TonB protein (Larsen *et al.*, 1993). Nevertheless, this domain functions to provide a spacer that allows TonB to span the periplasmic space. Furthermore, it has been speculated that the Pro-rich region adopts a relatively rigid conformation, which may

Fig. 9. Effect of iron limitation on the growth of *F. diplosiphon* strains. (a, b) Growth curves of *F. diplosiphon* WT and $\Delta tonB$ strains in BG11 or BG11_{-Fe} medium under (a) GL or (b) RL. Growth of cell cultures was measured as OD₇₅₀ at 3 day intervals. (c–j) Cellular morphologies of *F. diplosiphon* WT and $\Delta tonB$ mutant strains grown in BG11_{-Fe} medium under GL and RL. (c–f) Representative slices from a Z-series of DIC images of WT (c, d) and $\Delta tonB$ (e, f). (g–j) Maximum-projection phycobiliprotein autofluorescence images from a Z-series of images of GL-adapted filaments of WT (g, h) and $\Delta tonB$ (i, j) captured using a 40× oil immersion lens objective with 2× zoom setting.

help in the transduction of mechanical energy from the cytoplasmic membrane to the outer membrane (Evans *et al.*, 1986). These conserved regions were found not only in FdTonB but also in closely related cyanobacterial TonB-family proteins (Fig. 2).

Notably, the FdTonB protein also has a novel central glycine-rich domain spanning amino acids 184–325, a region with (Gly-X)_n repeats, and with more than 37 % Gly residues (Fig. 1c). A similar domain was found in two cyanobacterial TonB-family proteins, which exhibit significant similarity to FdTonB (Fig. 2). (Gly-X)_n repeats were proposed to be involved in protein–protein interaction (Sachetto-Martins *et al.*, 2000); however, the function of GRPs is still not fully understood. Recently, in *Arabidopsis thaliana*, a GRP that contains a similar (Gly-X)_n domain was purported to be involved in regulation of cellular elongation processes (Mangeon *et al.*, 2009). Our mutant strain, which is devoid of the proline-rich region, glycine-rich domain and C-terminal TBRD (i.e. amino acids 131–534), exhibited a specific light-dependent phenotype including both decreased cell length and significantly increased cell width as compared to the WT strain after 6 days of growth under GL (Fig. 8c, d). Thus,

the glycine-rich region in the FdTonB protein could play a similar role in mediating the regulation of cellular morphology in *F. diplosiphon*. However, in *F. diplosiphon*, the GRP-related function of FdTonB is presumably a unique light-dependent regulatory response.

During the process of CCA, the $\Delta tonB$ mutant cell shape is not significantly different from WT under RL. However, as described above, under GL the cell shape was strikingly different from the elongated WT cell shape, indicating that FdTonB protein is required for GL-dependent cellular morphology. Our analysis of the $\Delta tonB$:WT strain segregating a WT copy of the $\Delta tonB$ gene, which recovered a WT cellular morphology, provided evidence that the GL-dependent regulation of cellular morphology requires functional FdTonB in *F. diplosiphon*.

Although our results strongly suggest a role for FdTonB in the photoregulation of cellular morphology in *F. diplosiphon*, TonB proteins are well known classically for playing a crucial role in iron uptake. Based on what we know about TonB function in Gram-negative bacteria, if FdTonB is functioning as an iron-complex transporter, we would have expected to see a difference in our $\Delta tonB$ mutant strain relative to the WT strain when grown in iron-replete medium. However, under iron-replete conditions, the response of our mutant strain was no different from that of the WT strain (Fig. 9a, b). Furthermore, both WT and $\Delta tonB$ showed severe reductions in growth in iron-limited medium under RL, and limited reductions in growth in GL. The light-dependent response has been previously noted under iron-limited conditions for *F. diplosiphon* (B. Pattanaik, B. N. Sawyer & B. L. Montgomery, unpublished data). The indistinguishable response of the WT and mutant strains under grown in iron-replete vs iron-limited conditions suggests that FdTonB function is not correlated with the organism's response to iron limitation. Therefore, we suggest that FdTonB does not play a central role in iron transport, but possesses a novel function of regulating light-dependent cellular morphology in *F. diplosiphon*.

Morphological differences under iron-limited conditions are species specific. In the marine cyanobacterium *Trichodesmium*, no morphological difference was observed other than shortened and fragmented filaments under iron-limited conditions (Küpper *et al.*, 2008). Analyses of *Anacystis nidulans* R2 demonstrated that growth in iron-deficient conditions resulted in cells that were shorter by as much as half than cells growing in iron-replete conditions (Sherman & Sherman, 1983). Notably, the expression of genes known to be involved in the cell envelope pathway was also affected by iron limitation in *Synechocystis* sp. PCC 6803 (Singh *et al.*, 2003). In the present study, cellular morphology of the WT cells grown in iron-limited medium under GL conditions resembled that of the $\Delta tonB$ mutant; this is very interesting, but the mechanism is not yet understood. Under RL conditions, both strains formed vacuoles in the cell when grown in iron-limited conditions, which is indicative of a stress response. Based

on our results, FdTonB is not involved in the classical TonB-regulated iron transport system, which suggests either the presence of additional TonB-like proteins or alternatively different pathway(s) for iron transportation in *F. diplosiphon* that could be worthy of exploration. For example, the Feo system (Ferrous iron transport) is also used in bacterial systems for ferrous iron uptake (Cartron *et al.*, 2006). Some Feo proteins, for example a permease FeoB that is used for transportation of iron into a cell, have been identified in cyanobacterial systems including *Nostoc* sp. PCC 7120 and *Synechocystis* sp. PCC 6803 (Cartron *et al.*, 2006). Notably, although the definitive answer to whether additional TonB-related proteins or proteins such as FeoB exist in *F. diplosiphon* awaits the availability of the full genome sequence, TonB-family proteins appear to be present as single members, or rarely two members, in related cyanobacteria based on sequence similarity searches (data not shown; Mirus *et al.*, 2009).

In summary, our results indicate that the FdTonB protein is essential for correct regulation of cellular morphology during the process of CCA. Prior results indicated that the photoreceptor RcaE regulates cell shape via downstream effectors RcaF and RcaC in response to RL in *F. diplosiphon* (Bordowitz & Montgomery, 2008; Bordowitz *et al.*, 2010). Our current results lead us to suggest that FdTonB is important for GL-dependent regulation of cell shape. However, our RT-PCR results indicated that *FdtonB* is not regulated directly by RcaE. Thus, the regulatory factor that controls the previously reported GL-dependent upregulation of *tonB* remains to be identified. However, GL upregulation of *tonB* expression could depend upon recognized pathways such as the GL inductive Cgi pathway that has been established but not fully characterized at the molecular level in *F. diplosiphon* (Kehoe & Gutu, 2006). This first report of a biochemical function for a cyanobacterial TonB protein establishes FdTonB as a novel GL-regulated effector required for photoregulation of cellular morphology in *F. diplosiphon*.

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REFERENCES

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Bennett, A. & Bogorad, L. (1973). Complementary chromatic adaptation in a filamentous blue-green alga. *J Cell Biol* **58**, 419–435.
- Bogorad, L. (1975). Phycobiliproteins and complementary chromatic adaptation. *Annu Rev Plant Physiol* **26**, 369–401.
- Bordowitz, J. R. & Montgomery, B. L. (2008). Photoregulation of cellular morphology during complementary chromatic adaptation requires sensor-kinase-class protein RcaE in *Fremyella diplosiphon*. *J Bacteriol* **190**, 4069–4074.
- Bordowitz, J. R., Whitaker, M. J. & Montgomery, B. L. (2010). Independence and interdependence of the photoregulation of pigmentation and development in *Fremyella diplosiphon*. *Comm Integ Biol* (in press).
- Cai, Y. P. & Wolk, C. P. (1990). Use of a conditionally lethal gene in *Anabaena* sp. strain PCC 7120 to select for double recombinants and to entrap insertion sequences. *J Bacteriol* **172**, 3138–3145.
- Campbell, D. (1996). Complementary chromatic adaptation alters photosynthetic strategies in the cyanobacterium *Calothrix*. *Microbiology* **142**, 1255–1263.
- Cartron, M. L., Maddocks, S., Gillingham, P., Craven, C. J. & Andrews, S. C. (2006). Feo – transport of ferrous iron into bacteria. *Biomaterials* **19**, 143–157.
- Chu, B. C., Peacock, R. S. & Vogel, H. J. (2007). Bioinformatic analysis of the TonB protein family. *Biomaterials* **20**, 467–483.
- Cogley, J. G., Zerweck, E., Reyes, R., Mody, A., Seludo-Unson, J. R., Jaeger, H., Weerasuriya, S. & Navankasattusas, S. (1993). Construction of shuttle plasmids which can be efficiently mobilized from *Escherichia coli* into the chromatically adapting cyanobacterium, *Fremyella diplosiphon*. *Plasmid* **30**, 90–105.
- Cogley, J., Seneviratne, L., Drong, L., Thounaojam, M., Oda, J. F. & Carroll, J. (1999). Transposition of Tn5 derivatives in the chromatically adapting cyanobacterium, *Fremyella diplosiphon*. In *The Phototrophic Prokaryotes*, pp. 443–451. Edited by G. Peschek, W. Löffelhardt & G. Schmetterer. New York: Kluwer Academic.
- Cogley, J. G., Clark, A. C., Weerasurya, S., Queseda, F. A., Xiao, J. Y., Bandrapali, N., D'Silva, I., Thounaojam, M., Oda, J. F. & other authors (2002). CpeR is an activator required for expression of the phycoerythrin operon (*cpeBA*) in the cyanobacterium *Fremyella diplosiphon* and is encoded in the phycoerythrin linker-polypeptide operon (*cpeCDEST*). *Mol Microbiol* **44**, 1517–1531.
- Cserző, M., Wallin, E., Simon, I., von Heijne, G. & Elofsson, A. (1997). Prediction of transmembrane alpha-helices in prokaryotic membrane proteins: the dense alignment surface method. *Protein Eng* **10**, 673–676.
- Divakaruni, A. V., Loo, R. R. O., Xie, Y., Loo, J. A. & Gober, J. W. (2005). The cell-shape protein MreC interacts with extracytoplasmic proteins including cell wall assembly complexes in *Caulobacter crescentus*. *Proc Natl Acad Sci U S A* **102**, 18602–18607.
- Elhai, J. & Wolk, C. P. (1988). Conjugal transfer of DNA to cyanobacteria. *Methods Enzymol* **167**, 747–754.
- Evans, J. S., Levine, B. A., Trayer, I. P., Dorman, C. J. & Higgins, C. F. (1986). Sequence-imposed structural constraints in the TonB protein of *Escherichia coli*. *FEBS Lett* **208**, 211–216.
- Finn, R. D., Mistry, J., Schuster-Bockler, B., Griffiths-Jones, S., Hollich, V., Lassmann, T., Moxon, S., Marshall, M., Khanna, A. & other authors (2006). Pfam: clans, web tools and services. *Nucleic Acids Res* **34**, D247–D251.
- Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M. R., Appel, R. D. & Bairoch, A. (2005). Protein identification and analysis tools on the ExPASy server. In *The Proteomics Protocols Handbook*, pp. 571–607. Edited by J. M. Walker. Totowa, NJ: Humana Press.

- Kato, H., Hagino, N., Grossman, A. R. & Ogawa, T. (2001). Genes essential to iron transport in the cyanobacterium *Synechocystis* sp. strain PCC 6803. *J Bacteriol* **183**, 2779–2784.
- Kehoe, D. M. & Gutu, A. (2006). Responding to color: the regulation of complementary chromatic adaptation. *Annu Rev Plant Biol* **57**, 127–150.
- Keren, N., Aurora, R. & Pakrasi, H. B. (2004). Critical roles of bacterioferritins in iron storage and proliferation of cyanobacteria. *Plant Physiol* **135**, 1666–1673.
- Küpper, H., Šetlík, I., Seibert, S., Prášil, O., Šetlikova, E., Strittmatter, M., Levitan, O., Lohscheider, J., Adamska, I. & Berman-Frank, I. (2008). Iron limitation in the marine cyanobacterium *Trichodesmium* reveals new insights into regulation of photosynthesis and nitrogen fixation. *New Phytol* **179**, 784–798.
- Larsen, R. A. & Postle, K. (2001). Conserved residues Ser₁₆ and His₂₀ and their relative positioning are essential for TonB activity, cross-linking of TonB with ExbB, and the ability of TonB to respond to proton motive force. *J Biol Chem* **276**, 8111–8117.
- Larsen, R. A., Wood, G. E. & Postle, K. (1993). The conserved proline-rich motif is not essential for energy transduction by *Escherichia coli* TonB protein. *Mol Microbiol* **10**, 943–953.
- Larsen, R. A., Myers, P. S., Skare, J. T., Seachord, C. L., Darveau, R. P. & Postle, K. (1996). Identification of TonB homologs in the family *Enterobacteriaceae* and evidence for conservation of TonB-dependent energy transduction complexes. *J Bacteriol* **178**, 1363–1373.
- Maglott, D., Ostell, J., Pruitt, K. D. & Tatusova, T. (2007). Entrez Gene: gene-centered information at NCBI. *Nucleic Acids Res* **35**, D26–D31.
- Mangeon, A., Magioli, C., Menezes-Salgueiro, A. D., Cardeal, V., de Oliveira, C., Galvao, V. C., Margis, R., Engler, G. & Sachetto-Martins, G. (2009). AtGRP5, a vacuole-located glycine-rich protein involved in cell elongation. *Planta* **230**, 253–265.
- Marchler-Bauer, A., Anderson, J. B., Chitsaz, F., Derbyshire, M. K., DeWeese-Scott, C., Fong, J. H., Geer, L. Y., Geer, R. C., Gonzales, N. R. & other authors (2009). CDD: specific functional annotation with the Conserved Domain Database. *Nucleic Acids Res* **37**, D205–D210.
- Mirus, O., Strauss, S., Nicolaisen, K., von Haeseler, A. & Schleiff, E. (2009). TonB-dependent transporters and their occurrence in cyanobacteria. *BMC Biol* **7**, 68.
- Moock, G. S. & Coulton, J. W. (1998). TonB-dependent iron acquisition: mechanisms of siderophore-mediated active transport. *Mol Microbiol* **28**, 675–681.
- Montgomery, B. L. (2008). Shedding new light on the regulation of complementary chromatic adaptation. *Cent Eur J Biol* **3**, 351–358.
- Neilands, J. B. (1995). Siderophores: structure and function of microbial iron transport compounds. *J Biol Chem* **270**, 26723–26726.
- Nicolaisen, K., Moslavac, S., Samborski, A., Valdebenito, M., Hantke, K., Maldener, I., Muro-Pastor, A. M., Flores, E. & Schleiff, E. (2008). Alr0397 is an outer membrane transporter for the siderophore schizokinen in *Anabaena* sp. Strain PCC 7120. *J Bacteriol* **190**, 7500–7507.
- Pawelek, P. D., Croteau, N., Ng-Thow-Hing, C., Khursigara, C. M., Moiseeva, N., Allaire, M. & Coulton, J. W. (2006). Structure of TonB in complex with FhuA, *E. coli* outer membrane receptor. *Science* **312**, 1399–1402.
- Postle, K. & Good, R. F. (1983). DNA sequence of the *Escherichia coli* tonB gene. *Proc Natl Acad Sci U S A* **80**, 5235–5239.
- Postle, K. & Kadner, R. J. (2003). Touch and go: tying TonB to transport. *Mol Microbiol* **49**, 869–882.
- Postle, K. & Larsen, R. A. (2007). TonB-dependent energy transduction between outer and cytoplasmic membranes. *Biometals* **20**, 453–465.
- Sachetto-Martins, G., Franco, L. O. & de Oliveira, D. E. (2000). Plant glycine-rich proteins: a family or just proteins with a common motif? *Biochim Biophys Acta* **1492**, 1–14.
- Schalk, I. J., Yue, W. W. & Buchanan, S. K. (2004). Recognition of iron-free siderophores by TonB-dependent iron transporters. *Mol Microbiol* **54**, 14–22.
- Schwyn, B. & Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* **160**, 47–56.
- Seib, L. O. & Kehoe, D. M. (2002). A turquoise mutant genetically separates expression of genes encoding phycoerythrin and its associated linker peptides. *J Bacteriol* **184**, 962–970.
- Sherman, D. M. & Sherman, L. A. (1983). Effect of iron deficiency and iron restoration on ultrastructure of *Anacystis nidulans*. *J Bacteriol* **156**, 393–401.
- Singh, A. K., McIntyre, L. M. & Sherman, L. A. (2003). Microarray analysis of the genome-wide response to iron deficiency and iron reconstitution in the cyanobacterium *Synechocystis* sp. PCC 6803. *Plant Physiol* **132**, 1825–1839.
- Stowe-Evans, E. L., Ford, J. & Kehoe, D. M. (2004). Genomic DNA microarray analysis: identification of new genes regulated by light color in the cyanobacterium *Fremyella diplosiphon*. *J Bacteriol* **186**, 4338–4349.
- Thomas, C. M. & Smith, C. A. (1987). Incompatibility group P plasmids: genetics, evolution, and use in genetic manipulation. *Annu Rev Microbiol* **41**, 77–101.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

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