The competence gene, *comF*, from *Synechocystis* sp. strain PCC 6803 is involved in natural transformation, phototactic motility and piliation

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The gene *slr0388* was previously annotated to encode a hypothetical protein in *Synechocystis* sp. strain PCC 6803. When a positively phototactic strain of this cyanobacterium was insertionally inactivated at *slr0388*, the mutants were not transformable, and appeared to aggregate as a result of increased bundling of type IV pili. Also, these mutants were rendered non-phototactic compared to the wild-type. Quantitative real-time PCR revealed a 3.5-fold increase in *pilA1* transcript levels in the mutant over wild-type cells, while there were no changes in the level of *pilT1* and *comA* transcripts. Supernatant from mutant liquid culture contained more PilA1 protein, confirmed by mass spectrometric analysis, compared to the wild-type cells, which corresponded to the increase in *pilA1* transcripts. The increase in PilA1 subunits may contribute to the bundling morphology of pili that was observed, which in turn may act to retard DNA uptake by hindering the retraction of pili. This gene is therefore proposed to be designated *comF*, as it possesses a phosphoribosyltransferase domain, a distinguishing feature of other ComF proteins of naturally transformable heterotrophic bacteria. This report is the second of a competence-related gene from *Synechocystis* sp. strain PCC 6803, the product of which does not show homology to other well-studied type IV pili proteins.

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INTRODUCTION

The natural transformability of the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 has led to the establishment of protocols that have been used in studies of photosynthesis and phototaxis (Bhaya *et al.*, 2001; Fiedler *et al.*, 2005; Hubschmann *et al.*, 2005; Williams, 1988). Initial reports of the natural transformability of this strain also characterized the physiological factors that affected its transformation efficiency, including phase of growth and DNA concentration (Barten & Lill, 1995; Grigorieva & Shestakov, 1982). More recently, the molecular machinery of the type IV pili (Tfp) system has been implicated in the transformability of *Synechocystis* sp. strain PCC 6803 (Yoshihara *et al.*, 2001). A functional Tfp apparatus is also critical for phototaxis, while the regulatory signals and pathways underlying the cells' response to light are rapidly being unravelled (Bhaya, 2004; Bhaya *et al.*, 2001; Fiedler *et al.*, 2005; Yoshihara *et al.*, 2000; Yoshimura *et al.*, 2002). It is apparent that transformability and phototaxis in *Synechocystis* sp. strain PCC 6803 are intrinsically linked via a functional Tfp system, as the loss of function of Tfp components almost always affects these two processes.

As extracellular appendages found on the cell surface of many Gram-negative bacteria, Tfp are involved in physiological processes such as biofilm formation and twitching motility (Wall & Kaiser, 1999). Notably, many Tfp-possessing organisms are also naturally transformable, including *Neisseria gonorrhoeae*, *Pseudomonas stutzeri* and *Acinetobacter calcoaceticus* (Fussenegger *et al.*, 1997; Graupner *et al.*, 2000; Palmen & Hellingwerf, 1997). In *Synechocystis* sp. strain PCC 6803, at least 12 Tfp components have been shown to affect transformation (Nakasugi & Neilan, 2006; Okamoto & Ohmori, 2002; Yoshihara *et al.*, 2001). Many of these Tfp components are absolutely critical for transformability, such as the PilA1 and PilT1 proteins. PilA1 is the structural subunit of the pilus filament, whereas PilT1 is thought to be required for the disassembly of PilA1 subunits, thus generating the force necessary for the functionality of the Tfp apparatus via retraction of the pilus filament (Okamoto & Ohmori, 2002; Yoshihara *et al.*, 2001). Mutants deficient in PilA1 do not exhibit Tfp, while mutants of PilT1 are hyperpiliated.

In *Bacillus subtilis*, the regulation and development of competence is complex, involving a myriad of competence factors and pathways (Dubnau, 1991; Hamoen *et al.*, 2003; Piazza *et al.*, 1999; Sinderen *et al.*, 1995). Apart from one competence-specific factor, *slr0197* or *comA* (Yoshihara *et al.*, 2001; Yura *et al.*, 1999), virtually nothing is known of

Abbreviations: PRT, phosphoribosyltransferase; qPCR, quantitative real-time PCR; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

the competence proteins and pathways involved in transformation in *Synechocystis* sp. strain PCC 6803. As the pathways that dictate phototaxis in *Synechocystis* sp. strain PCC 6803 are rapidly being identified, there is a greater need to identify the particular factors that only affect transformability, so that the differentiating features of these processes can be characterized. Our investigations of competence-specific factors in *Synechocystis* sp. strain PCC 6803 have identified a hypothetical protein, encoded by the ORF *slr0388* in CyanoBase (Nakamura *et al.*, 1998), that has limited sequence similarity (<50%) to the ComF competence proteins of other naturally transformable bacteria. This report describes the phenotypic effects of this second competence factor found in *Synechocystis* sp. strain PCC 6803.

METHODS

Bacterial strains and culturing. The positively phototactic strain of *Synechocystis* sp. strain PCC 6803 was a kind gift from Devaki Bhaya (Department of Plant Biology, Carnegie Institution, Stanford, USA). Cells were grown either in BG-11 liquid medium (Rippka *et al.*, 1979), or on BGTS agar (1%, w/v) plates consisting of BG-11 supplemented with 10 mM TES (pH 8.2 with KOH) and 0.3% (w/v) sodium thiosulfate (Williams, 1988). Where appropriate, antibiotics were added (7 µg chloramphenicol ml⁻¹, 5 µg kanamycin ml⁻¹ or 5 µg spectinomycin ml⁻¹). Cultures were grown without shaking under continuous light (25 µmol photons m⁻² s⁻¹) supplied by cool white fluorescent lamps at 28 ± 1 °C. *Escherichia coli* DH5α was cultured at 37 °C on LB medium, and 100 µg ampicillin ml⁻¹ was added for use during cloning procedures.

Bioinformatics. A total of 96 nucleotide and amino acid sequences of competence-related genes from *Bacillus subtilis*, *Streptococcus pneumoniae*, *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Acinetobacter calcoaceticus* sp. strain BD413 and *Pseudomonas stutzeri* were compiled from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) and were individually compared against the *Synechocystis* sp. strain PCC 6803 genome using BLASTN and BLASTP (Altschul *et al.*, 1997), accessed through CyanoBase (Nakamura *et al.*, 1998). All potential homologues, including those with low sequence identities and similarities, were recorded and used in multiple alignments in CLUSTALW (Thompson *et al.*, 1994), as accessed through BioManager at the Australian National Genomic Information Service (<http://www.angis.org.au>). Protein motifs were identified using GenomeNet at the Bioinformatics Center, Institute for Chemical Research, Kyoto University (<http://motif.genome.jp/>).

Gene disruption. A 2.5 kbp fragment containing the ORF *slr0388* from *Synechocystis* sp. strain PCC 6803 genomic DNA was amplified by PCR using the primers *slr0388F* (5'-TGGATTAAACCGAGTTGGAAA-3') and *slr0388R* (5'-CGCATTTCTTCAGCAAAAATT-3'). The product was ligated into the pGEM-T Easy vector (Promega) according to the manufacturer's instructions. The resulting plasmid was then digested with *MunI* and ligated to a chloramphenicol resistance cassette derived from *Campylobacter coli* (Wang & Taylor, 1990). The final plasmid, p0388Cm, engineers a disruption of *slr0388* at residue 179 of the ORF, with 1 kb and 1.5 kb of *Synechocystis* sp. strain PCC 6803 genome sequence flanking either side of the chloramphenicol cassette. The inserted cassette contained two inverted repeats after a triple stop codon at the 3'-end of the sequence (Wang & Taylor, 1990). *Synechocystis* sp. strain PCC 6803 was transformed with p0388Cm, as described previously (Williams,

1988), with slight modifications. Exponential-phase cells were incubated for 6 h with a final DNA concentration of 2 µg ml⁻¹. Cells were spread onto surfactant-free nitrocellulose filters (0.45 µm pore size, 82 mm, Millipore) on 1% agar BGTS plates, and then transferred to selective plates after 24 h. Transformants were observed after 1 week. Complete chromosomal segregation of transformants was confirmed by PCR with primers *slr0388F* and *slr0388R*, as well as an additional primer, UP0388 (5'-TGGAAACGGAGCATCAAG-3'), situated upstream of the binding site of *slr0388F* in the *Synechocystis* sp. strain PCC 6803 genome.

Competency assay. To test the competency of *slr0388* inactivation mutants, a plasmid, p0161Km, was constructed with a kanamycin cassette disrupting the *pilT1* (*slr0161*) gene of *Synechocystis* sp. strain PCC 6803, essentially as described above. The primers used were 0161F (5'-GCCCCCAGTAATAAATCATC-3') and 0161R (5'-ATCATGACGGAAAACGTGCG-3'). Disruption of *pilT1* results in non-motile cells and a hyperpilated phenotype (Okamoto & Ohmori, 2002) that facilitates the confirmation of transformant status. Transformation of cells was performed as described above using a final concentration of 2 µg ml⁻¹ of p0161Km. Transformants were again confirmed for complete chromosomal segregation by PCR.

Complementation. The *slr0388* mutant was complemented with the wild-type *slr0388* gene. A vector was constructed in which the *slr0388* gene, ligated to the *psbA* promoter of *Synechocystis* sp. PCC 6803, was inserted into an integrative vector pKW1188 (a kind gift from Xudong Xu, Institute of Hydrobiology, Chinese Academy of Sciences) (Williams, 1988) at the *PstI* multiple cloning site. This construct, which encodes kanamycin resistance, was electroporated into the *slr0388* mutant as follows. Cells were grown in 100 ml BG-11 to an OD₇₃₀ of 0.4 (LKB Biochrom Ultraspec II), chilled on ice and harvested at 4 °C. The pellet was washed three times in 5 ml 1 mM HEPES buffer (pH 7.0) and resuspended in 200 µl of the 1 mM HEPES buffer. DNA was added (2 µg) to 40 µl of the resuspended cells to a final concentration of 10 µg ml⁻¹ and incubated on ice for 3 min. Electroporation was carried out in 0.2 cm cuvettes at 2.5 kV, 25 µF and 200 Ω settings, after which 500 µl BG-11 was added. Cells were then spread onto 0.45 µm pore-size nitrocellulose membrane filters (Millipore) placed onto BGTS plates. After 24 h recovery, filters were transferred onto selective BGTS plates (7 µg chloramphenicol ml⁻¹ and 5 µg kanamycin ml⁻¹). Transformants appeared after 2–4 weeks and were confirmed for complete chromosomal segregation by PCR. The competency of the complemented mutants was tested as described above, but with a construct in which the gene *slr0769* was insertionally inactivated with a spectinomycin resistance cassette.

Electron microscopy. Cells examined by transmission and scanning electron microscopy (TEM and SEM) were prepared either from exponentially grown liquid cultures or from agar plates, as described previously (Nakasugi & Neilan, 2005). For TEM, cells from agar plates were either resuspended in 100 µl BG-11, or applied directly to Formvar-coated copper grids.

Phototaxis motility assay. Phototaxis motility assays were carried out on 1% agar BGTS plates. Cells were streaked in a straight line and incubated perpendicular to a unidirectional light source. Movement of cells towards or away from the light source was observed after growth for 3 days and is typified by finger-like projections from the streak.

RNA extraction. Fifty millilitres of mid-exponential-phase cultures of OD₇₃₀ between 0.6 and 0.7 (LKB Biochrom Ultraspec II) were harvested, snap-frozen in liquid nitrogen and stored at -80 °C. RNA was extracted within a week of harvesting, as described elsewhere

(Schmidt-Goff & Federspiel, 1993). RNA extracts were treated with RQ DNase (Promega) and confirmation of successful DNase treatment was assessed by PCR.

RT-PCR. Specific primers were designed to amplify regions of approximately 200 nucleotides of the *pilA1* (*sll1694* in CyanoBase) (forward, 5'-GCTATCCAAGACGCAACG-3'; reverse, 5'-ACTTC-AGCACCACCACAATC-3'), *pilT1* (*slr0161* in CyanoBase) (forward, 5'-CACTTGGTTTTTGGCACTCTC-3'; reverse, 5'-TTGGGGGAC-TTTTCTTGAC-3') and *comA* (*slr0197* in CyanoBase) (forward, 5'-CACAGAAGATGGCAGTAAAG-3'; reverse, 5'-TGAAAAGAT-GGGCGACAGC-3') genes of *Synechocystis* sp. strain PCC 6803. Reverse transcription was performed with 200 units M-MLV reverse transcriptase, RNase H-minus (Promega), with 5 pmol reverse primer annealed to 150 ng RNA. Reaction conditions were 70 °C for 5 min, on ice for 5 min, 55 °C for 150 min and 70 °C for 15 min. One microlitre of each reaction was used for subsequent quantitative real-time PCRs (qPCR).

qPCR. Transcript levels were quantified by qPCR using the Rotor-Gene 3000 system (Corbett). Amplifications were performed using the Platinum SYBR green qPCR supermix UDG kit (Invitrogen). Two-step cycling was performed and comprised hold steps of 50 °C for 2 min and 95 °C for 2 min, and amplification for 50 cycles at 95 °C for 15 s and 60 °C for 30 s. Absolute and relative (normalized to wild-type) levels of each specific transcript were determined, as described elsewhere (Steunou *et al.*, 2006; Whelan *et al.*, 2003). Analyses were performed using triplicate technical replicates from triplicate biological cultures.

Extraction of pili. A simple method was adopted to investigate differences in the extracellular pili content of cells in order to compare the observed morphological and transcript level changes. Five millilitres of late-exponential-phase cultures at OD₇₃₀ 0.9 for each strain were first subjected to vortexing at the maximum setting for 1 min and then centrifuged at 2300 g for 15 min. Equal volumes of the supernatants were carefully taken without disturbing the cell pellet. The supernatants were then subjected to ammonium sulfate precipitation (final concentration 100 %) to remove proteins from solution. The supernatants were then centrifuged at 16 000 g for 15 min and the pellets resuspended in 50 µl 20 mM Tris/HCl (pH 8.0). Equal volumes of the pili extracts were electrophoresed on a 12 % SDS-PAGE gel to determine differences in protein-banding patterns and intensities. In addition, total protein concentration of the resuspended pellets was measured by the Nanodrop spectrophotometer ND-1000 (Nanodrop Technologies).

Mass spectrometric analysis. Protein bands that appeared to be differentially expressed between the wild-type and mutant cells were excised from the SDS-PAGE gels and the peptide sequence analysed by liquid chromatography tandem mass spectrometry (Gatlin *et al.*, 1998; Shevchenko *et al.*, 1996) at the Bioanalytical Mass Spectrometry Facility (BMSF), University of New South Wales. Mass spectra of the protein bands were subjected to the Mascot MS/MS ion search (Matrix Science) to enable peptide sequence determination.

The Mascot search was performed against the non-redundant National Center for Biotechnology Information (nr-NCBI) sequence database.

RESULTS

Identification of a *Synechocystis* sp. strain PCC 6803 gene involved in phototactic motility and transformation

Database searches and multiple sequence alignments revealed a hypothetical protein from *Synechocystis* sp. strain PCC 6803, gene designation *slr0388* in CyanoBase, that had moderate sequence identity to the competence proteins ComFC from *B. subtilis* (P39147), Com101A (also known as ComF in GenBank) from *H. influenzae* (P31773), ComF from *P. stutzeri* (CAB56477) and the putative competence protein ComFC from *S. pneumoniae* (AAL00814) (Table 1). These competence proteins also exhibited only moderate sequence identity to each other. Multiple alignments of the five competence proteins indicated two conserved regions (Fig. 1). The first conserved region (between residues 127 and 147) did not match any known protein motifs or domains in protein databases. The second conserved region (between residues 207 and 216) harboured a phosphoribosyltransferase (PRT) domain signature from the Pfam (PF00156) and Prosite (PS00103) databases. In addition, alignments showed that these proteins possess a putative tetracysteine motif (Fig. 1, between residues 30 and 48).

Since numerous genes involved in transformation in *Synechocystis* sp. strain PCC 6803 also affect phototactic motility, we investigated if this was also the case for *slr0388*. When the positively phototactic strain of *Synechocystis* sp. strain PCC 6803 was insertionally inactivated at *slr0388*, phototaxis was completely abolished after complete segregation at the gene locus (15 individual mutants were assayed) (Fig. 2B). Merodiploids, harbouring both the wild-type and insertionally inactivated *slr0388* gene (determined by PCR), still exhibited phototaxis. When the *slr0388* mutant was complemented with the wild-type *slr0388* gene, phototactic motility was restored (Fig. 2C), confirming that it is required for phototactic motility.

To investigate the involvement of *slr0388* in natural transformation, competency assays were performed on

Table 1. Sequence identities and similarities (parentheses) of the ComF group of proteins from the naturally transformable *Synechocystis* sp. strain PCC 6803, *B. subtilis*, *H. influenzae*, *P. stutzeri* and *S. pneumoniae*

	<i>B. subtilis</i> ComFC	<i>H. influenzae</i> Com101A	<i>P. stutzeri</i> ComF	<i>S. pneumoniae</i> ComFC
Slr0388	20 % (44 %)	24 % (48 %)	27 % (50 %)	21 % (40 %)
<i>B. subtilis</i> ComFC	–	21 % (45 %)	23 % (47 %)	35 % (61 %)
<i>H. influenzae</i> Com101A		–	33 % (64 %)	20 % (48 %)
<i>P. stutzeri</i> ComF			–	21 % (45 %)

Fig. 1. Multiple alignment of the ComF group of proteins from the naturally transformable *Synechocystis* sp. strain PCC 6803 (Slr0388), *B. subtilis* (Bs), *H. influenzae* (Hi), *P. stutzeri* (Ps) and *S. pneumoniae* (Sp). Conserved cysteine residues at positions 30, 33, 45 and 48 of the alignment indicate a putative tetracysteine motif. The region between positions 127 and 147 was not similar to any known conserved sequences from protein motif searches. The conserved region between positions 207 and 216 indicates a phosphoribosyl transferase (PRT) domain.

three individual mutants. Assays were carried out in triplicate, and in all cases the *slr0388* mutants were not transformable with p0161Cm, whereas the wild-type control displayed normal competence, typically between 800 and 1200 transformants per 1.4×10^8 cells when transformed with this plasmid. The transformation efficiency of wild-type *Synechocystis* sp. strain PCC 6803 appears to be highly variable, even when transformation is carried out on the same culture in multiple replicates. This has also been observed in another laboratory (Devaki Bhaya, personal communication). Nonetheless, it was clear that the *slr0388* mutants were not transformable with p0161Cm. When the *slr0388* mutant was complemented with the wild-type *slr0388* gene, transformability was restored to that of the wild-type. Thus, the results from the phototaxis and competency assays indicated that *slr0388* affects both phototactic motility and natural transformation, although the mechanistic level at which it is involved has yet to be determined.

Piliation characteristics of *slr0388* mutants

Phototaxis in *Synechocystis* sp. strain PCC 6803 is dependent on Tfp biogenesis and functionality (Bhaya *et al.*, 2000, 2001). As phototaxis appeared to be affected in the *slr0388* mutants, TEM and SEM were performed to investigate

changes in their piliation characteristics. TEM preparations did not indicate any notable differences in the complement of pili between wild-type and *slr0388* mutant cells (more than 50 cells were observed for each) (Fig. 3), although the number of pili observed here did not appear to be as profuse as that reported previously (Bhaya *et al.*, 2000). This could be due to the different methods of staining and variations in growth conditions. The diameter of individual pili on one cell was between 6 and 9 nm for both the wild-type and *slr0388* mutant. However, there were clear instances of thicker, 'bundled' pili comprising individual filaments, amongst other individual pili (Fig. 3, black arrows). This has also been reported in another study (Yoshihara *et al.*, 2001). The diameter of bundled pili ranged between 13 and 40 nm, depending on the number of individual filaments making up a given bundle. Although bundling of pili occurred in both wild-type and *slr0388* mutant strains (four individual mutants were observed under TEM), it appeared to occur more frequently in the mutant cells (27.5 ± 6.5 bundles per cell; mean $\pm$ SD) than in wild-type cells (19.9 ± 6.1 bundles per cell) (Fig. 3). The high standard deviations were due to the high variability of the occurrence of bundling in both wild-type (between 7 and 30 bundles per cell) and mutant cells (between 15 and 40 bundles per cell). A two-tailed, two-sample unequal variance *t*-test was carried out on the difference in the number of bundled pili between the

Fig. 2. Motility assays of *Synechocystis* sp. strain PCC 6803. The arrow indicates the unidirectional light source. (A, B) Wild-type and *slr0388* mutant of the positively phototactic strain, respectively. (C) The *slr0388* mutant complemented with the wild-type *slr0388* gene. Motility is indicated by finger-like projections, which represent cells moving toward the light source.

wild-type and mutant strains, which was found to be statistically significant ($P=0.02$, with an alpha level of 0.05). Bundles of pili appeared to be stretched outwards and were more rigid in mutant cells compared to the wild-type, and were most rigid and profuse when other cells were in close proximity (data not shown).

In scanning electron micrographs, wild-type cells exhibited thick fimbrial-like structures, some of which appeared to interconnect cells (Fig. 4A, C). These thick structures could equate to the bundled pili seen in TEM preparations. *slr0388* mutant cells also exhibited the bundled pili, which were in higher numbers. Some of the bundled pili were entangled, which appeared to facilitate the aggregation of cells (Fig. 4B, D, arrows).

When *slr0388* mutants were grown in liquid culture, the cells tended to aggregate to form string-like structures held together by a transparent matrix. This occurred during the

early- to mid-exponential phase of growth. When the cultures were shaken, these structures were difficult to disperse and further aggregated to form filaments, indicating that the cells were strongly adhered to each other. However, this characteristic was only observed in larger volumes of cultures (>50 ml). Wild-type cells in most instances did not display this property, and in the very rare cases where it was seen, the association was not as pronounced as in the mutants, and was easily dispersed by shaking. SEM preparations of the aggregated filaments of the mutants showed that the cells appeared to be held together in a matrix comprising bundled thick filaments (Fig. 5), similar to what was observed in the SEM preparations from agar plates.

Analysis of *pilA1*, *pilT1* and *comA* transcripts

The relative expression of three genes (*pilA1*, *pilT1* and *comA*) in the *slr0388* mutant, with respect to that of the

Fig. 3. TEM of *Synechocystis* sp. strain PCC 6803 wild-type (A, C) and *slr0388* mutant cells (B, D). Top and bottom panels represent cells from liquid and agar plate preparations, respectively. Top panels: magnification 80 000. Bottom panels: magnification 50 000. Scale bars, 200 nm. Black arrows indicate thick, bundled pili amongst other thinner, individual pili (white arrows).

Fig. 4. SEM of *Synechocystis* sp. strain PCC 6803 cells from agar plates. (A, C) Wild-type cells. (B, D) *slr0388* mutant cells. Top panels: magnification 12 000 (scale bars, 5 μ m). Bottom panels: magnification 24 000 (scale bars, 2 μ m). Arrows indicate complexes of bundled pili.

wild-type of the positively phototactic strain, was determined by qPCR. This genetic complement is known to be required for transformation in *Synechocystis* sp. strain PCC 6803 (Yoshihara *et al.*, 2001; Yura *et al.*, 1999). Triplicate cultures were harvested at mid-exponential phase ($OD_{730} \sim 0.6$ – 0.7) for RNA extraction. The levels of *pilT1* and *comA* transcripts in the mutant did not appear to alter from that of the wild-type. However, *pilA1*, which is translated into the subunits

Fig. 5. Representative SEM image of aggregated filaments isolated from liquid culture of the *slr0388* mutant. Filaments were formed only in larger volumes of culture (>50 ml). Wild-type cells did not display this property (refer to text). Magnification 12 000 (scale bar, 5 μ m).

Fig. 6. SDS-PAGE gel of supernatant extracts from the wild-type, *slr0388* mutant and *pilT1* mutant strains of *Synechocystis* sp. strain PCC 6803. Extracts were identically prepared for each strain from cultures at an identical OD_{730} of 0.9, and equal volumes of each resuspended extract were loaded. Lanes 1, 2 and 3: supernatant samples from wild-type, *slr0388* mutant and *pilT1* mutant liquid culture, respectively, before ammonium sulfate precipitation. Lanes 4, 5 and 6: supernatant samples from wild-type, *slr0388* mutant and *pilT1* mutant liquid culture, respectively, after ammonium sulfate precipitation and concentration. A differentially expressed protein band between the different cell extracts can be seen at approximately 21 kDa. Mass spectrometric sequencing of the 21 kDa bands from lanes 4, 5 and 6 showed that it is the PilA1 subunit of *Synechocystis* sp. strain PCC 6803 (see text for details).

that constitute the Tfp filaments, was transcribed at levels 3.5-fold higher in the mutant compared to the wild-type.

Differences in PilA1 expression between wild-type and mutant cells

Pili extraction from the culture supernatants of the *slr0388* mutant and wild-type (at equal OD_{730}) revealed the presence of an approximately 21 kDa protein band, which appeared to be present in higher concentration in the *slr0388* mutant supernatant (Fig. 6, lanes 4 and 5). Total protein concentration of the concentrated mutant supernatant (2.1 mg ml^{-1}) was three times higher than that of the wild-type concentrated supernatant (0.71 mg ml^{-1}), which correlates with the intensity of the 21 kDa bands observed on the SDS-PAGE gel. As a control, the same pili extraction was also carried out on a *pilT1* mutant, which is hyperpiliated and overexpresses the PilA1 subunit (Bhaya *et al.*, 2000; Okamoto & Ohmori, 2002). A highly expressed protein band of 21 kDa was also detected in the *pilT1* mutant (Fig. 6, lane 6). Mass spectrometric sequencing of the 21 kDa bands (Fig. 6, lanes 4–6) showed that all three bands significantly matched to *slr1694*, which encodes the PilA1 pili subunit of *Synechocystis* sp. strain PCC 6803, confirming the identity of this protein band.

DISCUSSION

We have determined that *slr0388*, encoding a hypothetical protein, is involved in natural transformation and

phototactic motility. We propose to designate this gene *comF* despite only moderate sequence identity to the ComF proteins of *B. subtilis*, *H. influenzae*, *P. stutzeri* and *S. pneumoniae*, used in its identification. The ComF proteins, including Slr0388, harbour a PRT domain, which is a highly conserved region in the PRT family of enzymes involved in the biosynthesis and salvage pathways of purines, pyrimidines and pyridines in bacteria and lower eukaryotes (Bashor *et al.*, 2002; Schumacher *et al.*, 2002). This domain is unique to this family of proteins, and is not found in other PRTases of the tryptophan, histidine and nicotinamide synthetic and salvage pathways (InterPro identifier IPR000836). The PRT domain forms the active site that catalyses the transfer of phosphoribosyl from α -D-5-phosphoribosyl-1-pyrophosphate to an acceptor base (e.g. adenine) (Bashor *et al.*, 2002; Craig & Eakin, 2000). Apart from this conserved region, the PRT family of enzymes have low sequence similarity, ranging from 20 to 45 % (Schumacher *et al.*, 2002). However, they also possess other regions that confer substrate selectivity and catalytic stability, which may correspond to the conserved region between residues 127 and 147 of the ComF group of proteins (Fig. 1). PRT enzymes also require metal ions for activity (Craig & Eakin, 2000; Heroux *et al.*, 1999). The tetracysteine motif at the N-terminus of the ComF group of proteins suggests that metal ions may be required for activity in these proteins, since this motif is very similar in organization to the zinc-binding motif of zinc-dependent enzymes (Possot & Pugsley, 1997).

Of the ComF proteins used to identify Slr0388 as a putative competence factor orthologue, the ComFC and Com101A proteins from *B. subtilis* and *H. influenzae*, respectively, have been shown to be involved in natural transformation (Larson & Goodgal, 1991; Londono-Vallejo & Dubnau, 1993). In addition, the ComF protein from *P. stutzeri* also appears to be involved in competency (GenBank accession no. CAB56477). ComFC of *S. pneumoniae* has not been investigated for its involvement in natural transformation, although the *comFC* gene is proximal to one encoding another competence factor (Lee *et al.*, 1999). *comFC* of *B. subtilis* and *com101A* of *H. influenzae* are also located at a competence locus (Londono-Vallejo & Dubnau, 1993; Tomb *et al.*, 1991).

It does not appear that *slr0388* is genomically linked to other competence genes. The gene upstream of *slr0388* is a cysteine desulfurase (*slr0387*, involved in amino acid synthesis), and is unlikely to be involved in competence. The gene downstream of *slr0388* appears to encode a hypothetical protein that shows similarities to the ErfK/YbiS/YcfS/YnhG class of proteins (Pfam identifier pfam03734.8), the functions of which are unknown. This gene, *slr0769*, possesses a putative promoter and ribosome-binding site. Disruption of this gene did not result in the loss of phototaxis (data not shown), although the competency of *slr0769* mutants has yet to be determined. Based on its own putative promoter and ribosome-binding site, *slr0769* is

probably transcribed independently of *slr0388*. Two other genes, *slr0770* and *slr0771*, encoding hypothetical proteins, are located downstream of *slr0769*. Their products did not reveal any homologues from BLASTP searches.

In *B. subtilis*, ComFC is the third ORF in a competence operon encoding two other competence proteins (Londono-Vallejo & Dubnau, 1993). Disruption of *comFC* led to only a fivefold decrease in transformation efficiency compared to a 1000-fold decrease after disruption of the first ORF. Nonetheless, *comFC* is required for the full development of competence in *B. subtilis*. In *H. influenzae*, *com101A* mutants are not transformable (Larson *et al.*, 1991), while Com101A has also been shown to be involved in the processing of single-stranded DNA (Larson & Goodgal, 1992). Mutants of *com101A* showed reduced levels of nucleoside release into the supernatant compared to the wild-type. Com101A did not possess any nuclease activity, which implied that it is not directly involved in DNA degradation. The presence of its PRT domain suggested that it could be involved in the salvage of degraded nucleosides, perhaps regulating another competence protein with nuclease activity. Not all PRT proteins display catalytic activity, with some displaying a regulatory function of other purine or pyrimidine synthesis enzymes (Sinha *et al.*, 2003). It seems likely that the ComF group of proteins are involved in the regulation of other genes in the competence pathway rather than being directly involved in a DNA-binding/processing apparatus.

In *Synechocystis* sp. strain PCC 6803, no nuclease has yet been identified that is involved in DNA degradation during transformation, although it has been shown that double-stranded DNA is converted to the single-stranded form during uptake (Barten & Lill, 1995). The gene *comA* may possess this nuclease activity due to its similarity with the *E. coli* *nuc* endonuclease (Yura *et al.*, 1999). Its disruption results in a loss of transformability (Yoshihara *et al.*, 2001). The transcript level of *comA* in the *slr0388* mutant was checked to determine if Slr0388 is involved in the transcriptional regulation of this putative nuclease. However, there was no significant difference in *comA* transcript levels between the mutant and wild-type as determined by qPCR analysis.

From transmission electron micrographs, the differences in the piliation characteristics between the wild-type and *slr0388* mutant were subtle. It did appear, however, that bundling of individual pilus filaments occurred more frequently in the mutant than in the wild-type, based on Student's *t*-test. The difference in bundling is substantiated by higher levels of *pilA1* transcripts (3.5-fold higher) and PilA1 subunits (Fig. 6) in the *slr0388* mutant. The increased bundling of pili in the *slr0388* mutant, likely due to the higher levels of PilA1 subunits, could impede phototaxis via the formation of a rigid scaffold, such as that observed in the SEM preparations (Figs 4 and 5), which retards the proper retraction of pili. Hence, the uptake of DNA could also be hindered due to a subfunctional Tfp apparatus. The

similarity of *pilT1* transcript levels between the *slr0388* mutant and wild-type suggests that increased bundling is not likely due to hyperpiliation, at least not as a result of changes in *pilT1* transcripts. It cannot be ruled out that *slr0388* could also affect the regulation of other Tfp components that result in the bundling of pili. In addition, *slr0388* may also be involved in other competence pathways, such as the proper processing or integration into the genome of foreign DNA introduced into the cell. In comparison, the *pilT2* (*slr1533*) mutant of the positively phototactic strain of *Synechocystis* sp. strain PCC 6803 displays normal piliation, but is negatively phototactic, hence affecting its motility towards light (Bhaya *et al.*, 2000). It was suggested that *pilT2* affects phototaxis through a regulatory influence on the Tfp apparatus. Thus *slr0388* could have a similar effect.

It has been theorized that in bacteria, the uptake of DNA from the environment may be for the acquisition of nucleotides (Solomon & Grossman, 1996). In *H. influenzae*, the phosphoenolpyruvate:glycose phosphotransferase system, which monitors carbon source availability and efficient catabolic gene expression, also regulates competence development (Macfadyen *et al.*, 1996). Furthermore, it has been shown that the Com101A protein of *H. influenzae* is involved in the processing of DNA and release of nucleosides (Larson & Goodgal, 1992). Therefore, it is intriguing that the ComF group of proteins, including Slr0388, harbours a PRT domain that is involved in the purine/pyrimidine salvage pathway. Another function ascribed to the natural transformation process is its role in the formation of biofilms (Petersen *et al.*, 2005). Again, it is interesting that inactivating *slr0388* results in an apparent aggregation of cells via a complex matrix of bundles of pili.

Our observations that *slr0388* is involved in both phototactic motility and natural transformation provide further evidence that these two processes are intrinsically linked in *Synechocystis* sp. strain PCC 6803. Apart from the Tfp genetic complement, the regulation of phototaxis and natural transformation is likely to involve other common genes that indirectly influence the biogenesis of the Tfp apparatus, as observed with *slr0388* in this study. To that end, the identification of other factors specific only to the regulation of natural transformation should facilitate the elucidation of pathways that distinguish this process from phototactic motility.

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