ctr1, a gene involved in a signal transduction pathway of the gliding motility in the cyanobacterium *Synechocystis* sp. PCC 6803

Young-Ho Chung[a,b], Mi-Sun Cho[a,c], Yoon-Jung Moon[a], Jong-Soon Choi[a], Yong-Cheol Yoo[a], Youn-Il Park[c], Kyun-Min Lee[b], Kye-Won Kang[b], Young Mok Park[a,*]

[a] *Biomolecule Research Team, Korea Basic Science Institute, 52 Yeoeun-dong, Yusung-ku, Taejon 305-333, South Korea*
[b] *Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 373-1 Kusong-dong, Yusung-ku, Taejon 305-701, South Korea*
[c] *Department of Biology, Chungnam National University, 220 Kung-dong, Yusung-ku, Taejon 305-764, South Korea*

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Abstract We generated random Tn*5* mutations in *Synechocystis* sp. PCC 6803 in search for genes involved in the signal transduction cascade for the cyanobacterial gliding motility. One of the non-gliding Tn*5* mutants, S1-105, had an insertional inactivation in the *slr1044* gene encoding a putative methyl-accepting chemotaxis protein. Interposon mutation on the *slr1044* (named *ctr1*) in the bacterium also eliminated gliding motility. In the interposon mutant, the expression of *pilA1* was 5-fold decreased compared with that of wild-type and thick pili, that are believed to be the motor for gliding, could not be observed by an electron microscope. Therefore, we suggest that the Ctr1 protein functions as a transducer that regulates the expression of *pilA1*, and thus is required for the biogenesis of thick pili.

Key words: Methyl-accepting chemotaxis protein; Gliding motility; Pilus production; Transposon mutagenesis; *Synechocystis* 6803

1. Introduction

Many motile photosynthetic microorganisms respond to the alterations of the quality and quantity of light and search actively for optimal environments for survival and growth. We reported previously that the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (henceforth referred to as Syn6803) could display phototactic and photokinetic gliding motility [1]. Syn6803 requires the contact of solid surface for gliding motility, as do filamentous cyanobacteria such as *Anabaena* and *Phormidium* species [2].

There have been only a few reports dealing with the mechanism of cyanobacterial photomovement. It was shown that an extracellular glycoprotein, oscillin, was responsible for the gliding movement in *Phormidium uncinatum* [3]. In addition, the marine unicellular cyanobacterium, *Synechococcus* sp. WH8102, can propel swimming motility by the outer membrane glycoprotein, SwmA, which possesses a similar feature to oscillin [4]. Also it was reported that slime secretion was a prerequisite for the gliding movement of filamentous cyanobacteria such as *Phormidium* and *Anabaena* [5]. Recently, it was addressed that type IV pili were required for phototactic gliding motility in Syn6803 and the synthesis of pili was controlled by an alternative sigma factor, SigF [6].

Many Gram-negative bacteria had type IV pili and utilized them as the motor system for gliding motility [7]. The actual mechanism of gliding motility and pili biogenesis was intensively studied in *Myxococcus xanthus* [8], *Escherichia coli* [9], and the pathogenic bacteria such as *Pseudomonas aeruginosa* [10] and *Neisseria gonorrhoeae* [11]. In *P. aeruginosa* and *M. xanthus*, the pil gene clusters have been shown to be required for pilus biogenesis [8]. Possible homologous genes corresponding to pil genes of *P. aeruginosa* and *M. xanthus* were inactivated in Syn6803 to confirm which genes were required for type IV pilus biogenesis [12]. From the genomic sequences of Syn6803, *pilA1* (pili structural subunit), *pilD* (bifunctional leader peptidase/methylase required for processing PilA subunits), *pilC* (polypeptide needed for pilus assembly) and two *pilT*-like genes (*pilT1* and *pilT2*, ATP binding polypeptides) were found by the similarity search [12]. Among pil homologous genes tested, *pilA1*, *pilT1*, *pilC* and *pilD* were required for gliding motility in Syn6803 whereas *pilA2* and *pilT2* were not involved in motility [12].

Here we isolated a transposon-generated mutant of Syn6803, S1-105, which did not show phototactic gliding motility. We identified that the S1-105 had an insertion in the open reading frame (ORF) of *slr1044* (Cyanobase, http://www.kazusa.or.jp/cyano/) by an inverse PCR method [13]. The *slr1044* gene, renamed *ctr1* (cyanobacterial transducer), was part of a gene cluster, which showed homology to the pil gene cluster of *P. aeruginosa*, and was required for the normal expression of *pilA1* and the biogenesis of thick pili.

2. Materials and methods

2.1. Strains and culture conditions

All mutants were derived from the wild-type motile Syn6803, which was previously described [1]. The media and culture conditions for growth and preparation of the cells for the observation of phototactic movement were previously described [1].

2.2. Tn*5* mutagenesis of Syn6803

Transposon mutagenesis of Syn6803 was performed as the previously described method in *Synechococcus* sp. PCC 7942 with minor

*Corresponding author. Fax: (82)-42-865 3405.
E-mail: ympark@comp.kbsi.re.kr

Abbreviations: Syn6803, *Synechocystis* sp. PCC 6803; MCP, methyl-accepting chemotaxis protein; RT-PCR, reverse transcriptase-mediated polymerase chain reaction

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modification [14]. In brief, Tn*5* transposon-bearing plasmid, pAM1037, which is a derivative of pRL1058, was introduced into Syn6803 cells by triparental conjugal transfer from *E. coli* strains, AM1452 and AM1460. Transconjugants were plated on BG11 agar (1.5%) plates containing 5% LB (v/v) and incubated under low intensity light (3 μmol/m^2/s) for 2 days at 28°C without antibiotic. Kanamycin was then underlaid beneath the plates to a final concentration of 50 μg/ml. The plates were incubated at a fluence rate of 15 μmol/m^2/s. Kanamycin-resistant colonies appeared after 4 weeks of incubation. The mutant colonies were streaked onto fresh BG11 plates supplemented with 10 μg/ml of kanamycin.

2.3. Motility assay

Cells were grown to OD_{730} of 0.8–1.2 in a shaking incubator under white fluorescent light of 15 μmol/m^2/s. Samples containing approximately 100–500 cells were spread on BG11 agar (0.4%) plates containing 5 mM glucose. The cells on agar plates were incubated under unidirectional light with an intensity of 10 μmol/m^2/s for 10–12 days until colony movement was detected.

2.4. DNA isolation, inverse PCR and direct DNA sequencing of Tn flanking sequence

Inverse PCR was performed as described previously in *E. coli* [13] with minor modifications. Genomic DNA was isolated by using the method of Porter [15], and completely digested by *Taq*I restriction enzyme. The digested DNA products were self-ligated using T4 DNA ligase (Intron, South Korea). PCR was performed for 36 cycles at 96°C for 30 s, 58°C for 30 s, 72°C for 2 min and extended at 72°C for an additional 15 min using a sense primer (5′-GCACGATGAAGAGCAGAAGT-3′) and an antisense primer (5′-GGATAAATCCCGCGGATGG-3′) with the self-ligated DNA products as template. DNA sequences were determined using Taq DyeDeoxy Terminator Cycle sequencing kit (ABI PRISM, Applied Biosystems, Foster, CA, USA) at Korea Basic Science Institute on a model 377 DNA sequencer (Applied Biosystems). Comparisons to database sequences were made using the PSI-blast algorithms [16]. Sequence alignments were performed with ClustalW program [17] and manual adjustments.

2.5. Cloning and inactivation

Restriction endonucleases were obtained from New England Biolabs (UK) and used according to the manufacturer's recommendations. The *ctr1* gene was amplified by PCR from the genomic DNA of wild-type Syn6803 using the primers 5′-TAAAACCGACTGAGGAAACC-3′ and 5′-CAGTGTATTTCCCCCAGCCT-3′. The resulting 2.7 kb product, containing the entire ORF of *ctr1* gene, was cloned into pGEM-T easy vector (Promega Co., USA). The *ctr1* gene was disrupted by excising an internal 1.7 kb *Kpn*I/*Sma*I fragment from coding region of the gene and replacing it with a cassette conferring spectinomycin resistance to the cells. Vectors containing the *ctr1* gene disruption were used to transform Syn6803 cells and antibiotic-resistant transformants were selected [15]. Genomic DNA was isolated from individual transformants and PCR analysis was performed to confirm that all chromosomal copies carried the disrupted gene.

2.6. RNA isolation, reverse transcriptase-mediated PCR (RT-PCR) and Northern hybridization

To examine expression of *ctr1* sequence, Syn6803 cells were grown under fluorescent light as described above, and total RNA was isolated as described by Mohamed [18]. After isolation, the RNA was treated with RNase-free DNase I. RT-PCR was carried out using Access RT-PCR kit (Promega Co., Madison, WI, USA) with total RNA and *ctr1*-specific primers 5′-GCCTACGCCGCCTATGGACA-3′ and 5′-GGATTAGTGGGGCTGGGGGC-3′ as recommended by the manufacturer. About 0.5 μg of total RNA was used for each reverse transcription in a 50 μl reaction volume. The amplification was performed for 30 cycles. PCR products were routinely analyzed on a 1.5% agarose gel.

Northern blotting was done as described by Burnett [19]. A fragment of *pilA1* was amplified from genomic DNA using the primers 5′-ACTCTCCAAAAAACGGGCAG-3′ and 5′-CTTCAGCCCGACAAACAACA-3′. The resulting 0.4 kb PCR product was labeled using *Bca*BEST labeling kit (Takara Shuzo Co., Shiga, Japan). Total RNA isolated from Syn6803 was transferred to a nylon membrane after agarose gel electrophoresis and was then hybridized to the ^{32}P-labeled *pilA1* DNA fragment using ULTRAhyb (Ambion Co., USA) as a hybridization buffer. Quantification of RNA abundance from Northern blot hybridization was conducted by using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA).

2.7. Electron microscopy

Syn6803 cells were negatively stained with 0.2% phosphotungstic acid for 1 min and examined using a Carl Zeiss EM912-omega microscope at Korea Basic Science Institute.

3. Results and discussion

3.1. Isolation of the non-motile mutants S1-105 in Syn6803

We have previously shown that Syn6803 cells displayed phototactic gliding motility both on 0.4% soft agar surface and in a cubic liquid chamber [1]. In an attempt to identify genes involved in cyanobacterial phototactic gliding motility, mutants of Syn6803, which showed altered gliding motility on agar plates, were isolated by Tn*5* transposon mutagenesis.

35 mutants that had non-motile phenotype were isolated among about 2000 kanamycin-resistant colonies generated by Tn*5* transposon mutagenesis. The non-motile mutants were streaked on 0.4% soft agar plates for complete segregation of mutant phenotype. One non-motile mutant, S1-105, was chosen for further study. Wild-type Syn6803 cells displayed positive phototaxis on the surface of 0.4% soft agar plate upon exposure of lateral incident light from a fluorescent lamp at 10 μmol/m^2/s (Fig. 1A). In contrast, none of S1-105 colonies showed any traces of motility on agar plate (Fig. 1B) under the same conditions.

3.2. Identifying the mutation site in S1-105

We performed inverse PCR [13] to determine the Tn*5* insertion site in S1-105. The 530 bp product was directly sequenced. DNA sequence analysis by Blast search in Cyanobase on the web (http://www.kazusa.or.jp/cyano/) showed that the Tn*5* transposon including two distal IS elements

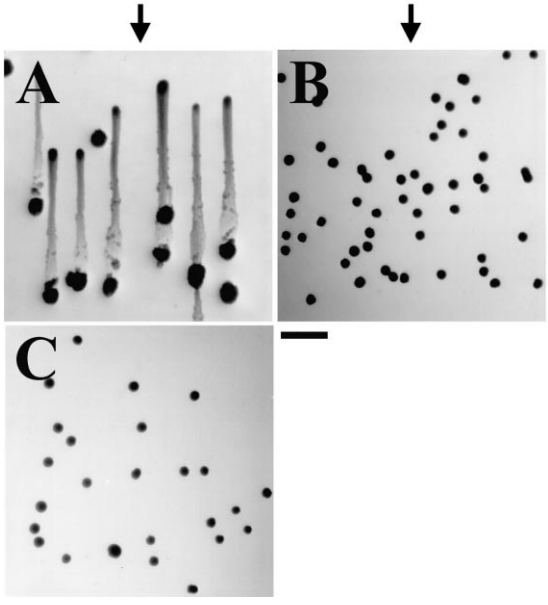

Fig. 1. Photographs of Syn6803 colonies. (A) Wild-type cells show active phototactic gliding movement toward the light source. The arrow indicates the direction of light. (B) Tn*5*-induced *ctr1* mutant, S1-105. (C) Interposon-generated mutant in the *ctr1* gene, CKN5. The bar represents 2 mm.

was inserted into the 1956th bp of the *slr1044* gene of Syn6803 (Fig. 2A).

PSI-blast search revealed that the *slr1044* gene product was homologous to a large group of bacterial proteins known as methyl-accepting chemotaxis proteins (MCPs) [20], such as Tsr of *E. coli* [21] and PilJ of *P. aeruginosa* [22]. Thus, we designated the *slr1044* gene as *ctr1* (cyanobacterial transducer 1). The alignment of the amino acid sequences between Syn6803 Ctr1 and *E. coli* Tsr [21] is shown in Fig. 3A. Interestingly, Ctr1 has significant similarity to the C-terminal half of enteric and *P. aeruginosa* MCPs. Ctr1 has 20% identity and 41% similarity with *E. coli* Tsr, and 31% identity and 53% similarity with *P. aeruginosa* PilJ.

The Ctr1 has several important characteristics as a MCP: first, the region of amino acids 694–755 of Ctr1 corresponds to the highly conserved domain (HCD), which is known to be important for the interaction between *E. coli* Tsr and CheW as well as CheA [23]. Ctr1 has 41% and 56% amino acid identity to *E. coli* Tsr and *P. aeruginosa* PilJ in the HCD, respectively. Second, the predicted amino acid sequence of Ctr1 also has two potential consensus methylation sites (K1 and R1) such as those seen in the enteric MCPs (Fig. 3A). Third, hydrophobicity plot [24] of Ctr1 shows the presence of two transmembrane domains (TM1 and TM2) at the central region (Fig. 3A,B). Fourth, Ctr1 has two HAMP domains, followed by TM2 domain in the central region (Fig. 3A,B). HAMP domain is an α helical domain composed of 50 amino acids in multidomain proteins that participate in a variety of signal transduction processes [25].

The periplasmic domain between TM1 and TM2 in most *E. coli* MCPs except Aer [26] was generally composed of more than 150 amino acid residues (Fig. 3B). Whereas the putative periplasmic domain of Ctr1 was relatively short (Fig. 3B), only 52 amino acid residues, and had no significant homology to any known proteins. The periplasmic domain of 150 amino acid residues in *E. coli* MCP was known to be responsible for receiving external chemical signals as a chemoreceptor. It is likely that the short external 52 amino acid residues of the Ctr1 do not have adequate periplasmic ligand binding domain and therefore are not involved in chemoreception of extracellular signal in Syn6803. In *E. coli*, Aer does not have a periplasmic ligand binding domain, instead its flavin adenine nucleotide binding domain is localized to the cytoplasm where it functions as a redox sensor [26]. Another variation of the MCPs can be found in *M. xanthus*. The receptor component of the Frz signal transduction system in *M. xanthus*, FrzCD, has homology to the enteric MCPs, but has no transmembrane domains [27]. The cytoplasmic location of FrzCD suggests that *M. xanthus* might respond to some internal metabolic intermediate(s) or other molecule(s) that reflect the nutritional state of the cell, rather than to specific extracellular nutrients. Therefore Ctr1 may recognize intracellular signal(s) or extracellular signal(s) in a different way from enteric bac-

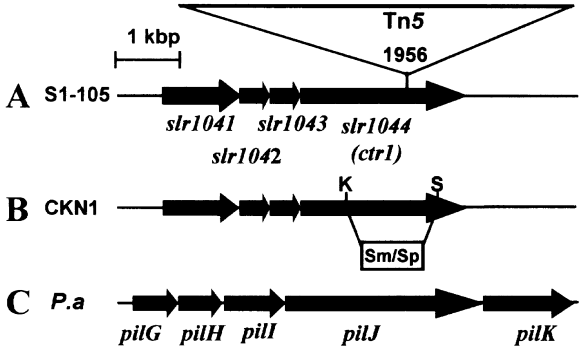

Fig. 2. Schematic representation of the *ctr1* gene locus in Syn6803 and the pil gene cluster in *P. aeruginosa*. (A) The site of the Tn*5* insertion in S1-105 is indicated by a vertical bar. Each arrow represents the location and direction of each gene transcript. (B) Strategy for construction of interposon mutagenesis in *ctr1* gene is shown. (C) Gene organization of the pil*GHIJK* gene cluster in *P. aeruginosa* [10]. *P.a.*, *P. aeruginosa*. Sm/Sp, cassette with non-polar spectinomycin and streptomycin resistance gene; K, *Kpn*I; S, *Sma*I. The horizontal bar represents 1 kb.

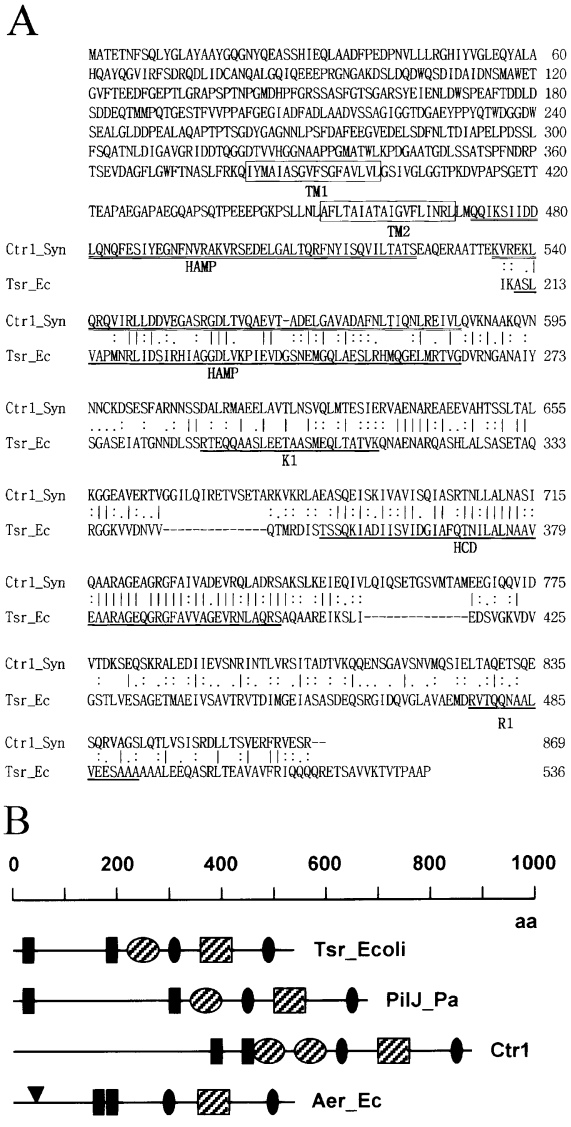

Fig. 3. Sequence alignment (A) and domain structures (B) of the Syn6803 Ctr1 compared with those of *E. coli* Tsr. (A) Sequence comparison of Ctr1 and *E. coli* Tsr. The vertical lines indicate identical amino acids, and colons and periods indicate two degrees of similarity of amino acids. (B) Domain structures. Tsr_Ec [21], Tsr from *E. coli*; PilJ_Pa [22], PilJ from *P. aeruginosa*; Ctr1, Ctr1 from Syn6803; Aer_Ec [25], Aer from *E. coli*. Black box indicates a transmembrane region. Hatched oval means a HAMP domain [24]. Black oval is a K1/R1 methylation region. Hatched box indicates a HCD [23]. Black inverted triangle is a flavin adenine nucleotide binding site.

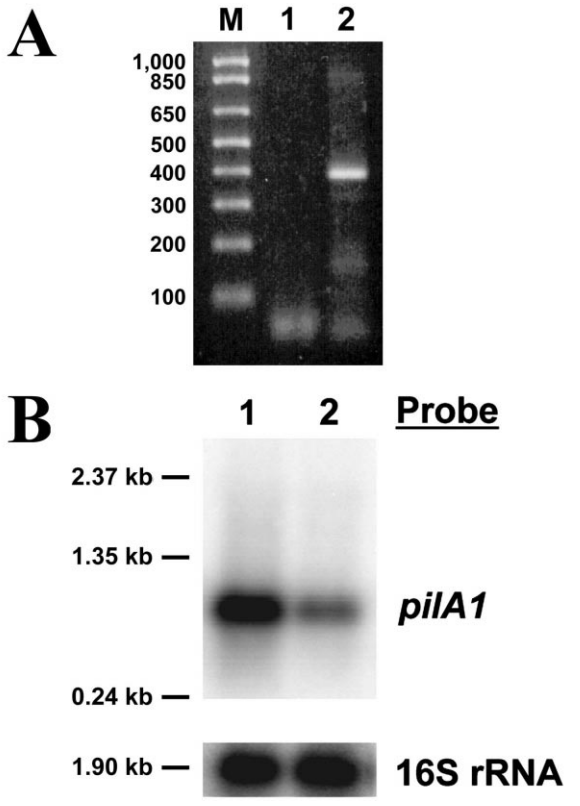

Fig. 4. Expression of *ctr1* and *pilA1* gene in Syn6803 cells. (A) RT-PCR products of negative control (lane 1) and total RNA of wild-type cell (lane 2) were shown. The size of RT-PCR product in wild-type was 385 bp. 1 kb Plus DNA ladder (Gibco/BRL, USA) was used as DNA size marker. (B) Total RNA from wild-type cells (lane 1) and, *ctr1* interposon mutant, CKN5 (lane 2), were hybridized with probes for *pilA1* gene (upper) or 16S rRNA (lower), which was used as a loading control. All lanes were loaded with 5 μg of total RNA.

teria. Specially, Ctr1 has a long N-terminal cytoplasmic tail, composed of about 380 amino acid residues, while most enteric MCPs have only several amino acid residues. This N-terminal cytoplasmic tail in Ctr1 might have roles in recognizing intracellular signal(s), like Aer or FrzCD.

3.3. Expression of ctr1 gene

To examine if expression of *ctr1* gene takes place in Syn6803 cells, total RNA was isolated from wild-type cells, and subjected to RT-PCR. The RT-PCR showed that *ctr1* gene was expressed in Syn6803 cells (Fig. 4A). Negative control experiment, which includes the entire component for RT-PCR, except reverse transcriptase, showed no band corresponding to the PCR product of *ctr1* gene.

Previously, Morgan [28] was able to detect the doublet band corresponding to MCP in Syn6803 by immunoblotting using antiserum raised to the highly purified Trg protein of *E. coli*. In addition, they detected MCP-like molecules in a gliding green non-sulfur bacterium, *Chloroflexus aurantiacus*. MacBride [29] has already characterized a MCP related to chemotactic transducer in the gliding bacterium *M. xanthus*. Detection of MCP-like molecules in these taxonomically distant gliding bacteria implies that MCPs may be involved generally in the sensory control of gliding motility.

3.4. Re-confirmation of the S1-105 locus for involvement in gliding motility

In order to find out that the Tn*5* insertion in *ctr1* gene is responsible for non-gliding phenotype, in S1-105 mutant strain, we adopted an interposon mutagenesis to create knockout mutation in *ctr1* gene. The *ctr1* gene was cloned by PCR in *E. coli* plasmid vector pGEM-T easy. A non-polar spectinomycin cassette was inserted in the *ctr1* gene for interposon mutagenesis (Fig. 2B). The interposon mutagenized *ctr1* gene was used to mutate the *ctr1* gene in the chromosome of Syn6803 by transforming wild-type cells. The spectinomycin-resistant transformants were selected to ensure the replacement of the wild-type gene by a double homologous recombination event. All the gene knockout mutants displayed non-motile phenotype (Fig. 1C) on the surface of 0.4% soft agar plates to lateral light stimulus with the intensity of 10 μmol/m^2/s, where wild-type Syn6803 cells showed active movement (Fig. 1A). One of the mutants was chosen for further characterization and named CKN5. The mutant CKN5 grew as compact colonies with well-defined edges like the previously described Tn*5* non-motile mutant, S1-105 (Fig. 1B). Also both S1-105 and CKN5 mutants did not show any photomovement on a quartz square chamber in a single cell level using a computerized video motion analysis system as previously described [1] (data not shown). PCR was used to verify that wild-type *ctr1* gene was replaced by the mutated one in the CKN5 mutant (data not shown). It was thus confirmed that the *ctr1* gene was involved in gliding movement in Syn6803.

3.5. The ctr1 gene is a part of a gene cluster, which is homologous to the gene clusters of P. aeruginosa pilus biogenesis and E. coli chemotaxis

In the Cyanobase, there are three ORFs (*slr1041*, *slr1042*, and *slr1043*) located upstream of the *ctr1* gene. We examined the three predicted ORFs using PSI-blast peptide similarity search program [16]. Interestingly, it was found that each

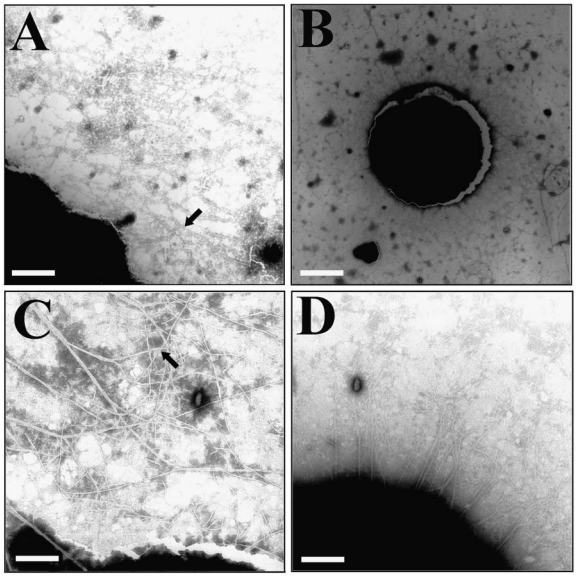

Fig. 5. Transmission electron microscopy of wild-type (upper left and lower left) and *ctr1* interposon mutant, CKN5 (upper right and lower right). The bars represent 0.71 μm (upper left and upper right), 0.27 μm (lower left and lower right). The arrows in wild-type point to a thick pilus.

protein of the three ORFs (*slr1041*, *slr1042*, and *slr1043*) had significant similarities to each of the proteins involved in bacterial pilus formation (the PilG, PilH, and PilI proteins of *P. aeruginosa*) and chemotaxis (the CheY, CheY, and CheW proteins in *E. coli*), respectively. The predicted translation product of *slr1041* (388 amino acids) shows similarity to a large group of bacterial proteins known as response regulators. C-terminal (120 amino acids residues) of Slr1041 reveals significant homology to the *P. aeruginosa* PilG [30] (32% identity) and the *E. coli* CheY protein (27% identity). The Slr1042 protein (147 amino acids) shows significant sequence homology to the *P. aeruginosa* PilH protein (36% identity) and to the *E. coli* CheY protein (34% identity) [22]. Also the predicted protein of the *slr1043* (187 amino acids) has a significant homology to the *P. aeruginosa* PilI (27% identity) and to the *E. coli* CheW (23% identity).

Like *P. aeruginosa*, Syn6803 cells have a *pilG*, *-H*, *-I* and *-J* homologous gene cluster (*slr1041, slr1042*, *slr1043* and *ctr1*), which is organized in the same manner [10] (Fig. 2C). While they do not have a gene corresponding to PilK (the enteric CheR homologue in *P. aeruginosa*) (Fig. 2A), they have two genes encoding PilL homologue (the enteric CheA homologue in *P. aeruginosa*), *slr0322* and *sll1296*. These genes are not closely linked to *ctr1*.

3.6. The ctr1 gene was involved in the expression of pilA1 gene and the biogenesis of thick pilus morphotype

It was demonstrated that the pil gene cluster (*pilG*, *-H*, *-I*, *-J*, *-K*) was a part of a signal transduction network involved in regulating pilus production and twitching motility in *P. aeruginosa* [22,30]. In order to test whether the *ctr1* gene participated in the production of Syn6803 pili, we, as a first step, observed the cyanobacterial cells by a transmission electron microscope. Wild-type Syn6803 had numerous, peritrichously arranged thick pili that were distributed over the entire cell surface (Fig. 5A,C). In contrast, no thick pili were observed on the surfaces of the *ctr1* interposon mutant, CTX5, although some thin pili were visible (Fig. 5B,D). These results suggested that *ctr1* mutant was either unable to make or assemble components of the thick pilus.

To determine whether *ctr1* gene affects the biosynthesis of type IV pilin that is a structural subunit of thick pilus in Syn6803, we examined the expression of the *pilA1* gene encoding pilin subunit of thick pilus in Syn6803 [12]. The total RNA was isolated from both Syn6803 wild-type cells and CKN5 and then the level of transcripts of the *pilA1* gene was measured by Northern hybridization using the *pilA1* gene as a probe. Wild-type cells accumulated a high level of *pilA1* transcript during growth under the white light with the intensity of 15 μmol/m^2/s. In contrast, the level of *pilA1* transcript in the CKN5 mutant was less than 20% of that in the wild-type Syn6803 (Fig. 4B). These results suggest that *ctr1* gene is essential for the normal expression of *pilA1*.

It has been reported that the *pilA1* transcripts accumulated to high levels in wild-type cells, and disruption of the *pilA1* gene encoding thick pili made loss of gliding motility in Syn6803 [12]. In *P. aeruginosa*, production of type IV pili has been shown to be controlled by environmental stimuli. The *P. aeruginosa pilA* gene is transcriptionally activated by the sensor–regulator pair proteins, PilS–PilR [31]. PilS, a sensory protein that resides in the cytoplasmic membrane, is known to be autophosphorylated when it encounters an environmental stimulus and transfers the phosphate to the response regulator, PilR. PilR, in turn, activates transcription of *pilA* by interacting with RNA polymerase and RpoN [32]. In Syn6803, we could not identify genes encoding possible homologues of RpoN, PilR, and PilS, based on the whole genome sequence.

In *P. aeruginosa*, it was proposed that ternary complex of PilJ, PilI, and PilL autophosphorylates PilL, in turn, the phosphate group of PilL is transferred to PilG or PilH. The phosphorylated PilG or PilH can play a critical role in the signal transduction system, which may regulate pilus production and twitching motility. In Syn6803, it is thus tempting to speculate that Ctr1 forms a ternary complex with Slr1043 and an unknown CheA homologous protein, and then transfers a signal to Slr1041 or Slr1042 (PilG or PilH homologue). The Slr1041 or Slr1042 may then, directly or indirectly, regulate pilus production by controlling *pilA1* gene expression.

In conclusion, our results imply that *ctr1* might be a member of the *slr1041*, *slr1042*, and *slr1043* gene cluster, which could be responsible for regulating pilus biogenesis and gliding movement in Syn6803. Ctr1 protein may act as a transducer whose function is to receive intracellular signal(s) and transduce these signal(s) to the pilus production machinery thus regulating the expression of the *pilA1* gene. We are currently involved in experiments to elucidate the roles of *slr1041*, *slr1042*, *slr1043*, and *ctr1* in a signal transduction pathway for pilus production and gliding movement in Syn6803.

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