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Biochemical and Biophysical Research Communications 315 (2004) 179–186
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The role of Slr1443 in pilus biogenesis in *Synechocystis* sp. PCC 6803: involvement in post-translational modification of pilins

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Received 9 January 2004

Abstract

We isolated a transposon-induced nonmotile mutant of the cyanobacterium *Synechocystis* sp. PCC 6803. The mutant was revealed to have a Tn*5* insertion in the *slr1443* gene that showed sequence similarity to a eukaryotic-type protein kinase. Thick pili were not observed on the mutant cell surface under the electron microscope. The *slr1443* gene was not involved in transcription or translation of the *pilA1* gene encoding pilin, the major component of thick pili. In the mutant, lower molecular mass pilin peptides were detected than in the wild-type. The pilin variant was not truncated at the N- or C-terminus of mature PilA1. The reduced molecular mass may have resulted from insufficient post-translational modification. The amounts of pilin variants were remarkably reduced in the periplasmic and surface fractions. The pilin variants were released into liquid media without being assembled into pili. Our finding suggests that Slr1443 plays an important role in pilus biogenesis at the level of the post-translational modification of pilin.

Keywords: Pilus biogenesis; *Synechocystis* sp. PCC 6803; Gliding motility; Post-translational modification

Cyanobacteria are prokaryotes capable of plant-like oxygenic photosynthesis and often move in response to light. Cyanobacterial movement in response to light might be a result of an adaptive mechanism to optimize photosynthesis [1]. We found that the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter named Syn6803) exhibits photomovement via gliding [2]. Recently, extensive molecular studies on the gliding motility of Syn6803 have been undertaken due in part to access to its entire genome sequence [3] and the natural competence of this strain to uptake DNA [4].

Wild type Syn6803 cells have two morphologically distinct types of appendages, thick and thin pili. Recently, the thick pili were shown to be type IV pili and to be required for gliding motility in Syn6803 [5]. Type IV pili are the common apparatus for twitching or gliding motility in various bacteria, of which the best studied are *Myxococcus xanthus*, *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, and *Vibrio cholerae* (for review, see [6]). Type IV pili biogenesis can be divided into four stages; (i) transcription of the gene encoding pilin, the main structural component of pili, (ii) translation of pilin peptide, (iii) membrane insertion and processing of pilin, and (iv) pili assembly. In *P. aeruginosa* and *M. xanthus*, many genes were found to be involved in each stage of pili biogenesis. Pilin is encoded by *pilA* in these bacteria, and it was reported that *pilS*, *pilR*, *fimS*, *algR*, and *rpoN* are involved in *pilA* transcription in *P. aeruginosa*. PilD is a bifunctional enzyme that cleaves the amino-terminal leader sequences of prepilin and catalyzes the N-methylation of mature pilin, which can be further processed into S-pilin by an unknown protease. S-pilin is released into media without being assembled into pili. *PilB*, *pilC*, *pilD*, *pilM*, *pilN*, *pilO*, *pilP*, and *pilQ* are known to be required for pili assembly. The *pil* genes are conserved and comprise the cellular machinery used for pili assembly in those bacteria.

The putative *pil* genes including the *pilA1* gene encoding pilin are well conserved in the Syn6803 genome,

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doi:10.1016/j.bbrc.2004.01.036

like those of other bacteria [5]. So far, three genes have been reported to be involved in the regulation of *pilA1* transcription; *ctr1* encoding a putative methyl-accepting chemotaxis protein [7], *sigF* encoding an alternative sigma factor [8], and *pilT1* [5]. Based on sequence homologies and electron microscope observations of the cell surface, it has been suggested that *pilB1*, *pilC*, *pilD*, *pilM*, *pilN*, *pilO*, and *pilQ* are involved in pili assembly [5,9]. To our knowledge, no report has produced experimental evidence that any genes are involved in the translational or post-translational modification of the *pilA1* gene during pili biogenesis in Syn6803. Furthermore, there is little information on the structural components for the pili fiber or the regulatory mechanism of cyanobacterial motility.

Recently, we established in vivo Tn*5* mutagenesis in Syn6803 in an effort to identify novel genes required for gliding motility [7]. Here we report on a nonmotile mutant isolated from the Tn*5* mutant library. The mutant was identified that Tn*5* was inserted in the *slr1443* gene encoding a protein homologous to eukaryotic Ser/Thr protein kinase. The *slr1443* gene may be the first found to be involved in the post-translational modification of pilin for pili assembly in Syn6803.

Materials and methods

Bacterial strains and culture conditions. The unicellular motile cyanobacterium *Synechocystis* sp. PCC 6803 from the Pasteur Culture Collection (Syn6803) was used for this study. Syn6803 was cultivated as previously described [2].

Isolation and identification of the Tn5-induced mutant. Tn*5* was introduced into wild-type Syn6803 as previously described [7]. Tn-induced mutants resistant to kanamycin were screened for photomovement on either 0.4% or 1.5% (w/v) agar-plates containing BG11 plus 5 mM glucose under unidirectional light with an intensity of 10 μmol/m^2/s for 3–4 days. To identify the transposon insertion site, Tn-flanking DNA was sequenced after inverse PCR of the genomic DNA, as previously described [7].

DNA manipulation, RNA isolation, and Northern blot analysis. Standard methods were used for the isolation and manipulation of DNA [10]. RNA preparation and Northern blotting were performed as previously described [7].

Gene inactivation. To inactivate the *slr1443* gene, a 2617 bp PCR fragment containing the entire ORF of *slr1443* was amplified using primers 5′-TGGGTAAAGTGGGACGAT-3′ and 5′-ACCAATGACC GCCGTTCTTA-3′ and cloned into pGEM-T easy vector (Promega, Madison, WI, USA). The *slr1443* gene was then disrupted by replacing an internal *Bal*I fragment with a nonpolar cassette conferring spectinomycin resistance. The resultant plasmid was used to transform motile Syn6803 wild-type cells and one of the spectinomycin resistant transformants, named PKN-1, was selected for further study. Complete segregation of the mutation was confirmed by PCR using the primers described above.

Transmission electron microscopy. Syn6803 cells were negatively stained with 0.1% phosphotungstic acid for 1 min, washed briefly with 0.02% phosphotungstic acid, and then examined under a Carl Zeiss EM912-omega microscope (Carl Zeiss, Thornwood, NY, USA).

Cell fractionation. Whole cell lysate was obtained by vortexing cells harvested from BG11 agar plates with 0.5 mm diameter glass beads (Biospec Products, Bartlesville, OK, USA). To prepare the cell surface fraction, cyanobacterial cells harvested from BG11 agar plates were resuspended in BG11 liquid media to an OD_{730} of 5.0 and sheared by vortexing. Suspensions were centrifuged twice at 10,000*g* for 20 min and the supernatants were collected. To purify periplasmic proteins by osmotic shock, cell pellets were resuspended in 30 mM Tris–HCl, pH 8, 20% sucrose, and 1 mM EDTA and incubated for 10 min. After centrifugation at 10,000*g*, 4 °C for 10 min, the resultant pellet was resuspended by vortexing in ice-cold 2 mM $MgSO_4$ and the cell suspension obtained was then stirred slowly for 10 min on ice. After re-centrifugation, supernatant was taken and used as the periplasmic fraction. The proteins secreted from cyanobacterial cells into liquid media were concentrated using the method described by Sergeyenko and Los [11].

Generation of polyclonal anti-sera against PilA1 peptide. Polyclonal antibodies were produced against synthetic peptides containing the internal sequence (amino acid residues 72–91) or the C-terminal sequence (149–168) of PilA1. Peptides were coupled to the immunogenic carrier protein keyhole limpet hemocyanin (KLH) via an additional C-terminal cysteine of each peptide by the *N*-γ-maleimidobutyryloxylsuccinimide (GMBS) conjugation method [12]. Immunization of each peptide and sampling of the anti-sera from rats were performed by a commercial facility (Peptron, Daejeon, Korea).

Gel electrophoresis. To detect pilin, 20 μg of total protein from each strain was loaded per lane on a 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel. Proteins derived from the same volume of bacterial suspension with the same turbidity (OD_{730} = 5.0) were loaded from the periplasmic and surface fractions. Total proteins for two-dimensional polyacrylamide gel electrophoresis (2-DE) were prepared as described by Sazuka et al. [13]. 2-DE was performed using the Amersham–Pharmacia IPG-phor and Hoefer DALT system (Amersham Biosciences, NJ, USA) according to manufacturer's instructions. Proteins (300 μg) were solubilized in 8 M urea, 2% (w/v) Chaps, 0.5% v/v IPG buffer, and 60 mM DTT, and loaded onto 18 cm, pH 4–7, immobilized pH gradient (IPG) strips. Isoelectrofocusing and SDS–PAGE were performed as previously described [14].

Immunoblot analysis. Proteins separated on the gels were transferred to nitrocellulose membrane. After blocking the membrane with 3% (w/v) nonfat dried milk in TBS buffer for 1 h, anti-serum against PilA1 peptide was added at a dilution of 1:1000 and allowed to bind for 4 h. The membrane was then washed three times in TBST (TBS, 0.5% Tween 20), incubated with a 1:2000 dilution of a goat anti-rat antibody conjugated with horseradish peroxidase (Sigma, St. Louis, MO, USA) for 1 h, and washed three times in TBST. The bound antibody was detected using ECL (Amersham Biosciences, NJ, USA).

In-gel digestion and mass spectrometry. Bands of interest were excised and digested with trypsin. Peptide MS/MS analysis was performed against the digested peptides by ESI-Q-TOF mass spectrometer as previously described [14].

N-terminal amino acid sequencing of protein bands. The proteins resolved on SDS–PAGE were electroblotted onto a PVDF membrane, as previously described [15]. N-terminal amino acid sequencing was performed using the Edman degradation method using an Applied Biosystems model 491A protein sequencer.

Results

Isolation of a Tn5-induced motility mutant and identification of the Tn insertion site

By in vivo transposon mutagenesis, we isolated several Tn*5*-induced mutants of Syn6803 that showed aberrant phototactic movement. One of these mutants, M32-8, was nonmotile on the surface of either 1.5% or

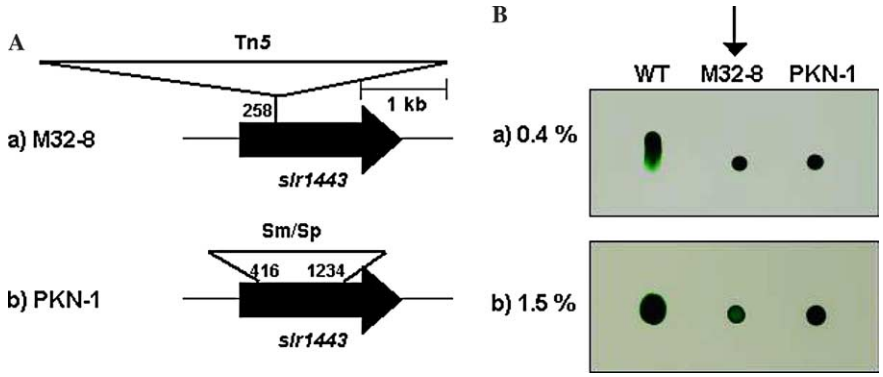

Fig. 1. (A) Gene map of the *slr1443* mutants (a) M32-8, the Tn*5*-induced mutant. (b) PKN-1, the interposon-generated *slr1443* mutant. The inserted sites of Tn*5* in M32-8 and a nonpolar spectinomycin resistance cassette in PKN-1 are shown. (B) Directional motility assay of wild-type cells (WT) and the *slr1443* mutants, M32-8 and PKN-1. Four microliter volumes of logarithmically growing cell suspension was spotted onto solid BG-11 agar plates containing 10 mM glucose. The cells were grown under unidirectional light (indicated by the arrow) of 10 μmol/m^2/s for 3 days. (a) 0.4% agar plate. (b) 1.5% agar plate.

0.4% agar plates and was chosen for further analysis. To identify the Tn*5* insertion site in the genome of M32-8, inverse PCR and DNA sequencing were performed as previously described [7]. Sequence search in CyanoBase (http://www.kazusa.or.jp/cyano/) revealed that the transposon was inserted at the 258th base pair of the *slr1443* gene of Syn6803 (Fig. 1A).

Sequence analysis of the slr1443 gene

The *slr1443* gene is predicted to code for a 68.7 kDa protein consisting of 615 amino acid residues with an estimated p*I* of 5.03. The deduced amino acid sequence of *slr1443* shares high similarity with a putative Ser/Thr protein kinase. BLAST search revealed that the Slr1443 peptide shares 33.4% identity and 50.6% similarity in the overlapped 605 amino acids with Alr2412 of *Anabaena* sp. PCC 7120 [16]. The N-terminal region of Slr1443 shows high similarity to PknA (28.6% identity and 45.4% similarity over 467 residues) of *Anabaena* sp. PCC 7120 [17] and SpkB (30% identity and 50% similarity over 159 residues) of Syn6803 [18]. Although the deduced amino acid sequence of *slr1443* has the 11 subdomains typical of eukaryotic-type Ser/Thr protein kinases [19], several key residues essential for the action of protein kinases are lacking as follows: Gly in the ATP binding site GXGXXGXV (subdomain I), Lys for phosphotransfer (subdomain II), Glu in subdomain III, a Ser/Thr-specific feature, DXKPXN motif (subdomain VI), DFG in subdomain VII, GYAAPE in subdomain VIII, Asp in subdomain IX, and Arg in subdomain XI (Fig. 2A).

The C-terminal region of Slr1443, amino acid residues 365–487, is homologous to an internal sequence of ubiquitin-protein ligase HUL4 (EMBL Accession No. Q873C0) from *Neurospora crassa* (31% identity and 44% similarity) (Fig. 2B). Ubiquitin-protein ligase is known to recognize specific substrates during ubiquitination reactions and to regulate the activity of plasma membrane proteins by endocytosis [20]. Another C-terminal region comprising the amino acid residues 241–477 of Slr1443 has 31% identity and 47% similarity to both the entire sequence of splicing factor 3a (SF3a) subunit 2 from *Brachydanio rerio* (EMBL Accession No. Q7ZVD4) and the N-terminal sequence of SF3a subunit 2 from *Homo sapiens* (EMBL Accession No. Q15428) (Fig. 2C). SF3a subunits are essential for the formation of active 17S U2 snRNP and prespliceosome [21].

Targeted inactivation of the slr1443 gene to confirm its involvement in gliding motility

The motility-related phenotype of the *slr1443* gene was confirmed by targeted gene inactivation. The region from nucleotides 416–1234 bp of the *slr1443* gene was replaced with a nonpolar spectinomycin cassette (Fig. 1A). One of the spectinomycin-resistant transformants, named PKN-1, was selected and segregated to ensure that the wild-type gene had been replaced with the mutagenized gene. The disruptant, PKN-1 was also nonmotile on the surface of both 1.5% and 0.4% agar plates (Fig. 1B). Hence, it was verified that the *slr1443* gene was involved in the gliding motility of Syn6803.

Observation of type IV pili on the cell surface

It was previously demonstrated that thick pili on the cell surface are type IV pili, which are responsible for phototactic gliding motility in Syn6803 [8]. Pili on cell surface were observed under a transmission electron microscope. Compared with the wild-type cells, which had numerous thick and thin pili on their surfaces,

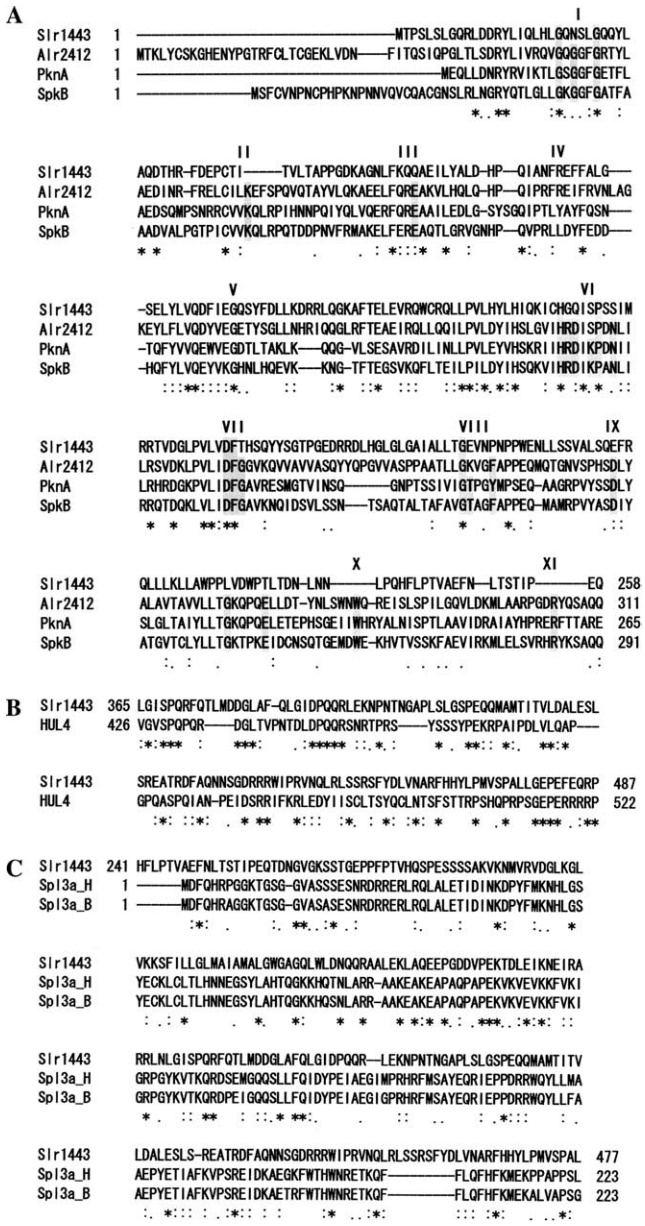

Fig. 2. Amino acid sequence comparison of Slr1443. (A) Sequence alignment of the amino terminal part of Slr1443 with a putative eukaryotic-type Ser/Thr protein kinase such as Alr2412 of *Anabaena* sp. PCC 7120, PknA of *Anabaena* sp. PCC 7120, and SpkB of Syn6803. Asterisks, colons, and periods indicate identical, highly similar, and similar residues, respectively. (A) Subdomains I–XI typical of eukaryotic-type protein kinases are shown above the alignment and highly conserved residues are shaded. (B) Sequence alignment of Slr1443 with a putative ubiquitin-protein ligase HUL4 from *N. crassa*. (C) Sequence alignment of Slr1443 with splicing factor 3a subunit 2 from *H. sapiens* (Spl3a_H) and *B. rerio* (Spl3a_B). Sequence comparisons were performed using ClustalW [36]. Asterisks, colons, and periods indicate identical, highly similar, and similar residues, respectively.

PKN-1 had few thick pili but had retained most of the thin pili (Fig. 3). This observation suggests that Slr1443 protein is required for thick pili biogenesis in Syn6803.

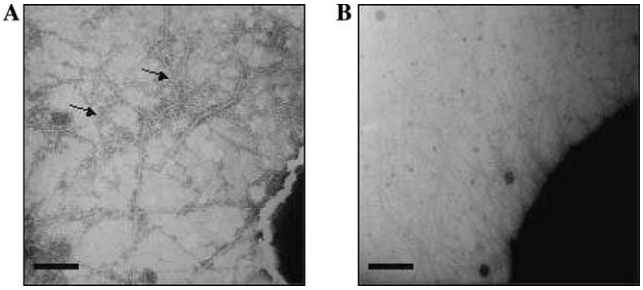

Fig. 3. Detection of pili on the cell surface by transmission electron microscopy. Logarithmic growing (A) wild-type and (B) PKN-1 cells. Cells are shown at a final magnification of 25,000×. The arrows in the wild type cell point to a thick pilus. The bars represent 0.71 μm.

A pilin variant of small size was produced by the slr1443 mutant

In order to determine how Slr1443 affects pili biogenesis, we investigated the expression level of *pilA1* encoding the pilin subunit of the thick pilus. First, the transcription level of *pilA1* was analyzed by Northern blotting against total RNA from wild-type and PKN-1 cells using the *pilA1* gene as a probe. PKN-1 was found to synthesize the same amount of *pilA1* mRNA as the wild-type (Fig. 4), showing that Slr1443 protein is not involved in the transcription of *pilA1*.

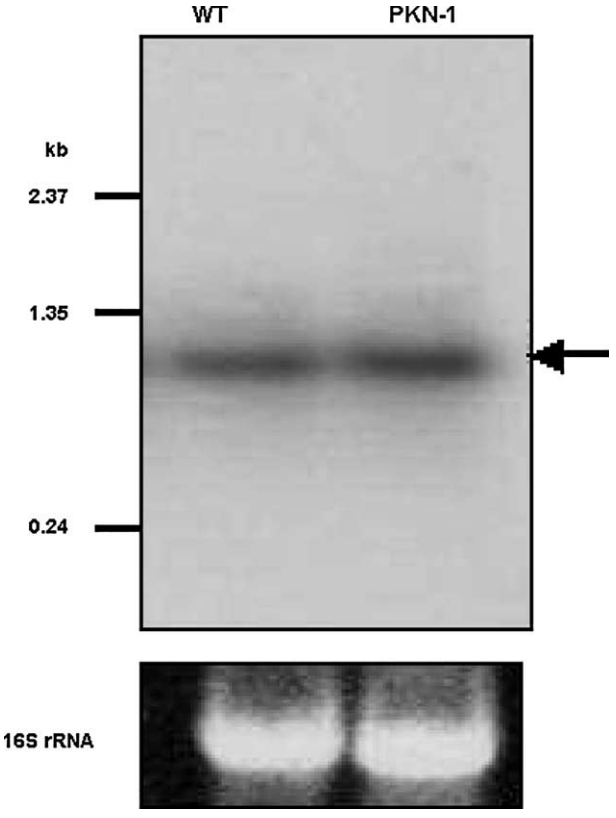

Fig. 4. Northern blot analysis of the *pilA1* gene. Total RNA from wild-type and PKN-1 cells was hybridized with a *pilA1* gene-specific probe. Ten micrograms of total RNA was loaded in each lane and 16S rRNA was used as a loading control. The arrow indicates *pilA1* mRNA.

The translation level of the *pilA1* gene was examined by immunoblotting with anti-PilA1 antibody. To produce anti-PilA1 antibody, overexpression of PilA1 protein with 6×His or intein tag was performed but failed. Thus antibodies, PIN72 and PC149, were generated against synthetic peptides of internal amino acid residues 72–91 or C-terminal residues 149–168 of PilA1. The peptide sequences for epitopes were first chosen as regions with low hydrophobicity and less homology with PilA1 homologues, to eliminate nonspecific binding. Compared with the tertiary structures already revealed for pilins from *P. aeruginosa* K124-4 [22] and *N. gonorrhoeae* MS11 [23], sequences expected to be exposed were finally selected and synthesized. Since both PIN72 and PC149 showed the same pattern on the immunoblot against wild-type cell lysate (data not shown), PC149 was used for further study.

By immunoblotting against total proteins, the presumed pilin peptide was detected at molecular weights (M_r) of 25 and 28 kDa in wild-type cells, while smaller bands, at 21 and 23 kDa, were detected in PKN-1 (Fig. 5A). All of the bands were confirmed as PilA1 by electrospray ionization quadrupole-time of flight mass spectrometry (ESI Q-TOF MS) after in-gel digestion with trypsin (data not shown). Pilin peptide quantities were the same in the wild type and in PKN-1 despite the different M_r.

For better analysis on the p*I* as well as M_r of pilin peptides, 2-DE was performed using total proteins prepared from liquid cultures of the cells (Fig. 5B). By immunoblot analysis, several spots of pilin peptides were detected at the M_rs of 25–28 kDa and the p*I*s of 4.0–4.2 in wild-type cells. In PKN-1, pilin peptides had M_rs of 21–23 kDa and p*I*s of 4.0–4.2 (Fig. 5C). The spots designated by the arrows 'a'–'d' on 2-DE seem to correspond to the bands 'a'–'d' on SDS–PAGE in Fig. 5A, respectively. As observed on the immunoblot of SDS–PAGE, the pilin peptides of PKN-1 migrated downward more rapidly than those of the wild-type cells on 2-DE. No significant change in the intensities of the pilin spots was observed for the wild-type and PKN-1 cells. This result suggests that variant types of pilin are synthesized in PKN-1 in the same quantities as in the wild-type, but subjected to insufficient post-translational modification. On the other hand, at least two types of pilin variants with different M_r and p*I* values were detected both in the wild-type and mutant cells (Figs. 5A and C). Therefore, there may be other type(s) of post-translational modifications for pilin in addition to the process involving Slr1443.

The pilin variant in the slr1443 mutant was not assembled into pili

To investigate whether the pilin variant in PKN-1 affected the assembly of functional pili, immunoblotting was performed. In both periplasmic (Fig. 6A) and surface fractions (Fig. 6B), pilin peptides were remarkably

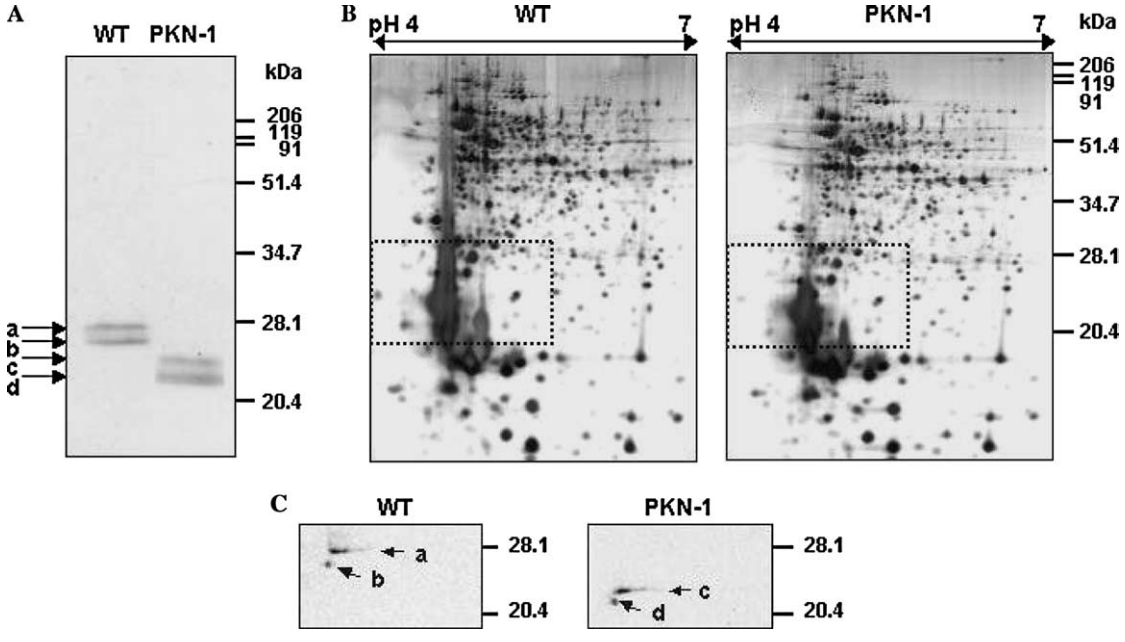

Fig. 5. Detection of pilin peptide by immunoblotting with anti-PilA1 antibody. (A) Immunoblot of the SDS–PAGE gel of whole cell lysate from wild-type (WT) and *slr1443* mutant (PKN-1) cells. Twenty micrograms of protein was loaded on a 15% SDS–PAGE gel. Arrows 'a'–'d' indicate that the PilA1 peptides were identified by peptide mass fingerprinting. (B) 2-DE of total proteins from the liquid cultures of each strain. Proteins were separated on pH 4–7 IPG strips and then on 12% SDS–polyacrylamide gel. Boxes indicate the regions corresponding to the following immunoblot images. (C) Immunoblot against the 2-DE gels. The spots designated by the arrows 'a'–'d' seem to correspond to the bands 'a'–'d' on the immunoblot of SDS–PAGE gel.

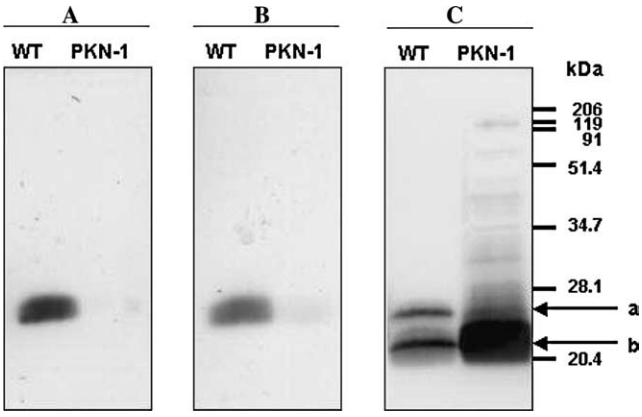

Fig. 6. Immunoblotting against each cell fraction using anti-PilA1 antibody. (A) Periplasmic fraction. (B) Surface fraction. (C) Secreted proteins into surrounding liquid media. Wild-type and *slr1443* mutant cells are designated as WT and PKN-1, respectively. Periplasmic and surface fractions were prepared from cells harvested from agar plates. Secreted proteins were prepared from liquid culture of bacteria as described by Sergeyenko and Los [11]. On both lanes in each fraction, proteins loaded were derived from the same volume of bacterial suspensions with the same turbidity. Arrows 'a' and 'b' indicate the PilA1 peptides.

reduced in PKN-1. This result strongly suggests that the smaller pilin variant was not assembled into pili. This is also in agreement with the finding that thick pili were not observed on the mutant cell surface by TEM analysis.

Immunoblotting was then performed against a liquid media fraction containing secretary proteins to determine why pilin peptides were absent from the periplasmic and surface fractions of PKN-1 (Fig. 6C). In the liquid media fraction, an intensive band was observed, which corresponded to the smaller pilin peptides, as shown in Fig. 5. It could be concluded that the pilin variant in the mutant was released into media and not assembled into pili.

Post-translational modification of pilin is required for assembly into functional pili

We investigated whether the decrease in the M_r of the pilin variant in PKN-1 was due to peptide truncation. First, the N-terminal amino acid region of the pilin variants was sequenced and found to be XTLIELLVVV (X indicates a modified amino acid), which is identical to the wild-type pilin. Second, sequence of C-terminus was identified by ESI-Q-TOF MS/MS analysis after tryptic digestion of pilin (data not shown). The existence of the C-terminus was also supported by the fact that the antibody PC149 bound to the C-terminus of mature PilA1. Taken together, we conclude that the reduction in M_r of the pilin variant in PKN-1 is not caused by truncation at either the N- or C-terminus. One possible explanation is that the insufficiency of the post-translational modification of pilin caused the M_r reduction and affected pili assembly.

Discussion

Syn6803 cells displayed gliding movement both on BG11 agar plates and in cubic liquid chamber [2]. Numerous genes have been characterized as motility-related genes by in vitro [24] and in vivo transposon mutagenesis [7]. However, neither the environmental factors nor the regulatory mechanisms of Syn6803 movement have been clearly defined. In view of the fact that various genes are involved in photomovement, cyanobacterial motility may be regulated in a complicated manner.

As a Pkn2 subfamily of eukaryotic-type protein kinases, seven putative genes are present in Syn6803 [3]. SpkA [25] and SpkB [18] are known as motility-related protein kinases. Interposon mutants of either *spkA* or *spkB* showed reduced motilities although they retained both thick and thin pili on their cell surfaces. Therefore, it was reported that both genes are not essential for motility or pili biogenesis, but rather that they stimulate motility via protein phosphorylation.

In the present study, we concluded that another protein kinase homologue, *slr1443*, was required for motility because both Tn*5* insertion and interposon mutagenesis of its gene resulted in a conclusive gliding motility defect. The *slr1443*-disrupted mutant had few thick pili on its cell surface, unlike *spkA* and *spkB* mutants which had a regular pili structure. This suggests that Slr1443 is involved in pili biogenesis, unlike SpkA and SpkB. The *slr1443* gene was previously reported as a pseudo-protein kinase irrelevant to gliding motility [26]. The reason why the *slr1443* mutant showed different phenotypes at the two laboratories is not understood, but we speculate as follows. To generate the *slr1443* interposon mutant in our study, an internal region from 416 to 1234 bp of the ORF was deleted and replaced by a cassette conferring spectinomycin resistance. On the other hand, Kamei et al. generated the *slr1443* mutant by inserting a kanamycin-resistance cassette at the 416th nucleotide of the ORF. A couple of reports found that distinct phenotypes of cyanobacterial mutants are generated by mutations at different sites in one gene, such as *taxAY1* which encodes a chemotaxis-like protein [27] or *rcaE* which encodes a response regulator to light [28].

To understand how *slr1443* is involved in the gliding motility of Syn6803, we studied the involvement of the gene in pili biogenesis by Northern and Western blot analyses. It was found that the *slr1443* gene is not involved in *pilA1* transcription. Pilin peptides were detected at M_rs of 25–28 and 21–23 kDa in wild-type and PKN-1 cells, respectively. It is not known why PilA1

with a theoretical M_r of 15250.94 Da exists as multiple forms at such high M_r. In *Neisseria*, some pilin variants with a theoretical M_r of 17 kDa showed apparent M_r of over 23 kDa on SDS–PAGE, although the cause was not clearly defined [29]. It is predicted that post-translational processing may be responsible for the apparent M_r increases of pilin in Syn6803.

The S-pilin in *Neisseria* and the pilin in PKN-1 are similar as both are secreted into liquid media without being assembled into pili. S-pilin is a pilin variant which is processed at an additional cleavage site, the 39th amino acid from the N-terminus of the mature pilin in *Neisseria* [30]. However, the pilin variant in PKN-1 has both the N- and C-termini of mature pilin. Taken together, the reduced M_r of the pilin variant in PKN-1 seems to be caused by insufficient post-translational modification compared with mature pilin in wild-type cells. Modifications that may affect M_r or p*I* on 2-DE include deamidation, glycosylation, phosphorylation, and oxidation [31].

In fact, various post-translational modifications of pilin have been observed in *Neisseria* and *Pseudomonas* species (for review, see [6]). First, mature pilin is processed from prepilin and methylated at the N-terminus by a prepilin peptidase PilD [32]. Second, in *Neisseria species*, pilin is phosphorylated at Ser-68 [23], glycosylated at Ser-63 with di- [33] and trisaccharides [34], and modified at Ser-93 with α-glycerophosphate [35]. As shown in our immunoblot results (Fig. 5), glycosylated pilin in *Neisseria species* migrated lately on high resolution SDS–PAGE [33]. The addition of glycerophosphate to Ser-93 was predicted as a prerequisite to anchor the assembled pilus fiber to outer membrane [35]. Since type IV pili from various species share a common subunit structure, architecture, and conserved cellular machinery for pilus assembly [6], pilin in Syn6803 is also expected to show post-translational modification similar to those shown by other bacteria [11].

The exact in vivo function of Slr1443 is not known. Although Slr1443 shares sequence similarity with eukaryotic-type Ser/Thr protein kinase, several key residues essential for the action of protein kinases are lacking. Moreover, in vitro protein kinase activity was not detected for the recombinant Slr1443 protein [26]. As described in Results (Figs. 2B and C), the C-terminal part of Slr1443 shares homology with a RNA spliceosome component SF3a subunit 2, and with a ubiquitination component ubiquitin-protein ligase in eukaryotes. The N-terminal domain of SF3a subunit 2 was found to bind with other subunits of SF3a and U2snRNP [21]. Ubiquitin-protein ligase is also required for binding to a substrate for ubiquitination [20]. Considering that both the SF3a subunit 2 and ubiquitin-protein ligase bind their substrate, the C-terminal part of Slr1443 might play a role as a substrate-binding region during pili biogenesis.

To the best of our knowledge, *slr1443* is the first gene reported to be required for the post-translational modification of pilin. It has been shown that Slr1443 plays an important role in gliding motility by regulating pilus biogenesis in Syn6803. Our findings strongly suggest that Slr1443 is involved in the post-translational modification of pilin for the successful assembly of functional pili. This study also provides a clue for the molecular analysis based on pili biogenesis such as synthesis and post-translational modification of pilin or assembly of pili. The characterization of the post-translational modifications of pilin is currently being undertaken by mass spectrometry to clarify the in vivo function of Slr1443 protein.

Acknowledgments

The Tn5-induced mutant, M32-8, of *Synechocystis* sp. PCC 6803 were provided by SMCC (*Synechocystis* sp. PCC 6803 Mutant Culture Collection funded by KOSEF. We thank Mi-Sun Cho for assistance with the generation of interposon mutant, Yong-Cheol Yoo for DNA sequencing, Kyoung Wook Kim and Jin-Young Kim for mass analysis, Seong Ho Yoon for N-terminal amino acid sequencing, and Young-Boo Lee for assistance with the electron microscopy. This work was supported by a grant to Y.-I. Park, Y.M. Park and Y.-H. Chung from the Korea Research Foundation (DP0361).

References

[1] D.-P. Häder, Photomovement, in: P. Fay, C.V. Baalen (Eds.), The Cyanobacteria, Elsevier, Amsterdam, 1987, pp. 325–345.
[2] J.-S. Choi, Y.-H. Chung, Y.-J. Moon, C. Kim, M. Watanabe, P.-S. Song, C.-O. Joe, L. Bogorad, Y.M. Park, The photomovement of the gliding cyanobacterium *Synechocystis* sp. PCC 6803, Photochem. Photobiol. 70 (1999) 95–102.
[3] T. Kaneko, S. Sato, H. Kotani, A. Tanaka, E. Asamizu, Y. Nakamura, N. Miyajima, M. Hirosawa, M. Sugiura, S. Sasamoto, T. Kimura, T. Hosouchi, A. Matsuno, A. Muraki, N. Nakazaki, K. Naruo, S. Okumura, S. Shimpo, C. Takeuchi, T. Wada, A. Watanabe, M. Yamada, M. Yasuda, S. Tabata, Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions, DNA Res. 3 (1996) 109–136.
[4] V.A. Dzelzkalns, L. Bogorad, Stable transformation on the cyanobacterium *Synechocystis* sp. PCC 6803 induced by UV irradiation, J. Bacteriol. 165 (1986) 964–971.
[5] D. Bhaya, N.R. Bianco, D. Bryant, A. Grossman, Type IV pilus biogenesis and motility in the cyanobacterium *Synechocystis* sp. PCC 6803, Mol. Microbiol. 37 (2000) 941–951.
[6] J.S. Mattick, Type IV pili and twitching motility, Annu. Rev. Microbiol. 56 (2002) 289–314.
[7] Y.-H. Chung, M.-S. Cho, Y.-J. Moon, J.-S. Choi, Y.-C. Yoo, Y.-I. Park, K.-M. Lee, K.W. Kang, Y.M. Park, Ctr1, a gene involved in a signal transduction pathway of the gliding motility in the cyanobacterium *Synechocystis* sp. PCC 6803, FEBS Lett. 492 (2001) 33–38.
[8] D. Bhaya, N. Watanabe, T. Ogawa, A.R. Grossman, The role of an alternative sima factor in motility and pilus formation in the cyanobacterium *Synechocystis* sp. strain PCC 6803, Proc. Natl. Acad. Sci. USA 96 (1999) 3188–3193.

[9] S. Yoshihara, X.X. Geng, S. Okamoto, K. Yura, T. Murata, M. Go, M. Ohmori, M. Ikeuchi, Mutational analysis of genes involved in pilus structure, motility and transformation competency in the unicellular motile cyanobacterium *Synechocystis* sp. PCC 6803, Plant Cell Physiol. 42 (2001) 63–73.

[10] J. Sambrook, D.W. Russell, Molecular Cloning: A Laboratory Manual, third ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001.

[11] T.V. Sergeyenko, D.A. Los, Identification of secreted proteins of the cyanobacterium *Synechocystis* sp. strain PCC 6803, FEMS Microbiol. Lett. 193 (2000) 213–216.

[12] K. Fujiwara, M. Yasuno, T. Kitagawa, Novel preparation method of immunogen for hydrophobic hapten, enzyme immunoassay for daunomycin and adriamycin, J. Immunol. Methods 45 (1981) 195–203.

[13] T. Sazuka, M. Yamaguchi, O. Ohara, Cyano2Dbase ubdated: linkage of 234 protein spots to corresponding genes through N-terminal microsequencing, Electrophoresis 20 (1999) 2160–2171.

[14] M.H. Nam, E.J. Heo, J.Y. Kim, S.I. Kim, K.-H. Kwon, J.B. Seo, O. Kwon, J.S. Yoo, Y.M. Park, Proteome analysis of the responses of *Panax ginseng* C.A. Meyer leaves to high light: use of electrospray ionization quadrupole-time of flight mass spectrometry and expressed sequence tag data, Proteomics 3 (2003) 2351–2367.

[15] J.-S. Choi, D.-S. Kim, J. Lee, S.-J. Kim, S.-I. Kim, Y.H. Kim, J. Hong, J.-S. Yoo, K.H. Suh, Y.M. Park, Proteome analysis of light-induced proteins in *Synechocystis* sp. PCC 6803: identification of proteins separated by 2D-PAGE using N-terminal sequencing and MALDI-TOF MS, Mol. Cells 10 (2000) 705–711.

[16] T. Kaneko, Y. Nakamura, C.P. Wolk, T. Kuritz, S. Sasamoto, A. Watanabe, M. Iriguchi, A. Ishikawa, K. Kawashima, T. Kimura, Y. Kishida, M. Kohara, M. Matsumoto, A. Matsuno, A. Muraki, N. Nakazaki, S. Shimpo, M. Sugimoto, M. Takazawa, M. Yamada, M. Yasuda, S. Tabata, Complete genomic sequence of the filamentous nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120, DNA Res. 8 (2001) 205–213.

[17] C.C. Zhang, A gene encoding a protein related to eukaryotic protein kinases from the filamentous heterocystous cyanobacterium *Anabaena* PCC 7120, Proc. Natl. Acad. Sci. USA 90 (1993) 11840–11844.

[18] A. Kamei, S. Yoshihara, T. Yuasa, X.X. Geng, M. Ikeuchi, Biochemical and functional characterization of a eukaryotic-type protein kinase, SpkB, in the cyanobacterium, *Synechocystis* sp. PCC 6803, Curr. Microbiol. 46 (2003) 296–301.

[19] S.K. Hanks, A.M. Quinn, T. Hunter, The protein kinase family: conserved features and deduced phylogeny of the catalytic domains, Science 241 (1988) 42–52.

[20] R. Dunn, L. Hicke, Domains of the Rsp5 ubiquitin-protein ligase required for receptor-mediated and fluid-phase endocytosis, Mol. Biol. Cell 12 (2001) 421–435.

[21] D. Nesic, A. Krämer, Domains in human splicing factors SF3a60 and SF3a66 required for binding to SF3a120, assembly of the 17S U2 snRNP, and prespliceosome formation, Mol. Cell. Biol. 21 (2001) 6406–6417.

[22] D.W. Keizer, C.M. Slupsky, M. Kalisiak, A.P. Campbell, M.P. Crump, P.A. Sastry, B. Hazes, R.T. Irvin, B.D. Sykes, Structure of a pilin monomer from *Pseudomonas aeruginosa*: implications for the assembly of pili, J. Biol. Chem. 276 (2001) 24186–24193.

[23] K.T. Forest, S.A. Dunham, M. Koomey, J.A. Tainer, Crystallographic structure reveals phosphorylated pilin from *Neisseria*: phosphoserine sites modify type IV pilus surface chemistry and fibre morphology, Mol. Microbiol. 31 (1999) 743–752.

[24] D. Bhaya, A. Takahashi, P. Shahi, A.R. Grossman, Novel motility mutants of *Synechocystis* strain PCC 6803 generated by in vitro transposon mutagenesis, J. Bacteriol. 183 (2001) 6140–6143.

[25] A. Kamei, T. Yuasa, K. Orikawa, X.X. Geng, M. Ikeuchi, A eukaryotic-type protein kinase, SpkA, is required for normal motility of the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803, J. Bacteriol. 183 (2001) 1505–1510.

[26] A. Kamei, T. Yuasa, X. Geng, M. Ikeuchi, Biochemical examination of the potential eukaryotic-type protein kinase genes in the complete genome of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803, DNA Res. 9 (2002) 71–78.

[27] D. Bhaya, A. Takahashi, A.R. Grossman, Light regulation of type IV pilus-dependent motility by chemosensor-like elements in *Synechocystis* PCC 6803, Proc. Natl. Acad. Sci. USA 98 (2001) 7540–7545.

[28] D.M. Kehoe, A.R. Grossman, Similarity of a chromatic adaptation sensor to phytochrome and ethylene receptors, Science 273 (1996) 1409–1412.

[29] M. Marceau, X. Nassif, Role of glycosylation at Ser63 in production of soluble pilin in pathogenic *Neisseria*, J. Bacteriol. 181 (1999) 656–661.

[30] R. Haas, H. Schwarz, T.F. Meyer, Release of soluble pilin antigen coupled with gene conversion in *Neisseria gonorrhoeae*, Proc. Natl. Acad. Sci. USA 84 (1987) 9079–9083.

[31] D.C. Liebler, Introduction to Proteomics: Tools for the New Biology, Humana Press, Totowa, NJ, 2002.

[32] M.S. Strom, S. Lory, Structure–function and biogenesis of the type IV pili, Annu. Rev. Microbiol. 47 (1993) 565–596.

[33] H.E. Parge, K.T. Forest, M.J. Hickey, D.A. Christensen, E.D. Getzoff, J.A. Tainer, Structure of the fibre-forming protein pilin at 2.6 Å resolution, Nature 378 (1995) 32–38.

[34] E. Stimson, M. Virji, K. Makepeace, A. Dell, H.R. Morris, G. Payne, J.R. Saunders, M.P. Jennings, S. Barker, M. Panico, I. Blench, R.E. Moxon, Meningococcal pilin: a glycoprotein substituted with digalactosyl 2,4-diacetamido-2,4,6-trideoxyhexose, Mol. Microbiol. 17 (1995) 1201–1214.

[35] E. Stimson, M. Virji, S. Barker, M. Panico, I. Blench, J. Saunders, G. Payne, E.R. Moxon, A. Dell, H.R. Morris, Discovery of a nevel protein modification: α-glycerophosphate is a substituent of meningococcal pilin, Biochem. J. 316 (1996) 29–33.

[36] J.D. Thompson, D.G. Higgins, T.J. Gibson, 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 (1994) 4673–4680.