FEBS Letters 479 (2000) 72–77 FEBS 23988

Involvement of an FtsH homologue in the assembly of functional photosystem I in the cyanobacterium *Synechocystis* sp. PCC 6803

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Received 16 May 2000; revised 12 July 2000; accepted 12 July 2000

Edited by Gunnar von Heijne

Abstract The *Synechocystis* sp. PCC 6803 genome encodes four putative homologues of the AAA protease FtsH, two of which (slr0228 and sll1463) have been subjected to insertional mutagenesis in this study. Disruption of sll1463 had no discernible effect but disruption of slr0228 caused a 60% reduction in the abundance of functional photosystem I, without affecting the cellular content of photosystem II or phycobilisomes. Fluorescence and immunoblotting analyses show reductions in PS I polypeptides and possible structural alterations in the residual PS I, indicating an important role for slr0228 in PS I biogenesis.

Key words: AAA protease; Cyanobacterium; Photosynthesis; Photosystem I; *Synechocystis*

1. Introduction

Photosynthetic electron transport and photophosphorylation are carried out by large protein–pigment complexes in the thylakoid membranes of cyanobacteria and chloroplasts, and these complexes contain some of the most abundant proteins in nature. Despite their abundance and complexity (photosystem (PS) II alone contains over 20 different polypeptides) astonishingly little is known about their assembly.

The AAA (ATPases associated with a variety of cellular activities) proteases are widely, if not universally, distributed in prokaryotes and eukaryotes and have been implicated in processes as diverse as cell cycle regulation, protein degradation, vesicle-mediated transport, and organelle biogenesis (reviewed in [1,2]). Members of the AAA protease superfamily are characterised by one or two copies of a highly conserved 230–250 amino acid AAA motif [1] also referred to as the conserved ATPase domain or CAD, which evolutionary analysis suggests was present in the last common ancestor of eubacteria, archaebacteria and eukaryotes [3]. The AAA motif itself contains three boxes of particularly highly conserved residues, two of which can be assigned to motifs A and B of the Walker-type ATPases and the third may be a portion of the catalytic centre [4]. FtsH is an archetypal member of the AAA protease superfamily. The *ftsH* gene was first identified by the isolation of a temperature-sensitive *Escherichia coli* mutant defective in septation [5] and encodes a polypeptide of 71 kDa with two potential membrane-spanning α-helices towards the N-terminus with the rest of the polypeptide, including the ATPase domain, being presumably cytoplasmic [6]. A further characteristic of the cytoplasmic domain of FtsH is a zinc metallo-protease signature [7]. FtsH is involved in a number of processes in *E. coli* (for review see [8]) including the degradation of a number of unassembled proteins and the control of phospholipid and lipopolysaccharide biosynthesis [9].

Recently, using an antibody against the *E. coli* FtsH protein, Lindahl et al. [10] demonstrated the presence of a cross-reacting protein in the thylakoids of spinach that appeared to be an integral membrane protein with a hydrophilic portion exposed to the stroma. Isolation and characterisation of an *Arabidopsis* cDNA revealed a protein with a high degree of similarity to FtsH, which was capable of import into isolated chloroplasts where it was processed to its mature form and targeted to the thylakoid membrane. Genes encoding FtsH homologues have also been characterised in the chloroplast genomes of red and brown algae [11,12]. Light-stimulated degradation of an unassembled Rieske FeS protein has been shown to involve a FtsH-like thylakoid-associated protease [13] and FtsH has been implicated in the secondary degradation steps of the PS II D1 protein [14].

Sequencing of the complete genome of the cyanobacterium *Synechocystis* sp. PCC 680 revealed the presence of four open reading frames (ORFs) encoding polypeptides which appeared to be homologues of FtsH [15] (http://www.kazusa.or.jp/cyano/). The purpose of the research described was to establish whether any of these four cyanobacterial proteins plays a role in the assembly of the thylakoid-associated photosynthetic protein-complexes.

2. Materials and methods

2.1. Growth of cyanobacteria

The laboratory strain of *Synechocystis* sp. PCC 6803, which exhibits a spreading colonial morphology and does not utilise glucose [16], was grown in BG11 medium [17] supplemented with 10 mM sodium bicarbonate at 30°C and at a light flux of 30 μE m^{-2} s^{-1} (PAR) in an illuminated shaking incubator. Solid media were prepared by the addition of 1.5% Difco Bacto-agar.

2.2. DNA manipulations, PCR and insertional mutagenesis

Restriction enzymes and T4 DNA ligase were used under the conditions recommended by the manufacturers. Standard molecular biological procedures were carried out as described by Sambrook et al. [18]. PCR primers were designed using Primer Designer version 3

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0014-5793/00/$20.00
PII: S0014-5793(00)01871-8

(Educational and Scientific Software). For each of the four ORFs to be mutated the primers were designed such that the forward primer was upstream of the ORF startpoint and the reverse primer was in the C-terminal portion of the ORF and that a convenient restriction site compatible with one of the drug resistance cassettes was roughly central to the fragment to be amplified. The primers for the four ORFs are as follows: slr1604 (F) 5′-GGCCACTAGCACCGTCATTA-3′ (R) 5′-ACCGGTGGTGACTTCCTCTT-3′ (1678 bp); slr1390 (F) 5′-CTGATGGCCACCATGGCTAA-3′ (R) 5′-GTGGCCGCAATGACAATGAT-3′ (1397 bp); slr0228 (F) 5′-GCCGTTCCGTCTTGCAATTC-3′ (R) 5′-ATGGCGTGGCCTACTTCGTG-3′ (1684 bp); (sll1463) 5′-TACAAGCCGCCTGTGAACTG-3′ (R) 5′-ACCAATACCTGGCGATCGAA-3′ (1416 bp). The PCR products for the four ORFs were cloned into pCR2.1 using a TA cloning kit (Invitrogen). ORF slr1604 was mutated by insertion into a *Sma*I site of the 3.7 kb *Sma*I fragment from plasmid pUIDC1 [19]. The 2 kb Ω fragment from plasmid pHP45 [20] was inserted into the *Acc*I site and *Nhe*I site of slr0228 and sll1463 respectively. ORF slr1390 received the 1.4 kb kanamycin resistance fragment from plasmid pKRP-11 [21] into a *Bsg*I site. *Synechocystis* sp. PCC 6803 was transformed with each of the constructs and selection made for the appropriate antibiotic. Representative colonies from each transformation were streaked out three times to allow for segregation and their genotype was confirmed by both PCR and Southern blotting.

2.3. Transformation of Synechocystis sp. PCC 6803

Synechocystis sp. strain PCC 6803 was transformed as described elsewhere [22]. Cells were grown to a density of $2–5\times10^8$ cells ml^{-1} and collected by centrifugation at room temperature. After resuspension in fresh growth medium to a density of 1×10^9 cells ml^{-1} they were used immediately for transformation. 150 μl cell suspension was mixed with plasmid DNA and incubated for 1 h. After incubation the mixture was spread onto the non-selective BG-11 plates. After incubating the plates under standard conditions for 18 h to allow for expression of antibiotic resistance in the transformed cells, the appropriate antibiotic was placed underneath the agar. Antibiotics were used at the following concentrations: kanamycin (5 μg ml^{-1}), chloramphenicol (7.5 μg ml^{-1}), spectinomycin (25 μg ml^{-1}). Colonies of transformed cells were usually seen after a week of incubation.

2.4. Preparation of total membranes, SDS–PAGE and Western blotting

Synechocystis membranes were isolated according to England and Evans [23]. Membrane fractions corresponding to 1 μg chlorophyll and 15 μg protein were analysed by SDS–PAGE and immunoblotting using antibodies to PsaF, PsaD and PS I-9. Detection was made using Enhanced Chemiluminescence according to the manufacturers instructions (Amersham).

2.5. Measurement of absorption and fluorescence spectra

Cell absorption spectra were measured in an Aminco DW2000 spectrophotometer. Fluorescence emission spectra were measured at 77 K in a Perkin-Elmer LS50 luminescence spectrometer. Cells were resuspended in growth medium to a concentration of 5 μM chlorophyll, injected into 4 mm diameter silica tubes, dark-adapted for 5 min and then frozen in liquid nitrogen. The excitation and emission slit-widths were 5 nm.

2.6. Estimation of the cell content of PS I, PS II and phycobilisomes

Cell density of cultures was estimated from light-scattering at 750 nm. A haemocytometer was used to calibrate the measurement for *Synechocystis* cells. Chlorophyll *a* concentrations were estimated from the absorbance of methanol extracts at 665 nm [24]. The ratio of phycocyanin to chlorophyll was estimated from the cell absorption spectrum, using the formulas of Myers et al. [25]. Thylakoid membranes were isolated as described by Mullineaux [26]. An absorption spectrum of the phycobilin-containing supernatant from the preparation was used to calculate the phycocyanin/allophycocyanin ratio [27]. Phycobilisome content was estimated on the assumption that there are 66 allophycocyanin subunits per phycobilisome core [28]. PS I content was estimated spectrophotometrically. Thylakoid membranes were homogenised and resuspended to a chlorophyll concentration of 10 μM. An absorption difference spectrum was then recorded for membranes in the presence of ascorbate or ferricyanide (2 mM). The concentration of P_{700} was estimated from the maximum absorption difference (at approximately 702 nm) using an extinction coefficient of 64 mM^{-1} cm^{-1} [29]. PS II was assayed from the binding of ^{14}C-labelled atrazine [30]. Whole cells were incubated for 5 min in the dark with different concentrations of the labelled atrazine. The samples were then centrifuged and the supernatants were separated from the cell pellets. The supernatants were mixed with a scintillation cocktail and counted in a scintillation counter. The amounts of atrazine bound by the cells were estimated by reference to the counts from a series of known dilutions of the atrazine. The PS II content of the samples was assumed to be equivalent to the saturating amount of atrazine bound. This was estimated by weighted linear regression on a double-reciprocal plot of atrazine added versus atrazine bound.

3. Results

The genome of the cyanobacterium *Synechocystis* sp. PCC 6803 has been completely sequenced and shown to encode four ORFs which represent putative FtsH homologues [15]. An alignment (Fig. 1) of these four ORFs with FtsH from *E. coli*, together with the chloroplastic FtsH-homologue from *Arabidopsis thaliana*, reveals that the six proteins exhibit extensive similarity/identity and that the four cyanobacterial proteins contain the highly conserved ∼230 amino acid AAA protease domain, including the two nucleotide binding motifs and the SRH motif, as well as a zinc-binding motif characteristic of the active site of zinc-metalloproteases. Furthermore, the Pedant database (http://pedant.mips.biochem.mpg.de/) [31] indicates that the proteins encoded by slr1390 and sll1463 are likely to have one N-terminal transmembrane region and ORFs slr1604 and slr0228 are likely to have two.

The sequence information available for the cyanobacterial genes presents the opportunity to investigate the involvement of their protein products in the assembly of components of the photosynthetic apparatus. The four genes encoding putative FtsH homologues were cloned and insertionally mutated with drug resistance cassettes (for details see Section 2). Transformation into *Synechocystis* sp. PCC 6803 of each of the four mutated genes would be expected to primarily yield recombinants in which double crossovers replaced the chromosomal copy of the gene with the mutated copy via marker exchange. The pCR2.1 vector is unable to replicate in cyanobacteria and consequently after segregation the transformants should carry only the insertionally mutated copy of the gene in question. Since cyanobacteria contain multiple copies of the genome, representative transformants for each construct were streaked out three times to permit complete segregation of cells carrying mutant genomes. Thus, each mutant strain would contain one mutated *ftsh*-like gene and three wild type *ftsH*-like genes. Analysis by both PCR and Southern hybridisation of representative clones for mutated ORFs slr0228::Ω and sll1463::Ω for confirmed complete segregation (data not shown), thus ruling out the presence of any residual copies of the unmutated gene. Since the transformants were able to segregate clones which lacked the wild type copy of the gene it can be inferred that neither of these genes by themselves are essential, in contrast to the *E. coli ftsH* gene [7]. In contrast, analysis of representative clones carrying mutations in ORFs slr1390 and slr1604 revealed incomplete segregation even after multiple rounds of restreaking which would suggest that these ORFs encode proteins essential for cell viability. Visual screening of the transformants indicated that only in the case of clones carrying the mutant allele slr0228::Ω was there a marked change in pigmentation with colonies appearing more blue than blue-green. This change in pigmentation was confirmed

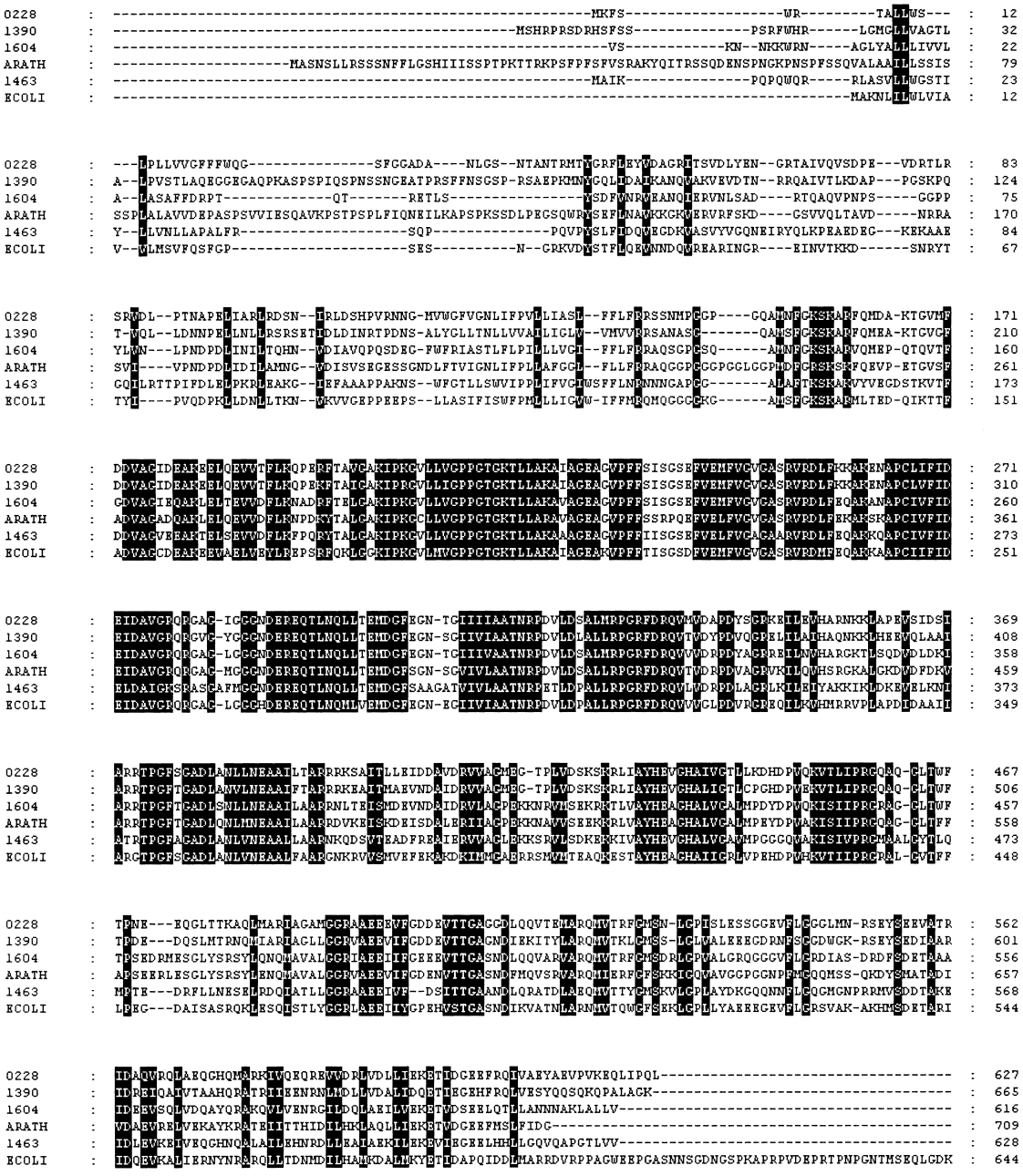

Fig. 1. A Clustal W 1.74 alignment of *E. coli* FtsH (ECOLI) and the homologues from *Synechocystis* sp. PCC 6803 (0228, 1390, 1604, 1463) and *A. thaliana* (ARATH). Groups of conserved or identical residues are shown in inverse video.

by the whole cell absorption spectrum (Fig. 2), which revealed a marked increase in the phycocyanin/chlorophyll ratio. The apparent alteration in the abundance of PS I was confirmed and extended by assays for the cell content of PS II, PS I and phycobilisomes, the results of which are summarised in Table 1. These results show that the cell content of PS II and phycobilisomes in mutant slr0228::Ω is largely unchanged compared to the wild type, but there is a 60% reduction in the number of functional PS I reaction centres per cell. The analysis of PS II–chlorophyll obtained by measurement of the binding of ^{14}C-atrazine is shown in Fig. 3. Under normal laboratory conditions the growth rate of mutant slr0228::Ω was only slightly reduced compared to that of the wild type (data not shown).

Fig. 4 shows low-temperature fluorescence emission spectra for cells of mutant slr0228::Ω and the wild type. Excitation was at 435 nm (light absorbed primarily by chlorophyll *a* in the PS II and PS I core complexes). The peaks at about 685 and 692 nm arise from PS II, and the peak at about 725 nm from PS I. In comparison to the wild type, the mutant shows a decrease in fluorescence emission from PS I relative to PS II (the spectra are normalised to the PS I peak because the absolute amplitudes of fluorescence spectra from frozen samples are unreliable). This is consistent with the decreased PS II/PS I ratio in the mutant (Table 1). The spectrum for the 0228 mutant also shows a blue-shift in the PS I fluorescence emission maximum, from 725 to 722.5 nm. This shift is not simply a consequence of the altered PS I/PS II ratio, because

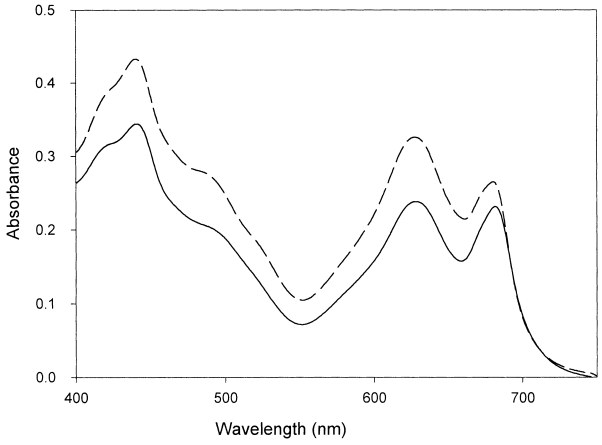

Fig. 2. Whole cell absorption spectra of wild type *Synechocystis* sp. PCC 6803 (solid line) and the slr0228::Ω mutant (broken line).

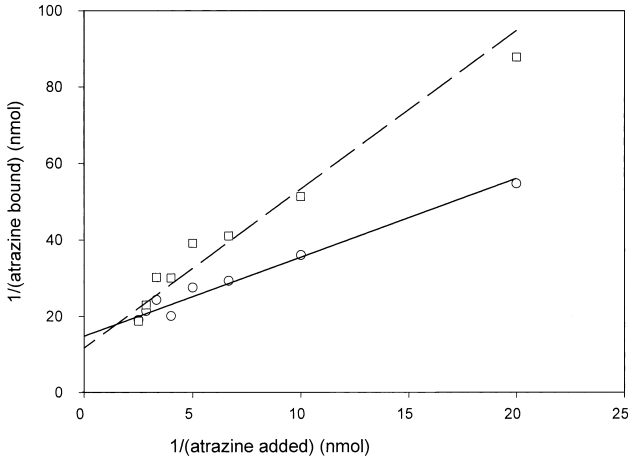

Fig. 3. PS II assay of wild type *Synechocystis* sp. PCC 6803 (circles, solid line) and the slr0228::Ω mutant (squares, broken line) presented as a double reciprocal plot of atrazine added versus atrazine bound. Both samples were at the same chlorophyll concentration. The lines are derived from weighted linear regression; the intercept with the *y*-axis gives the concentration of PS II.

it has not been observed in other cases where the PS I/PS II ratio changes. It suggests that the PS I centres present in the 0228 mutant are structurally different from those in the wild type. In contrast, there was no alteration in the abundance of PS II and there was no detectable shift in the absorbance difference spectrum for P_{700} (data not shown).

The spectrophotometric analysis cannot distinguish between the possibilities of a general reduction in normally functional PS I, a heterogeneous population of PS I with a proportion of inactive or partially active complexes, or a homogeneous population of partially active PS I complexes. Western blotting using antibodies raised against components of PS I was used to establish whether PS I polypeptides were present in normal or reduced abundance (Fig. 5). The SDS–PAGE profile of total membranes from the wild type and mutant (Fig. 5A) shows few differences with the exception of unidentified new, diffuse bands representing proteins of about 32 kDa and 23–25 kDa. However, differences in the content of PS I proteins are revealed by immunoblotting carried out with samples loaded at either constant chlorophyll or constant protein. Since the mutant has a reduced chlorophyll content, the logical comparison is likely to be on the basis of constant protein. Antibodies raised against *Synechocystis* sp. PCC 6803 PsaD and PsaF revealed a reduction in both polypeptides, which was more marked under loadings of constant protein and consistent with the 65% reduction in PS I observed by difference spectroscopy. An antibody raised against whole PS I from barley recognised a 15 kDa polypeptide in *Synechocystis* membranes, which was likewise reduced in the mutant.

Table 1
A summary of the effects of the slr0228::Ω mutation on the light-harvesting structures of *Synechocystis* sp. PCC 6803

	Wild type	Mutant slr0228::Ω
Chlorophyll/cell	1.70×10^7	1.39×10^7
Phycocyanin/chlorophyll	0.519	0.649
Phycocyanobilin/cell	8.82×10^6	9.02×10^6
Phycocyanin/allophycocyanin	4.35	3.81
Phycocyanobilin/phycobilisome	431	377
Phycobilisomes/cell	20 500	23 900
Chlorophyll/PS I	187 ± 5	390 ± 19
PS I/cell	91 000 ± 2 000	36 000 ± 1 500
Chlorophyll/PS II	370 ± 33	293 ± 60
PS II/cell	46 000 ± 4 000	47 000 ± 10 000
PS I/PS II	2.0 ± 0.2	0.79 ± 0.17
PS I/phycobilisome	4.4 ± 0.1	1.5 ± 0.1
PS II/phycobilisome	2.2 ± 0.2	2.0 ± 0.4

4. Discussion

The results demonstrate that inactivation of the slr0228 gene causes a marked reduction in the abundance of functional PS I and a probable alteration in its structure, with no apparent significant effects on the content of PS II or phycobilisomes. Western blotting indicated a reduced abundance of PsaD and PsaF in thylakoids from mutant slr0228::Ω which was consistent with the spectrophotometric data. Since other PS I polypeptides have been shown not to accumulate in the absence of the PsaA/B reaction centre proteins [32,33] it may be assumed that PsaA/B were present in at least the same abundance as the non-core polypeptides. Thus,

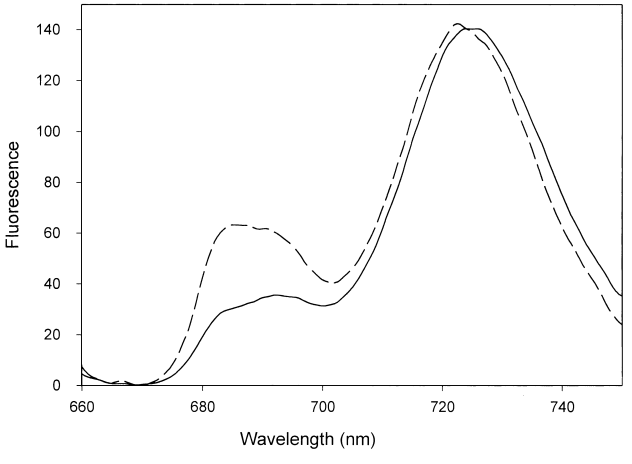

Fig. 4. Low-temperature fluorescence emission spectra at 77 K (normalised at 725 nm) of wild type *Synechocystis* sp. PCC 6803 (solid line) and the slr0228::Ω mutant (broken line). Excitation was at 435 nm.

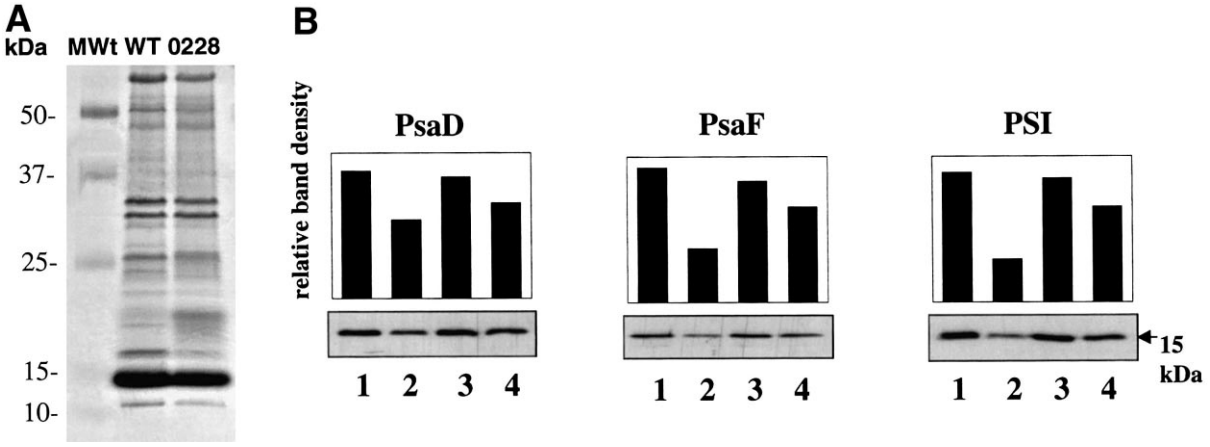

Fig. 5. Protein analysis of total membrane preparations from wild type *Synechocystis* sp. PCC 6803 and the slr0228::Ω mutant. A: Coomassie-stained gel of total protein from wild type (WT) and 0228 mutant cells (0228). Mobilities of molecular weight markers (in kDa) are indicated. B: Western blots probed with antibodies against (a) PsaD, (b) PsaF and (c) barley PS I. The tracks are as follows: wild type (1) and slr0228::Ω mutant (2) loaded at constant protein, and wild type (3) and slr0228::Ω mutant (4) loaded at constant protein. The relative band intensity, measured by laser scanning densitometry, is shown in the upper panel.

it would appear that either (a) there is a reduced abundance of fully functional PS I complexes, or (b) that there is a reduced, but heterogeneous population of partially active PS I. There is also the question of the residual assembly of PS I, albeit with slightly altered properties as indicated by the blue shift in the low temperature fluorescence emission spectrum. It would seem reasonable to assume that one or more of the other three cyanobacterial FtsH homologues can, to a restricted extent, replace the function of the slr0228 gene product in the assembly of PS I. This is being tested by the construction of multiple *ftsH* mutants.

The effect of the slr0228::Ω mutation on the assembly of PS I may be direct or indirect. The ORFs on either side of the slr0228 gene are on the opposite strand of the DNA and therefore slr0228 is not part of an operon. Consequently there is unlikely to be a polarity effect of the interposon, but also no clue to the function(s) of the gene can be obtained from examining the homologies of co-transcribed genes. Indirect effects of the mutation might be to cause a reduction in the rate of transcription of PS I genes or the translation of PS I mRNAs. There are a number of points at which the slr0228 gene product might be directly involved in the assembly of mature, functional PS I complexes that are consistent with the presence of PS I complexes in the thylakoid in reduced abundance. The mutation might lead to a reduced or incorrect insertion of PS I polypeptides into, or through, the thylakoid membrane. These possibilities can be considered in the light of the activities of closely related proteins. The gene product of slr0228 falls into the SF6 sub-family of AAA proteases as defined by Beyer [4]. This subfamily, which includes the *E. coli* FtsH, the FtsH-like proteins from *Synechocystis* sp. PCC 6803 and *A. thaliana*, as well as Yta10p and Yta12p proteins from *Saccharomyces cerevisiae*, is apparently restricted to eubacteria, chloroplasts and mitochondria. *E. coli* FtsH is involved in the proteolytic degradation of specific proteins (for review see [2,8]), but has also been suggested to function as a molecular chaperone [34]. This idea is supported by the observation that over-production of GroEL/GroES and HtpG can suppress some of the pleiotropic effects of *ftsH* mutations [35].

Further support for the role of SF6 sub-family members acting as molecular chaperones comes from the observation that the Yta10-12 complex of *S. cerevisiae* is required, independently of its proteolytic function, for the assembly of the mitochondrial membrane-associated ATP synthase [36]. Most recently, a chaperone-like activity has been associated with the AAA domain of the yeast Yme1 AAA protease [37]. This evidence would support the notion that the slr0228 gene product may function as a molecular chaperone in the correct assembly of PS I complexes, a topic which is currently being investigated in this laboratory. Given the similarity of the cyanobacterial and *A. thaliana* proteins (Fig. 1), a similar molecular chaperone role may be suggested for in the chloroplastic FtsH. This proposed role would not exclude additional functions relating to thylakoid protein turnover, such as a quality control function degrading incorrectly assembled polypeptides/complexes. The *E. coli* FtsH can degrade both the transmembrane and periplasmic domains of several integral membrane proteins, provided they are not tightly folded [38]. There is already evidence for the protein turnover function, given the apparent role of the FtsH in the light-stimulated degradation of unassembled Rieske FeS protein [13] and secondary degradation of D1 [14].

Acknowledgements: We would like to thank Professor. P.G. Chitnis and Professor H. Scheller for the kind gift of the antibodies used in this study.

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