Degradation of Phycobilisomes in Synechocystis sp. PCC6803

EVIDENCE FOR ESSENTIAL FORMATION OF AN Nbla1/Nbla2 HETERODIMER AND ITS CODEGRADATION BY A Clp PROTEASE COMPLEX

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Background: In cyanobacteria, starvation-induced phycobilisome degradation is caused by Nbla.

Results: Synechocystis expresses two Nbla proteins that form a heterodimer. The heterodimer binds phycobiliproteins and ClpC and is degraded by a ClpC-ClpP1ClpR protease in vitro.

Conclusion: Nbla1/Nbla2 is an adaptor protein that mediates degradation of phycobilisomes by the ATP-dependent protease ClpC-ClpP1ClpR.

Significance: These findings improve our understanding of the mechanisms by which cyanobacteria adapt to changing environmental conditions.

When cyanobacteria acclimate to nitrogen deficiency, they degrade their large (3–5-MDa), light-harvesting complexes, the phycobilisomes. This massive, yet specific, intracellular degradation of the pigmented phycobiliproteins causes a color change of cyanobacterial cultures from blue-green to yellow-green, a process referred to as chlorosis or bleaching. Phycobilisome degradation is induced by expression of the nbla gene, which encodes a protein of ~7 kDa. Nbla most likely acts as an adaptor protein that guides a Clp protease to the phycobiliproteins, thereby initiating the degradation process. Most cyanobacteria and red algae possess just one nbla-homologous gene. As an exception, the widely used “model organism” Synechocystis sp. PCC6803 expresses two such genes, nbla1 and nbla2, both of whose products are required for phycobilisome degradation. Here, we demonstrate that the two Nbla proteins heterodimerize in vitro and in vivo using pull-down assays and a Förster energy-transfer approach, respectively. We further show that the Nbla proteins form a ternary complex with ClpC (the HSP100 chaperone partner of Clp proteases) and phycobiliproteins in vitro. This complex is susceptible to ATP-dependent degradation by a Clp protease, a finding that supports a proposed mechanism of the degradation process. Expression of the single nbla gene encoded by the genome of the N2-fixing filamentous cyanobacterium Nostoc sp. PCC7120 in the nbla1/nbla2 mutant of Synechocystis sp. PCC6803 induced phycobilisome degradation, suggesting that the function of the Nbla heterodimer of Synechocystis sp. PCC6803 is combined in the homodimeric protein of Nostoc sp. PCC7120.

Cyanobacterial cultures growing in a nutrient-rich medium exhibit a typical blue-green color due to the photosynthetic pigments chlorophyll a and phycobiliproteins. Together with mostly uncolored linker proteins, phycobiliproteins form the phycobilisome (PBS)2. The PBS is a large (3–5 MDa) multiprotein complex associated with the cytoplasmic side of the thylakoid membranes (reviewed in Refs. 1–4). In addition to the non-pigmented linker proteins, the complex is composed of the chromophorylated phycobiliproteins phycocyanin (PC; λmax ≈ 620 nm), allophycocyanin (λmax ≈ 650 nm), and, in some cyanobacteria, phycoerythrin (λmax ≈ 565 nm) or phycoerythrocyanin (λmax ≈ 590 nm). The linker proteins are necessary for the assembly and stabilization of the PBS structure through their interactions with the phycobiliprotein subunits. They also play a role in energy transfer from the PBS to photosystem II and, in some cyanobacteria, to photosystem I as well.

PBSs are classified according to their morphology. The most common PBS structure is the hemidiscoidal type, which is made up of a core complex that is anchored in the thylakoid membrane and peripheral rods (1, 3, 5). Size, composition, and position of PBSs on the thylakoid membrane vary according to light quality and quantity so as to optimize light absorption (6–9). Nearly complete degradation of PBSs can be observed in unicellular, non-diazotrophic cyanobacteria upon nitrogen starvation and, in some species, also upon sulfur depletion. Degradation begins with the sequential loss of the peripheral rods and terminates with degradation of the core complex (10, 11). Because phycobiliproteins constitute up to 50% of the total soluble protein of a cyanobacterial cell (2), a considerable amount of cellular protein becomes degraded within the first 24 h of nitrogen deprivation. The loss of phycobiliproteins results in a color change of cyanobacterial cultures from blue-green to yellow-green, a process referred to as bleaching or chlorosis (12). PBS degradation is not essential for acclimation to nutrient deprivation but is rather thought to prevent photo damage under stress and to provide substrates for the de novo

2 The abbreviations used are: PBS, phycobilisome; Nostoc 7120, Nostoc sp. PCC7120; Synechocystis 6803, Synechocystis sp. PCC6803; Synechococcus 7942, Synechococcus elongatus PCC7942; PCC, Pasteur Culture Collection; PC, phycocyanin; IPTG, isopropyl β-D-thiogalactopyranoside; Cer, curculean; CFP, cyan fluorescent protein; Ni-IDA, nickel-iminodiacetic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

[1] This article contains supplemental Table S1 and Fig. S1.

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synthesis of proteins required for the acclimation process (11, 13).

The small protein NblA (non-bleaching A) plays a key role in the degradation process (14). Cyanobacterial mutants lacking NblA degrade virtually none of their PBS under nitrogen starvation and thus exhibit a non-bleaching phenotype (14–17). The exact function of NblA has been the subject of numerous studies. Binding experiments have shown that NblA interacts with the α-subunits of phycobiliproteins (18, 19). In pull-down experiments, NblA was found to bind to ClpC, an HSP100 chaperone partner of a Clp protease, in an ATP-dependent manner (20). This result led to a proposed model of PBS degradation in which NblA acts as a so-called adaptor protein of a Clp protease (20). Clp degradation complexes consist of two functional elements: a cylinder-like proteolytic core of two heptameric rings and an AAA+ chaperone (21, 22). The hexameric chaperone ring is responsible for substrate recognition, unfolding, and threading of the extended polypeptide chain through a narrow pore into the protease compartment, where the proteolytically active sites are sequestered from the cytoplasm (23, 24). The substrate specificity of a Clp protease is determined by its chaperone partner. Substrate recognition occurs by binding to the chaperone partner of the Clp protease or is mediated by adaptor proteins (25, 26). Adaptor proteins simultaneously bind the substrate and the chaperone, thereby forming a ternary complex that causes degradation of the substrate. In other words, adaptor proteins modulate the substrate specificity of a protease, enabling the degradation of particular proteins at the proper time. This means that substrate degradation is solely regulated by the expression of the adaptor protein. This is precisely what is observed in PBS degradation, which is induced by the expression of the small protein NblA (14). Because the Clp proteases require NblA to recognize phycobiliproteins as substrates, strains lacking NblA degrade the PBS at a low rate, even under conditions of nitrogen starvation (14, 15, 17).

Most phycobiliprotein-containing cyanobacteria and red algae possess nblA genes. The NblA proteins are rather small, consisting of ~60 amino acids. Sequence alignment of NblA proteins reveals a low homology (about 30% sequence identity on average) (27). Short stretches of highly conserved amino acid residues located near the N and C termini of the proteins are involved in phycobiliprotein and ClpC binding (19, 20). NblA proteins from Nostoc sp. PCC7120 (Nostoc 7120), Thermosynechococcus vulcanus, and Synechococcus elongatus sp. PCC7942 (Synechococcus 7942) have been crystallized (19, 27). All three NblA proteins share a similar structure. They are homodimers that consist of two α-helices: one shorter N-terminal helix and a longer C-terminal helix. The biologically active form of NblA is thus a four-helix bundle.

Cyanobacteria and red algae usually possess one nblA gene. However, two genes have been found in Nostoc 7120 and Synechocystis sp. PCC6803 (Synechocystis 6803). Whereas only one of the two nblA genes is expressed upon nitrogen deprivation in Nostoc 7120 and is sufficient for PBS degradation (17), both genes are expressed in Synechocystis 6803, and both are necessary for induction of the degradation process (15, 16).

In this study, we investigated why the two NblA proteins, NblA16803 and NblA26803, are necessary for PBS degradation in Synechocystis 6803. We present evidence that NblA16803 and NblA26803 act as a heterodimer in which NblA16803 mediates the binding to ClpC6803 and phycobiliproteins. Both NblA proteins are degraded by a Clp protease in vitro, a finding that strongly supports the proposed model of the role of NblA in PBS degradation. We further show that expression of the single nblA gene from Nostoc 7120 complements the non-bleaching phenotype of the nblA1/nblA2 double mutant of Synechocystis 6803.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—Strains are listed in supplemental Table S1. Unless stated otherwise, sources of plasmids, antibiotics, medium, restriction enzymes, and chemicals were as described previously (20).

**Synechocystis** 6803 and its mutants were grown photoautotrophically under constant illumination essentially as described before (17) except that the growth temperature was 28 °C, and the internal diameter of the culture vessels was 3.5 cm. Whole-cell absorbance spectra were recorded from 550 to 750 nm on a Specord® 200 PLUS spectrophotometer (Analytik Jena, Jena, Germany) and were corrected for cell scattering at 750 nm.

Strains of *Escherichia coli* were grown under standard conditions (28). When appropriate, antibiotics were added to the medium to final concentrations of 50 μg ml⁻¹ ampicillin, 50 μg ml⁻¹ kanamycin, or 25 μg ml⁻¹ chloramphenicol, respectively.

**Plasmid and Mutant Constructions**—Plasmids and oligonucleotides are listed in supplemental Table S1 and Fig. S1.

**Construction of Plasmids for Protein Overexpression in *E. coli***—Vectors for expression of GST-tagged NblA16803 and NblA26803 in *E. coli* were constructed as follows. The plasmid pGEX-2TK/Ndel (20) was digested with NdeI and SmaI in order to obtain the fragment encoding GST, the fragment was ligated into the expression vector pACYCDuet-1, which was digested with NdeI and EcoRV prior to ligation. The resultant plasmid pACYC/GST was digested with Ndel and, after dephosphorylation, was ligated to a fragment harboring nblA16803 (ssl0452) or nblA26803 (ssl0453), respectively, yielding plasmids pACYC/A1_GST and pACYC/A2_GST. Fragments encoding nblA16803 and nblA26803 were generated by PCR from genomic *Synechocystis* 6803 DNA using primers nbl7 and nbl28 (amplification of nblA16803) or nbl24 and nbl31 (amplification of nblA26803). Each primer sequence contained an Ndel restriction site for cloning. The primers nbl28 and nbl31 additionally contained a sequence encoding the PreScission™ protease recognition sequence, enabling cleavage of the NblA16803/NblA26803-GST fusion protein. For coexpression of His-tagged NblA16803 and GST-tagged NblA26803, the nblA16803 gene was amplified by PCR using primers nbl32 (EcoRI site incorporated) and nblA-4 (HindIII site incorporated) and was ligated into plasmid pACYC/A2_GST to yield plasmid pACYC/His_A1 and A2_GST. For simultaneous expression of His-tagged NblA26803 and GST-tagged NblA16803, nblA26803 was amplified by PCR using primers nbl33 (HindIII site inserted) and nbl34 (EcoRI site inserted). The PCR fragment was cloned into the plasmid pACYC/A1_GST, generating plasmid pACYC/His_A2 and A1_GST.
For coexpression of NblA26803 and GST-tagged NblA16803, the nblA26803 gene was amplified by PCR using the primer nblI4 (NcoI site incorporated) and nblI21 (EcoRI site incorporated). The resulting PCR fragment was ligated into plasmid pACYC/ A1_GST, generating plasmid pACYC/A2 and A1_GST. For binding experiments, the ClpC6803 protein was expressed without any affinity tag. For construction of the expression plasmid, clpC6803 (sI0020) was amplified by PCR from total Synechocystis 6803 DNA using primers clpC_3 (NdeI site inserted) and clpC_5 (BamHI site inserted). The PCR fragment was digested with NdeI (partial digestion) and BamHI and cloned into plasmid pET11a, yielding plasmid pET11/ClpC. For degradation experiments, the ClpC6803 protein was expressed with a N-terminal His tag. ClpC6803 was amplified by PCR with clpC_3 (NdeI site inserted) and clpC_5 (BamHI site inserted) and ligated into plasmid pET22b, resulting in plasmid pET22b/HisClpC6803. For expression of the phycocyanin α-subunit N-terminally fused to GST, the chromosomal gene cpcA (sll1578) from Synechocystis 6803 was amplified by PCR using primers cpcA_1 (BamHI site inserted) and cpcA_2 (EcoRI site inserted) and cloned into plasmid pGEX-2TK via BamHI and EcoRI to yield plasmid pGEX/cpcA. For expression of HisClpP1 and ClpR, the clpP1 (srl0542) gene was first amplified with primers clpP1_1 (NcoI site incorporated) and clpP1_2 (NcoI site incorporated) from total Synechocystis 6803 DNA. The PCR product was digested with NcoI cloned into plasmid pACYC/Duet-1, yielding the plasmid pACYC/HisClpP1. The clpR (srl0164) gene was then PCR-amplified with primers clpR_1 (NdeI site inserted) and clpR_2 (XhoI site inserted). The PCR fragment was digested with NdeI and XhoI and cloned into the plasmid pACYC/HisClpP1, resulting in the plasmid pACYC/HisClpP1, ClpR.

All DNA constructs were checked by DNA sequencing. All plasmids and oligonucleotides are listed in supplemental Table S1.

**PBS Isolation from Synechocystis 6803 Crude Extract**—The PBS preparation was performed as described before (29).

**Coexpression and Purification of HisClpP1 and ClpR and Expression of His-tagged ClpC**—After induction with isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 h at 30 °C, cells were pelleted and resuspended in buffer A (10 mM Tris-HCl buffer (pH 7.5), 75 mM KCl, 1 mM DTT). Cells were disrupted by sonication with a homogenizer (Sonopuls) for 2 × 2 min (settings: KS76, pulsation 2, 55% power), and the extract was clarified by centrifugation and incubated with Protino® Ni-IDA resins (Macherey-Nagel). Bound proteins were purified according to the manufacturer’s protocol (GE Healthcare). The NblA16803 and NblA26803 proteins were further purified by size exclusion chromatography on a Superdex 75TM column (GE Healthcare).

**Coexpression and Purification of the NblA16803 and NblA26803 Heterodimers**—GST-NblA16803 and His-NblA26803 or His-NblA16803 and GST-NblA26803 were coexpressed after induction with IPTG (overnight, 18 °C). Crude extracts were divided; one half was incubated with glutathione-agarose (Macherey-Nagel) for binding of GST-tagged NblA, and the other half was incubated with Protino® Ni-IDA resins (Macherey-Nagel) for binding of His-tagged NblA. Purification was performed as described above. Eluted proteins were immediately analyzed by Tricine-SDS-PAGE with 6 m urea (30).

**Protein Expression, Purification, and in Vitro Binding Assays Using GST-tagged PC or GST-tagged NblA**—The N-terminally GST-tagged α-subunit of PC (GST-PC) was expressed after induction with IPTG for 3 h at 30 °C and purified according to the manufacturer’s protocol (GE Healthcare). Expression of the C-terminally GST-tagged NblA16803 (NblA16803-GST) was induced with IPTG for 80 h at 18 °C. NblA26803-GST was expressed with IPTG overnight at 18 °C. GST fusion proteins were purified as described above. GST-free NblA16803 and NblA26803 were obtained by on-column cleavage with PreScission™ protease (GE Healthcare). The NblA16803-GST and His-tagged NblA26803 were purified as described above. The expression of ClpC6803 was induced by IPTG for 3 h at 30 °C. The soluble fraction of the crude extract containing overexpressed ClpC6803 without affinity tag was used for the in vitro binding assays.

**In vitro binding assays** were started by adding glutathione-agarose to the soluble cell extract containing the overexpressed GST-PC fusion protein. After incubation for 30 min at room temperature with gentle agitation, unbound proteins were removed by three washes with five column volumes of buffer B. The PC-loaded agarose was divided into four equal aliquots and incubated with crude extract containing overexpressed ClpC6803. Additionally ATP, NblA16803, NblA26803, or NblA16803 and NblA26803 were added in surplus. After incubation for 30 min at room temperature with gentle agitation, unbound proteins were removed by washing with buffer B containing 1 mM ATP as described above. Bound proteins were eluted by the addition of 40 mM glutathione in 50 mM Tris-HCl (pH 8.0). Eluted proteins were directly analyzed by Tricine-SDS-PAGE with 6 m urea.

**In Vitro Degradation of a-Casein, PBS, NblA16803 and NblA26803 by ClpC6803**—Each purified protein was used in a final concentration of 2 μM in buffer C (50 mM Tris-HCl (pH 7.5), 75 mM KCl, 1 mM DTT, 5 mM MgCl2). All reactions were performed at 37 °C with an ATP-regenerating system consisting of 20 ng of pyruvate kinase (Roche Applied Science), 4 mM phosphoenolpyruvate, and 2 mM ATP. Degradation of a-casein (Sigma-Aldrich), the NblA16803/NblA26803 heterodimers, and the PBSs was analyzed by Tricine-SDS-PAGE in the presence of 6 m urea.

**Cloning and Expression of Fluorescent Proteins in Synechocystis 6803**—For FRET measurements, the donor cerulean (Cer) and the acceptor YFP were used. Due to the spectral overlap between donor (Cer) emission (excitation/emission maxima,
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433/475 nm) and acceptor (YFP) absorbance (excitation/emission maxima, 514/527 nm), excitation of the donor molecule leads to emission from the acceptor molecule when the proteins are in close proximity (1–10 nm). For labeling, we used the monomeric forms of YFP and Cer. These forms were generated by a single amino acid exchange (A206K) in order to eliminate self-association of the proteins and thus avoid false positive FRET signals (31).

For expression of fluorescent NblA proteins in Synechocystis 6803, a ~1480-bp fragment containing nblA1_6803 and nblA2_6803, flanked by putative promoter and terminator regions, was amplified using primers nbl11 (XhoI incorporated) and nbl27 (XbaI incorporated). The PCR product was digested by the restriction enzymes XbaI and XhoI and ligated into the XbaI/XhoI-digested plasmid pET22b, yielding plasmid pET/A1 and A2, which served to introduce an NcoI site at the 3' - or 5' -end of the nblA1_6803 coding region and an NdeI site at the 3' - or 5' -end of the nblA2_6803 coding region. The restriction sites were successively generated by mutagenesis (QuickChange® site-directed mutagenesis kit; Stratagene) using primers nblA1QCM1 and nblA1QCM2 (3'-end) or nblA1QCM3 and nblA1QCM4 (5'-end) as well as nblA2QCM1 and nblA2QCM2 (3'-end) or nblA2QCM3 and nblA2QCM4 (5'-end). Mutagenesis at the 3'-end of the nblA1_6803 gene resulted in the change of the base triplet for Leu61 to a methionine residue and the concomitant loss of the base triplet for one amino acid (Gly62). The amino acid sequence of the NblA2_6803 protein was altered by site-directed mutagenesis from Leu59 to a histidine residue and from Pro60 to a methionine residue, whereby the mutagenesis of both 5'-ends did not result in any changes. The resultant plasmids were restricted with NcoI or NdeI and, after dephosphorylation, ligated to a PCR fragment harboring the coding region by QuikChange® mutagenesis using primers nblA1QCM3 and nblA1QCM4. The resultant plasmid pVZ/Y and A2, pVZ/A1 and A2, pVZ/A1 and A2, pVZ/A1 and A2, pVZ/A1 and A2, pVZ/Y_A1 and A2, pVZ/Y_A1, pVZ/Y_A1 and Cer_A2, and pVZ/Y_A2 and A2_C were transferred to the nblA1/nblA2 double mutant of Synechocystis 6803, and the plasmid pVZ/YFP and A2_Cer was transferred to Δnbl2 mutant cells of Synechocystis 6803 by conjugation (32). Exconjugants were selected on BG11 agar containing 50 μg ml⁻¹ kanamycin and 14 μg ml⁻¹ chloramphenicol.

FRET Measurements—The FRET signal was detected with a fluorescence spectrometer (Fluoromax-4-Horiba) in a suspension of living Synechocystis 6803 cells with an A570 of ~0.3. Cer expressed in the cells was excited by monochromatic 458-nm light, and fluorescence emission spectra were recorded in the wavelength range from 500 to 600 nm.

Fluorescence Lifetime Measurements—Fluorescence lifetime measurements were performed using a FluTime 200 instrument (PicoQuant) in suspension of living Synechocystis 6803 cells with an A750 of ~0.3 as described before (33).

Complementation Experiments with NblA of Nostoc 7120—The plasmid for expression of nblA from Nostoc 7120 was constructed as follows. A 669-bp fragment, bearing upstream sequences of nblA1_6803 (ssl0452) and nblA2_6803 (ssl0453) and a 417-bp fragment containing downstream sequences of nblA1_6803 and nblA2_6803 was amplified by PCR from total Synechocystis 6803 DNA using primers nbl11 (XhoI site inserted) and nbl13 (Ncol site inserted) and primers nbl26 (Ndel site inserted) and nbl27 (XbaI site inserted), respectively. The fragments were cloned into the plasmid pET22b, yielding plasmid pET22/P6803NblA7120. Subsequently, the plasmid pET22/P6803NblA7120 was digested with NcoI and NdeI and ligated to a fragment harboring the nblA gene from Nostoc 7120, which was amplified by PCR from total Nostoc 7120 DNA using primers nblana2.23 (Ncol site inserted) and nblana2.24 (Ndel site inserted), respectively. The fragment harboring the nblA gene from Nostoc 7120 was excised by digestion with XbaI and XhoI and inserted into the conjugative, self-replicating plasmid pVZ321. The plasmid pVZ/A1/nblA2_6803/Nostoc 7120 was transferred to nblA1/NblA2 double mutant cells by conjugation, and exconjugants were selected on BG11 agar containing 50 μg ml⁻¹ kanamycin and 14 μg ml⁻¹ chloramphenicol.

Zinc-induced Fluorescence—For zinc-induced fluorescence, the Tricine-SDS-PAGE was run with 1 mM zinc acetate in cathode buffer (30). The fluorescence was visualized by a UV transilluminator (Bio-Rad).
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Detecting the Interaction between NblA1\textsubscript{6803} and NblA2\textsubscript{6803} in Vivo by FRET—The biologically active form of NblA from Nostoc 7120 is a homodimer (19). Dimer formation seems to be a characteristic feature of NblA proteins (27) and is assumed to be essential for their function. To monitor the dimerization of the two NblA proteins during the PBS degradation process, we used a method based on FRET. As a donor-acceptor pair, we chose Cer (35), a variant of enhanced cyan fluorescent protein (CFP), and YFP.

Plasmids containing the monomeric forms of YFP and Cer (31), designated Y and C, respectively, were used for labeling. The NblA-fluorophore fusion proteins were expressed in the nblA1/nblA2 double mutant under the control of the NblA promoter and terminator region in the self-replicating vector pVZ321. The following controls were used for evaluation of FRET measurements: (i) nblA1/nblA2 double mutant transformed with plasmids expressing only a single fluorophore fused to NblA1\textsubscript{6803} or NblA2\textsubscript{6803}; (ii) nblA1/nblA2 double mutant transformed with plasmid pVZ/A2\_Y and A2\_C expressing two NblA2\textsubscript{6803} proteins, one tagged with YFP and one tagged with Cer; (iii) nblA2 single mutant (15) transformed with plasmid pVZ/Y and A2\_C, which allows expression of the YFP protein and C-terminal Cer-tagged NblA2\textsubscript{6803} and (iv) nblA2 single mutant transformed with plasmid pVZ/A2\_Y and A2\_C (see above).

In preliminary experiments, we determined whether NblA-fluorophore fusion proteins were functional in vivo. Mutants grown in nitrogen-containing medium (BG11) were transferred to medium without combined nitrogen (BG11\textsubscript{0}), and whole-cell absorbance spectra were recorded after 24 h (Fig. 2, A–C). PBS degradation after nitrogen deprivation took place in all strains expressing only a single fluorophore, regardless of whether the fluorophore was fused to the C or N terminus of the respective NblA protein (Fig. 2, A and B). Complementation was also observed in mutants expressing C-terminally tagged NblA1\textsubscript{6803} and NblA2\textsubscript{6803} (A1\_Y and A2\_C) (Fig. 2A). However, expression of N-terminally tagged NblA proteins (Y\_A1 and C\_A2) did not induce PBS degradation (Fig. 2B). This observation can be explained by our previous finding that NblA triggers PBS degradation by binding to ClpC via its N-terminal helix (20). Accordingly, fluorophores fused to the N terminus of NblA presumably sterically hinder the interaction with ClpC, thereby interfering with the in vivo function of NblA. In this context, it is remarkable that complementation occurred when only one of the two NblA proteins was N-terminally fused to a fluorophore, whereas it did not matter which NblA protein was labeled or what fluorophore it was labeled with (Fig. 2B).

Furthermore, as shown in Fig. 2C, expression of YFP and C-terminally tagged NblA2\textsubscript{6803} (Y and A2\_C) and simultaneous expression of two NblA2\textsubscript{6803} proteins with C-terminal YFP or Cer (A2\_Y and A2\_C) complemented the non-bleaching phenotype of the nblA2-deficient mutant.

On the basis of the results of complementation experiments, we used the mutant expressing functional C-terminally tagged NblA1\textsubscript{6803} and NblA2\textsubscript{6803} for our initial FRET measurements. To monitor possible interactions between NblA1\textsubscript{6803}-YFP and NblA2\textsubscript{6803}-Cer, we used fluorescence spectroscopy. We excited the donor molecule (Cer) by actinic light with a wavelength of

RESULTS

Homodimeric NblA of Nostoc 7120 Complements the Non-bleaching Phenotype of the nblA1/nblA2 Double Mutant of Synechocystis 6803—Genomes of most cyanobacteria, such as Synechococcus 7942 and Nostoc 7120, encode just one nblA gene, whose expression suffices to initiate the proteolytic degradation of the PBS. The intensively studied unicellular strain Synechocystis 6803, however, simultaneously expresses two sequence-related nblA genes, nblA1\textsubscript{6803} and nblA2\textsubscript{6803} to induce PBS degradation (15, 16).

Complementation experiments were performed to determine whether the two NblA proteins from Synechocystis 6803 act in the same manner as NblA from Nostoc 7120 (20). To this end, the coding region of Nostoc 7120 nblA (ORF asr\_4517) and a DNA fragment encoding Synechocystis 6803 nblA1\textsubscript{6803} and nblA2\textsubscript{6803} were amplified by PCR. Both fragments were ligated into the self-replicating plasmid pVZ321 containing the predicted promoter and terminator regions of Synechocystis 6803 nblA1\textsubscript{6803} and nblA2\textsubscript{6803}, which are cotranscribed from a common promoter (15). The generated plasmids were subsequently transferred to the Synechocystis 6803 nblA1/nblA2 double mutant by triparental mating (34).

When grown in nitrate-containing medium, the pigmentation of the resulting exconjugants was indistinguishable from that of the wild type, as judged from whole-cell absorbance spectra (Fig. 1). After transfer to nitrogen-free medium, nblA1\textsubscript{6803} and nblA2\textsubscript{6803} expressed in trans in the nblA1/nblA2 double mutant, complemented the mutant phenotype, visible in the absorbance spectra shown in Fig. 1 as a decrease in the PC absorbance peak at ~625 nm. A similar complementation effect was obtained with nblA from Nostoc 7120. This clearly demonstrates that the homodimeric NblA from Nostoc 7120 is able to replace the function of NblA1\textsubscript{6803} and NblA2\textsubscript{6803} in Synechocystis 6803.
PBS Degradation Depends on NbIA1/NbIA2 and a Clp Protease

Expression of NbIA proteins was induced by nitrogen depletion and monitored by immunoblot analysis (Fig. 2, D and E). PBS degradation was verified by recording emission spectra of whole cells immediately as well as 3, 5, 8, and 24 h after nitrogen deprivation. There were no significant differences in emission spectra among analyzed mutants grown on nitrate-containing medium (Fig. 3, A and B; 0 h); the emission peak at ~540 nm corresponds to Synechocystis 6803 autofluorescence. Mutants were then switched to nitrogen starvation conditions, and fluorescence emission was monitored for 24 h. The mutant expressing C-terminally tagged NbIA16803 and NbIA26803 (A1_Y and A2_C) showed an increase in the amplitude of acceptor (YFP) emission at ~530 nm over the first 8 h. After 24 h of nitrogen starvation, acceptor emission was still detected, albeit with a lower amplitude (Fig. 3, A1_Y, A2_C). A decrease in absorbance at 620 nm indicates degradation of PBS. D, immunoblot analysis of the NbIA fusion proteins in crude extracts of Synechocystis 6803 (Fig. 2E, A2_C) and anti-GFP antibodies (Fig. 2E). Both fluorophores were presumed to be expressed, and FRET was measured as described above. The emission spectrum of the mutant expressing YFP and Cer coupled to NbIA26803 showed no FRET signal (Fig. 3B, Y and A2_C), and its spectrum did not differ significantly from that of mutants containing only a single fluorophore (Fig. 3, A and B). The A2_Y and A2_C mutant also showed no FRET signal during the first 8 h after nitrogen depletion (Fig. 3A). After 24 h, however, an increase in acceptor fluorescence indicative of FRET was detected, albeit to a lesser extent than in the A1_Y and A2_C mutant. Interestingly, immunoblot analyses revealed an induction of protein expression within the first hours after nitrogen step-down and a decrease in protein levels after 24 h, a pattern inconsistent with the temporal changes in the FRET signal (Fig. 2D).

In order to attribute the observable changes in steady-state fluorescence characteristics to changes in the donor fluorescence lifetime, we performed time-resolved measurements of the donor (Cer) lifetime in addition to measuring steady-state FRET. Donor fluorescence lifetime might decrease, for exam-
ple, when the acceptor molecule is in close proximity to the donor molecule and absorbs energy through FRET. These measurements were performed 8 h after nitrogen step-down, the time at which the strongest FRET signal occurred (Fig. 3A).

The average fluorescence lifetime of Cer fused to NblA26803 was 2.29 ns (Fig. 3C). In the presence of YFP fused to NblA16803, the fluorescence lifetime of Cer decreased to 1.94 ns, a difference of 0.35 ns. This faster decay of Cer fluorescence is attributable to the energy transfer (FRET) from Cer to YFP that occurred when the two fluorophores were brought close together by interactions between NblA16803 and NblA26803. When YFP was linked to NblA26803 instead of NblA16803, the fluorescence lifetime of Cer fused to another NblA26803 molecule also decreased, albeit to a lesser extent (ΔτAV = 0.15 ns; Fig. 3C). The possibility that the reduced fluorescence lifetime is due to nonspecific energy transfer from the donor to the acceptor cannot be excluded. However, the resulting difference in fluorescence lifetimes between Cer and Cer expressed together with YFP (ΔτAV = 0.2 ns) still indicates a clear FRET signal.

Taken together, the results from time-resolved fluorescence measurements confirm our steady-state measurements, indicating that FRET occurred when Cer and YFP were connected to NblA16803 and NblA26803, respectively. The much weaker FRET signal that occurred when both fluorophores were coupled to NblA26803 suggests a weak tendency of NblA2 to form homodimers.

**FIGURE 3. Formation of NblA16803–NblA26803 heterodimers in vivo.** The nblA1/nblA2 double mutant of Synechocystis 6803 was complemented in trans, under the control of the nblA promoter and terminator, with one NblA protein C-terminally fused to the FRET donor Cerulean (C) and the other fused to the FRET acceptor YFP (Y). For expression of NblA fusion proteins, the Synechocystis 6803 mutants were starved of combined nitrogen. A, fluorescence emission spectra of Synechocystis 6803 cells. Emission spectra of A1_Y, A2_C, A1_Y, A2, A1, A2_C, and A2_Y, A2_C mutants were recorded on cultures adjusted to an A750 of 0.3. Cerulean was excited at 458 nm, and emission of the FRET partner YFP was recorded between 500 and 600 nm after 0, 3, 5, 8, and 24 h of nitrogen depletion. B, fluorescence emission spectra of Synechocystis 6803 cells. Emission spectra of A1_Y and A2_C, A1 and A2_C, and A2_Y and A2_C mutants were recorded at cell densities corresponding to an A750 of 0.3. Cerulean expressed in the cells was excited at 458 nm, and emission of the FRET partner YFP was recorded between 500 and 600 nm after 0, 7, and 24 h of nitrogen depletion. C, fluorescence lifetime spectroscopy of the A1_Y and A2_Cer, A2_C and A2_Y, and A1 and A2_C mutants. Cells at a density corresponding to an A750 of 0.3 were analyzed after 8 h of nitrogen starvation. Fluorescence intensity measurements were carried out in 1-cm cuvettes with excitation at 440 nm and emission of cerulean-based fluorescence decay recorded at 475 nm. Only a single photon every 100 laser pulses was registered (1 count); therefore, measurements were repeated for a total of 10,000 counts, and the measured time differences were sorted into a histogram. Each measurement was repeated seven times. The resulting decay curves were analyzed by fitting to the sum of two exponential terms using a non-linear least squares iterative procedure (FluoFit, PicoQuant). For each measurement, the amplitude-weighted lifetime (τAV) of cerulean was calculated. The mean values of τAV for each biological replicate are shown in the diagram, and the S.D. for each value is indicated. Furthermore the mean value of τAV for cerulean-based fluorescence of each mutant is shown.
Detecting the Interaction between NblA1 and NblA2 in Vitro by Pull-down Experiments—To validate our in vivo results, we performed in vitro binding studies. We first cloned nblA1 and nblA2 into the expression vector pACYC-Duet-1, which is designed for coexpression of two target proteins. This vector was chosen to imitate expression in Synechocystis 6803, where nblA1 and nblA2 are adjacent cotranscribed genes (15).

Affinity purification of NblA1-GST invariably resulted in coprecipitation of HisNblA2 and vice versa (Fig. 4), clearly demonstrating an interaction between NblA1 and NblA2 in vitro. These findings corroborate the results obtained with in vivo binding studies, described above. Taken together, in vivo and in vitro experiments provide evidence for an interaction between NblA1 and NblA2, indicating that the biologically active form is a heterodimer.

NblA1 and NblA2 Heterodimers Interact with ClpC and the α-Subunit of PC in Vitro—Previous investigations of NblA function in Nostoc 7120 revealed that formation of a ternary complex among NblA, phycobiliproteins, and ClpC is indispensable for PBS degradation (20). Here, we performed binding experiments with NblA1, NblA2, ClpC, and the apoprotein of the α-subunit of PC from Synechocystis 6803. The N-terminally GST-tagged apoprotein of the PC α-subunit was immobilized on glutathione-agarose. The loaded matrix was then divided into two equal aliquots and incubated with crude extracts of ClpC6803-overexpressing E. coli in the presence or absence of NblA1 and NblA2. As expected, copurification of ClpC6803 was observed only when both NblA1 and NblA2 were present (Fig. 5A). Similar results were obtained using another experimental approach. In this case, heterodimers consisting of NblA1 and His-tagged NblA2 were immobilized on nickel-nitrilotriacetic acid, incubated first with a crude extract of E. coli expressing ClpC6803 and then with a crude extract of Synechocystis 6803. As shown in Fig. 5 (B and C), ClpC6803 and phycobiliproteins (PC and apophycocyanin) copurified with NblA1 and His-tagged NblA2.

NblA1 Mediates the Interaction of ClpC6803 with Phycobiliproteins—As shown previously, a highly conserved stretch of amino acids near the N terminus of NblA from Nostoc 7120 is involved in ClpC binding (20), whereas residues near the C terminus are involved in phycobiliprotein binding (19). Assuming that the biologically active form of NblA in Synechocystis 6803 is a heterodimer, we would expect that both NblA1 and NblA2 would be able to bind ClpC6803 and phycobiliproteins and that both NblA proteins would be essential for formation of a complex with ClpC6803 and phycobiliproteins. To verify this hypothesis, we first analyzed whether NblA1 and NblA2 interact with ClpC6803 in vitro. To this end, we immobilized C-terminally GST-tagged NblA1 and NblA2 on glutathione-agarose and incubated each with a crude extract of E. coli expressing ClpC6803. After washing, proteins were eluted and separated by Tricine-SDS-PAGE (Fig. 6A). ClpC6803 copurified only with GST-tagged NblA1. There was no interaction between ClpC6803 and NblA2.
indicate that the presence of NblA16803 is sufficient for the formation of the ternary complex with ClpC6803 and phycobiliproteins to the PC apoprotein and ClpC6803 (Fig. 6A). We conclude that the ClpC6803 binding site on the heterodimer is located on NblA16803.

To demonstrate that NblA16803 is sufficient to induce formation of the ternary complex with ClpC6803 and phycobiliproteins in vitro, we first immobilized the GST-tagged apoprotein of α-PC on glutathione-agarose, and then incubated the protein-agarose conjugate with a crude extract from E. coli cells expressing ClpC6803. After washing, NblA1-GST complexes were eluted with glutathione, resolved by Tricine-SDS-PAGE, and stained with Coomassie Blue R-250. GST protein served as a control.

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FIGURE 6. NblA16803 mediates binding to ClpC6803 and phycobiliproteins.

A, binding of ClpC6803 to GST-tagged NblA16803 in vitro. NblA16803 and NblA26803 were expressed with a C-terminal GST tag, immobilized on glutathione-agarose, and incubated (in the presence of 5 mM Mg-ATP) with soluble crude extract from E. coli cells expressing ClpC6803. After washing, NblA1-GST complexes were eluted with glutathione, resolved by Tricine-SDS-PAGE, and stained with Coomassie Blue R-250. GST protein served as a control.

B, NblA-GST complexes were eluted with glutathione, separated by Tricine-SDS-PAGE, and stained with Coomassie Blue R-250. GST protein served as a control.

To determine whether ClpP copurifies with HisClpR, we performed size-exclusion chromatography3 and native PAGE.3 The apparent molecular mass of the protein complex was 200 kDa, indicating that ~3–4 copies of ClpR and HisClpP1 assemble into an oligomeric structure.

Hydrolysis of α-casein by the ClpP1-ClpR Core Complex Depends on ClpC6803 and ATP—To analyze the proteolytic activity of the recombinant ClpP1-HisClpR complex, we tested its capacity to degrade α-casein. Accordingly, we incubated the complex with α-casein, HisClpC6803, and ATP in the presence of an ATP-regenerating system (38). With numerous exposed hydrophobic amino acids on its surface (39), α-casein from cow’s milk is a nonspecific substrate for Clp proteases (40, 41). ClpC6803 is the putative chaperone partner of the ClpP1-ClpR proteolytic core complex and is required for substrate recognition, ATP-dependent unfolding, and translocation into the proteolytic chamber. At intervals after combining reaction components, aliquots of the assay mixture were withdrawn, proteins were separated by Tricine-SDS-PAGE, and α-casein concentration was estimated by Coomassie Blue staining (Fig. 7A). The ClpC6803·ClpP1-ClpR complex completely degraded α-casein within 10 min (Fig. 7A). As expected, the functional roles of ClpC6803, no degradation occurred in the absence of this putative chaperone (Fig. 7A, lanes 7 and 8). Moreover, no degradation of α-casein was observed upon incubation with ClpC6803 alone (Fig. 7A, lanes 9 and 10), demonstrating that all three proteins (ClpP1, ClpR, and ClpC6803) are required to form a proteolytically active Clp complex.

3 A. Baier, W. Winkler, T. Korte, W. Lockau, and A. Karradt, unpublished data.
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![Diagram A](image)

**Figure 7.** ClpC<sub>6803</sub> forms an active proteolytic complex with ClpP1-ClpR and degrades the adaptor proteins NblA<sub>16803</sub> and NblA<sub>26803</sub>. A. In vitro degradation of α-casein. α-Casein was incubated in the presence of pyruvate kinase (20 ng/μl), 4 mM phosphoenolpyruvate, 2 mM ATP, and purified ClpC<sub>6803</sub> and ClpP1/ClpR (2 μM each) at 37 °C. Samples were taken at the indicated time points, separated by SDS-PAGE, and visualized by Coomassie Blue R-250 staining.

B. In vitro degradation of purified NblA<sub>16803</sub> and NblA<sub>26803</sub>. NblA<sub>16803</sub>/NblA<sub>26803</sub> (5 μM each) were incubated in the presence of pyruvate kinase (20 ng/μl), 4 mM phosphoenolpyruvate, 2 mM ATP, purified ClpC<sub>6803</sub> and ClpP1-ClpR (2 μM each), and purified PBS from *Synechocystis* 6803 (29) at 37 °C. Samples were taken at the indicated time points, separated by SDS-PAGE, and visualized by Coomassie Blue R-250 staining.

A Clp Protease Composed of ClpC<sub>6803</sub>, ClpP1, and ClpR Degradates NblA<sub>16803</sub> and NblA<sub>26803</sub> In Vitro—Previous investigations of the interaction between NblA and ClpC from *Nostoc* 7120 showed that exchanging amino acids in the ClpC binding site of NblA results in accumulation of NblA in vivo. From this we inferred that NblA acts as an adaptor protein that is codegraded with its substrate as part of the overall regulatory mechanism. We therefore asked whether NblA proteins are degraded in vitro by recombinant *Synechocystis* 6803 Clp protease.

To test the hypothesis that NblA itself may be codegraded by the Clp protease, we performed degradation experiments with NblA<sub>16803</sub> and NblA<sub>26803</sub> essentially as described above. Degradation of NblA<sub>16803</sub> and NblA<sub>26803</sub> was monitored by Coomassie Blue staining of samples resolved on Tricine-SDS-polyacrylamide gels. Fig. 7B illustrates the simultaneous degradation of NblA proteins within 30 min. No degradation was observed in the absence of ClpC<sub>6803</sub> or ATP. These results demonstrate that the putative Clp protease ClpC<sub>6803</sub>-ClpP1-ClpR degrades NblA<sub>16803</sub> and NblA<sub>26803</sub> in vitro at similar rates. Attempts to degrade the GST-tagged PC apoprotein or PBS isolated from *Synechocystis* 6803 in vitro have not yet proved successful (see below).

**DISCUSSION**

Complementation of the Non-bleaching Phenotype of the nblA<sub>1</sub>/nblA<sub>2</sub> Double Mutant of *Synechocystis* 6803—In this study, we solved part of the mystery of why *Synechocystis* 6803 possesses two nblA genes, which are both essential for PBS degradation (15), when other cyanobacteria accomplish this by expressing only one nblA gene (14, 17, 18).

The complementation experiment shown in Fig. 1 demonstrates that the NblA protein from *Nostoc* 7120 is capable of restoring the wild-type phenotype to the non-bleaching *nblA<sub>1</sub>/nblA<sub>2</sub> double mutant of *Synechocystis* 6803, suggesting that the functions of NblA<sub>6803</sub> and NblA<sub>26803</sub> are combined in one protein in *Nostoc* 7120. A similar result was obtained by Dines et al. (27), who reported successful complementation of the non-bleaching phenotype of the nblA mutant of *Synechococcus* 7942 by expression of the *Nostoc* 7120 nblA gene. These results support the conclusion that NblA proteins are functionally interchangeable among different cyanobacteria. Consistent with this, the various NblA proteins contain highly conserved regions near the N and C termini that mediate binding to phycobiliproteins and ClpC<sub>6803</sub> (19, 20), and the three existing crystal structures are very similar (19, 27).

**Formation of NblA<sub>16803</sub>/NblA<sub>26803** Heterodimers—FRET measurements, fluorescence lifetime measurements, and in vitro pull-down experiments demonstrated that NblA<sub>16803</sub> and NblA<sub>26803</sub> form a heterodimer. In contrast, the tendency toward homodimer formation was observed only under conditions that do not reflect those that exist in the cell, where nblA<sub>6803</sub> and nblA<sub>26803</sub> are cotranscribed (15, 16). On the basis of our results, we postulate that the biologically active form is a heterodimer, and we assume that the structure of the heterodimer is similar to that of other NblA proteins that have been crystallized to date.

**Heterologous Expression of the Putative Protease ClpC<sub>6803</sub>-ClpP1-ClpR and Degradation of NblA<sub>16803</sub> and NblA<sub>26803**—To provide evidence that PBSs are degraded by a Clp protease, we performed in vitro degradation assays. Investigations of Clp proteases in *Synechococcus* 7942 have demonstrated that cyanobacteria possess two distinct Clp proteases in the soluble fraction of the cell and probably a third that is associated with the thylakoids. This membrane-associated protease appears to be involved in PBS degradation because (i) the protease has been reported to interact with PBS (37), and (ii) ClpC, the putative HSP100 chaperone partner of this protease, interacts with NblA (20).

The membrane-associated protease consists of a unique proteolytic core complex, composed of the Clp subunits, ClpP1 and ClpR, and the HSP100 chaperone partner ClpC, as noted above. To heterologously express the protease, we first identified orthologs of ClpP1, ClpR, and ClpC in *Synechocystis* 6803. We then successfully cloned, expressed, and purified the corre-
sponding proteins. As expected, coexpression of the two proteolytic subunits, ClpR and ClpP1, enabled the purification of a multiprotein complex. The complex had a molecular mass of about 200 kDa, which is similar to that of the ClpR-ClpP3 proteolytic core complex of Synechococcus 7942 (41), and appeared to correspond to a single mixed heptameric ring of ClpR and ClpP1, as expected. The protease activity of the complex, determined by analyzing the degradation of \( \alpha \)-casein, was also similar to that of the proteolytic core ClpP3-ClpR from Synechococcus 7942 (41). Both cyanobacterial proteases are thus much slower than the model protease ClpA-ClpP from \( E. \) coli (41). The lower activity of cyanobacterial Clp proteases compared with that of \( E. \) coli may be due, at least in part, to differences in the structures of the core complexes (41, 42). We further demonstrated that Nbla1\(_{6803}\) and Nbla2\(_{6803}\) are degraded by the recombinant Clp protease ClpC-ClpP1-ClpR.

The Clp protease-mediated degradation of Nbla1\(_{6803}\) and Nbla2\(_{6803}\) represents an elegant Nbla regulatory mechanism. During the first hours after nitrogen starvation, the heterodimer is highly expressed, forming a ternary complex with ClpC\(_{6803}\) and the PBS that leads to ClpC\(_{6803}\) and PBS degradation. When all PBSs are degraded, the remaining Nbla proteins are also degraded by the protease, eliminating the need for further regulation. This finding that the protease degrades Nbla reinforces previous reports by our group that Nbla accumulates when the Clpc binding site on Nbla is inactivated by mutagenesis. Collectively, these observations indicate that the Nbla protein acts like other identified adaptor proteins that undergo protease-mediated degradation, even when they are not delivering a substrate (43, 44).

Despite the fact that all conditions necessary for PBS degradation were fulfilled, including demonstrable protease activity and formation of a ternary complex between Nbla1\(_{6803}\)/Nbla2\(_{6803}\) phycobiliproteins, and ClpC, we were unable to demonstrate in vitro degradation of PBS isolated from Synechocystis 6803 or the GST-tagged PC apoprotein. In further attempts to measure PBS degradation, we used a crude extract of wild-type cells grown under nitrogen or harvested immediately after nitrogen deprivation and also tried adding recombinant ClpC, Nbla1\(_{6803}\), Nbla2\(_{6803}\), ClpP1-ClpR, ATP, and an ATP regeneration system. Both strategies failed to induce degradation. One possible explanation for these unexpected results might be that dephosphorylation of PBS linker proteins is necessary for the degradation process. In this context, Piven et al. (45) reported that nitrogen starvation and long term exposure to higher light intensities resulted in an increase in dephosphorylated linker proteins and partial disassembly of the PBS. Even studies in Nostoc 7120, which exhibit a bottom-to-top disassembly of the PBS in early heterocysts, suggest that dephosphorylation of linker proteins may act as a signal for degradation (46). Accordingly, one possible scenario could be that, when the V-shaped Nbla-dimers penetrate the gap formed by four back-to-back-assembled PBS \( \beta \)-subunits (27), the PBS structure disassembles (27), enabling dephosphorylation of the linker proteins by a phosphatase and opening the door for subsequent degradation. However, this explanation cannot account for the observation that the PC apoprotein is not degraded in vitro despite binding to Nbla1\(_{6803}\)/Nbla2\(_{6803}\) (Fig. 6B). It is also possible that a bilin lyase could be involved in the degradation process. Bilin lyases catalyze the covalent ligation of chromophores to specific binding sites of phycobiliproteins and also control subsequent detachment (47). In Synechococcus 7942, the bilin lyase NlbB participates in the degradation of PBS (48); in contrast, the two homologs of NlbB in Synechocystis 6803 are not essential for PBS degradation in this strain (16). Thus, it may be that another unidentified lyase remains to be found. A further possibility could be that Nbla1\(_{6803}\) and Nbla2\(_{6803}\) must be activated by phosphorylation or dephosphorylation. Examples in which adaptor proteins must be activated are known from other organisms, including Bacillus subtilis, where the tyrosine kinase MecB is activated by phosphorylation (49). In this context, it is conceivable that light could induce Nbla1/ Nbla2 activation, perhaps through a light-dependent phosphatase like NlbS (50, 51). Clearly, further biochemical analyses will be required to establish the relevance of the mechanisms discussed here and close out the topic of PBS degradation.

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