Molecular bases of an alternative dual-enzyme system for light color acclimation of marine *Synechococcus* cyanobacteria

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Marine *Synechococcus* cyanobacteria owe their ubiquity in part to the wide pigment diversity of their light-harvesting complexes. In open ocean waters, cells predominantly possess sophisticated antennae with rods composed of phycocyanin and two types of phycoerythrins (PEI and PEII). Some strains are specialized for harvesting either green or blue light, while others can dynamically modify their light absorption spectrum to match the dominant ambient color. This process, called type IV chromatic acclimation (CA4), has been linked to the presence of a small genomic island occurring in two configurations (CA4-A and CA4-B). While the CA4-A process has been partially characterized, the CA4-B process has remained an enigma. Here we characterize the function of two members of the phycobilin lyase E/F clan, MpeW and MpeQ, in *Synechococcus* sp. strain A15-62 and demonstrate their critical role in CA4-B. While MpeW, encoded in the CA4-B island and up-regulated in green light, attaches the green light-absorbing chromophore phycoerythrobilin to cysteine-83 of the PEII α-subunit in green light, MpeQ binds phycoerythrobilin and isomerizes it into the blue light-absorbing phycourobilin at the same site in blue light, reversing the relationship of MpeZ and MpeY in the CA4-A strain RS9916. Our data thus reveal key molecular differences between the two types of chromatic acclimaters, both highly abundant but occupying distinct complementary ecological niches in the ocean. They also support an evolutionary scenario whereby CA4-B island acquisition allowed former blue light specialists to become chromatic acclimaters, while former green light specialists would have acquired this capacity by gaining a CA4-A island.

light regulation | marine cyanobacteria | photosynthesis | phycobilin lyase | phycoerythrin

Marine *Synechococcus* are the second most abundant phototrophs in the oceans, significantly contributing to oceanic primary production and carbon cycling (1, 2). These picocyanobacteria occupy the upper sunlit layer of marine waters from the equator up to 80 °N (3, 4) and exhibit a broad range of photosynthetic pigments, allowing them to optimally exploit a large variety of light environments, from particle-loaded estuaries to the optically clear open ocean waters (5). This pigment diversity arises from a large flexibility in the composition of their light-harvesting antennae, called phycobilisomes (PBS). Structurally, these complexes comprise a central core from which radiate six or eight rods made of α/β heterodimers of phycobiliproteins that first assemble into $(\alpha\beta)_6$ hexamers and then stack into rods with the assistance of linker proteins (6). In the open ocean, virtually all *Synechococcus* cells belong to pigment type 3 (PT 3), in which PBS rods are made of the three phycobiliproteins, phycocyanin (PC), phycoerythrin-I (PEI), and phycoerythrin-II (PEII), which can covalently bind three chromophore types: the red light-absorbing phycocyanobilin (A_{max} = 650 nm), the green light (GL)-absorbing phycoerythrobilin (PEB; A_{max} = 550 nm), and the blue light (BL)-absorbing phycourobilin [PUB; A_{max} = 495 nm (7, 8)]. Different pigment subtypes have been defined within PT 3, based on the relative ratio of fluorescence excitation at 495 and 545 nm with emission at 580 nm ($Exc_{495:545}$), a proxy for their molar PUB:PEB ratio. This ratio is constitutively low ($Exc_{495:545} < 0.6$), intermediate ($0.6 \leq Exc_{495:545} < 1.6$), or high ($Exc_{495:545} \geq 1.6$) in PT 3a, 3b, and 3c, respectively (5, 9). Furthermore, in PT 3d strains, which appear to make up nearly half of the PT 3 population globally (9), the PUB:PEB ratio can reversibly vary with ambient light color, a process called type IV chromatic acclimation (CA4) that allows cells to optimally absorb either GL or BL, depending on the ambient ratio of these light colors (10, 11). While most genes coding for PBS rod components are localized in a large genomic region, called the PBS region (5), strains capable of CA4 additionally possess a small genomic island existing in one of two distinct configurations

Significance

Of all cyanobacteria on Earth, marine *Synechococcus* are those displaying the greatest pigment diversity. The most sophisticated pigment type is cells able to reversibly modify their color by a phenomenon called type IV chromatic acclimation or CA4. Two genetically distinct CA4 types (CA4-A and CA4-B) have evolved in different lineages. Together, they represent almost half of all *Synechococcus* cells in oceanic areas and are equally abundant but occupy complementary ecological niches. While the molecular mechanism of CA4-A has recently started to be deciphered, the CA4-B mechanism was so far uncharacterized. Here, by unveiling this mechanism and demonstrating its singularity relative to CA4-A, we provide highlights on the evolutionary history of *Synechococcus* acclimation to light color in the oceans.

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(12). Each contains the two regulators encoded by *fciA* and *fciB* (13) and one gene coding for a putative phycobilin lyase family member, which can be either *mpeZ* or *mpeW*, defining the two distinct genotypes CA4-A and CA4-B [the corresponding PTs are named 3dA and 3dB, respectively (12)]. Phycobilin lyases are enzymes responsible for the covalent attachment of chromophores at specific cysteine residues of phycobiliprotein subunits and are thus key players in *Synechococcus* pigmentation. Indeed, chromophore ligation takes place prior to phycobiliprotein subunit assembly, and proper phycobilin arrangement is crucial for efficient energy transfer along the rods (14). However, among the dozen (putative) phycobilin lyases encoded in marine *Synechococcus* genomes (5), only a few have been biochemically characterized. RpcG from *Synechococcus* sp. WH8102 (PT 3c) was shown to attach PEB to cysteine-84 (C84) of the α-subunit of PC and to isomerize it to PUB (15). Such dual-function enzymes are called phycobilin lyase-isomerases. More recently, the biochemical characterization of the CA4-A strain *Synechococcus* sp. RS9916 (clade IX) revealed the specific chromophore changes that occur during CA4; namely, one out of five chromophore-binding sites on PEI and two of the six sites of PEII change from PEB in GL to PUB in BL. Two enzymes implicated in CA4-A and belonging to the E/F structural clan (16) have been characterized: MpeZ is the lyase-isomerase adding PUB at C83 of the PEII α-subunit (MpeA) in BL (17), whereas its paralog MpeY is the PEB lyase acting on the same site in GL (18). While *mpeZ* was found to be expressed more highly under BL (12, 13, 17), *mpeY* is constitutively expressed whatever the ambient light color. This led to the suggestion that the cellular MpeZ:MpeY protein ratio ultimately controls the chromophore bound at MpeA-C83 (18). By contrast, the identity and function of the corresponding enzymes in CA4-B strains were so far unknown.

Here we characterize the *mpeW* gene located in the CA4 genomic island of the CA4-B strain *Synechococcus* sp. A15-62 (subclade IIc). We show that it encodes a phycobilin lyase acting on MpeA-C83 in GL that vies with a previously unidentified lyase-isomerase (MpeQ) acting on the same cysteine position in BL. Our results highlight the key role of the diversification of the MpeY enzyme family in the adaptation of marine *Synechococcus* to different light color niches and provide insights to explain the occurrence of two mutually exclusive CA4 systems in marine *Synechococcus*.

Results

The MpeY Phycobilin Lyase Family Is Polyphyletic. Based on its conserved genomic position in the PEII-specific subregion of the PBS region (Fig. 1*A*) and on sequence similarity, the gene located immediately upstream of the *mpeBA* operon (encoding the two subunits of PEII) was previously named *mpeY* in all *Synechococcus* strains containing PEII (5, 9, 18). However, the phylogenetic analysis of the different members of this family revealed that *mpeY* is in fact polyphyletic, with three distinct clusters, each corresponding to a different pigment subtype (Fig. 1*C*). PT 3a strains (i.e., GL specialists) possess the MpeY^{3a} variant encoded by the PT 3a-specific *mpeY* allele, and PT 3dA strains contain the MpeY^{3dA} variant recently characterized in the PT 3dA strain *Synechococcus* sp. RS9916 as a PEB lyase acting on C83 of the α-PEII subunit (MpeA) (18), while a third variant is found in PT 3c (i.e., BL specialists) and 3dB strains. Strikingly, sequences from the latter two pigment subtypes were intermingled in the phylogenetic tree, as was previously observed for *mpeBA* or *cpeBA* phylogenies, indicating that PBS genes of these pigment subtypes are phylogenetically too close to be distinguishable (9, 12). Tandem mass spectrometry (MS/MS) analysis of the chromophorylation of MpeA from the PT 3a strain *Synechococcus* sp. WH7803 revealed that MpeA-C83 binds a PEB, while PUB is bound to both MpeA-C75 and 140 (*SI Appendix*, Fig. S1). This suggests that MpeY^{3a} must have a PEB lyase function, like MpeY^{3dA}. In contrast, as demonstrated below, the function of the MpeY-like protein found in PT 3c and 3dB differs from that of both MpeY^{3a} and MpeY^{3dA}, and we therefore renamed it "MpeQ" in the tree (Fig. 1*C*) and in the text hereafter. The protein sequences of MpeW, MpeZ, MpeQ, MpeY^{3a}, and MpeY^{3dA} have on average 34 to 58% amino acid identity over their whole length, compared to an average of 70 to 82% identity among members of each of these subfamilies (Fig. 1*B* and Dataset S1).

Chromophore Changes Occurring during CA4-B Are the Same as Those Occurring in the CA4-A Process. Although the Exc$_{495:545}$ ratios of CA4-A and CA4-B strains have previously been shown to be similar in both BL (1.6 to 1.7) and GL (0.6 to 0.7) (12), it was unclear what specific changes in chromophorylation were taking place during the CA4 process in CA4-B strains. To investigate this, PBS from wild-type (WT) *Synechococcus* sp. A15-62 grown under BL or GL were purified, and PEI and PEII subunits were separated by high performance liquid chromatography (HPLC). Absorption spectra of individual subunits (Fig. 2 and *SI Appendix*, Fig. S2) suggested that the PUB:PEB molar ratio changed between GL and BL from 0:2 to 1:1 for the α-PEI subunit (CpeA) and from 1:2 to 3:0 for the α-PEII subunit (MpeA), while the chromophorylation of β-PEI (CpeB) and β-PEII (MpeB) subunits did not vary with light color (1:2 in both conditions; Table 1). The chromophorylation of the two α-subunits was further characterized by MS/MS, confirming the above-mentioned molar ratios and demonstrating that the three cysteine positions that change chromophorylation (from PEB in GL to PUB in BL) are MpeA-C83 and C140 and CpeA-C139 (Table 1, Dataset S2, and *SI Appendix*, Figs. S3–S5), i.e., the same chromophores and positions as in the CA4-A strain RS9916 (17).

MpeW and MpeQ Are Required for PEB and PUB Attachment on MpeA, Respectively. Since the biochemical characterization of MpeZ and MpeY^{3dA} in RS9916 showed that they were a PEB lyase-isomerase and a PEB lyase, respectively, both acting on MpeA-C83 (17, 18), we hypothesized that the corresponding enzymes in CA4-B strains, MpeW and MpeQ (Fig. 1*A*), could be a PEB lyase and PEB lyase-isomerase, respectively, both also acting on MpeA. To test this hypothesis, we created *mpeW*$^-$ and *mpeQ*$^-$ insertion mutants in the CA4-B strain A15-62. Comparison of fluorescence excitation spectra revealed that for GL-grown cultures, Exc$_{495:545}$ was increased in the *mpeW*$^-$ mutant compared to the WT, whereas no difference was observed under BL (Fig. 3 *A* and *B*). Conversely, no difference could be seen between Exc$_{495:545}$ of *mpeQ*$^-$ mutants and WT under GL, while the Exc$_{495:545}$ ratio dramatically decreased in BL-grown *mpeQ*$^-$ mutants compared to WT (Fig. 3 *C* and *D*). Both mutants still exhibited some levels of chromatic acclimation since the Exc$_{495:545}$ ratio changed from 1.17 ± 0.01 in GL to 1.68 ± 0.07 in BL for *mpeW*$^-$ and from 0.74 ± 0.02 in GL to 0.91 ± 0.01 in BL for *mpeQ*$^-$, as compared to 0.74 ± 0.01 in GL and 1.72 ± 0.09 in BL for WT cells (all data are average ± SE; $n = 2$). Based on these results, we hypothesized that 1) MpeW is required for PEB attachment, while MpeQ is required for PEB attachment and isomerization to PUB, and 2) both are acting on one or possibly two chromophore binding cysteine(s) but seemingly not all three sites that change chromophorylation during the CA4 process. The CA4 phenotype of both mutants could be restored with the reintroduction of WT *mpeW* or *mpeQ* genes in *trans* (Fig. 3 and *SI Appendix*, Fig. S6).

Since in the CA4-A strain RS9916, the transcript levels of the lyase-isomerase *mpeZ* were found to be about 32-fold higher in BL than GL (12, 13), while the *mpeY* lyase levels were similar in both colors (18), we also checked the light color response of the counterparts *mpeQ* and *mpeW* genes in the CA4-B strain A15-62. Real-time PCR expression data showed that while *mpeQ* is not

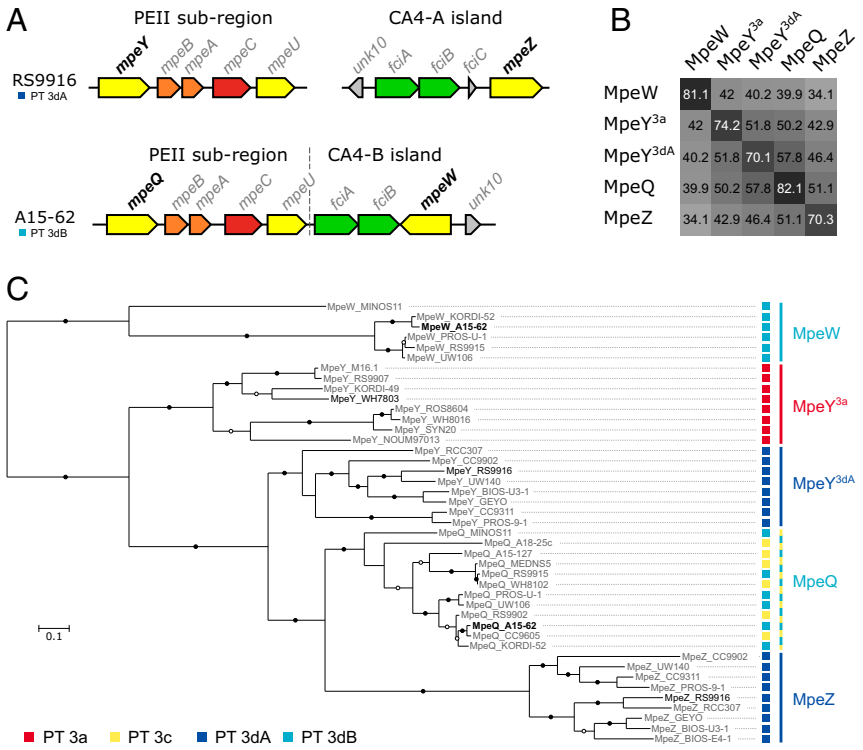

Fig. 1. Phylogeny and similarity of the different members of the MpeQWYZ phycobilin lyase enzyme family and genomic organization of the corresponding genes. (*A*) Comparison of the genomic regions involved in CA4-A and CA4-B processes. The CA4-A genomic island and the PEII subregion of the PBS genomic region are located at two different loci on the chromosome of *Synechococcus* strain RS9916 (*Top*), whereas in strain A15-62 the CA4-B genomic island is located in the middle of the PBS genomic region, immediately downstream the PEII subregion (*Bottom*). The two genes characterized in the present study (*mpeW* and *mpeQ*) are shown as underlined black bold letters, their functional homologs in the CA4-A strain RS9916 (*mpeY* and *mpeZ*) in black bold letters, and other genes in gray. (*B*) Mean pairwise percentage of identity between the protein sequences of MpeW, MpeQ, MpeZ, and MpeY^{3a} and MpeY^{3dA} (encoded by the two *mpeY* alleles), calculated based on full-length alignments. (*C*) Maximum likelihood phylogeny (protein sequences, LG + I + G + F model) of the MpeQWYZ enzyme family. CpeY sequences were used as outgroup to root the tree. Nodes with bootstrap support >70 and >90% are indicated by empty and filled black dots, respectively. Sequences of A15-62 used in this study are highlighted in bold black letters, and other sequences discussed in the text are shown in black, while all other sequences are shown in gray.

significantly differentially regulated between GL and BL, *mpeW* is 44.26 ± 1.34-fold (average ± SD; $n = 3$) more highly expressed in GL than BL (*SI Appendix*, Fig. S7).

***mpeW*$^-$ and *mpeQ*$^-$ Mutants Differ from the WT by the Chromophore Attached to MpeA-C83 in GL and BL, Respectively.** To identify the biochemical changes responsible for the differences in whole-cell fluorescence, PBS were purified from the *mpeW*$^-$ and *mpeQ*$^-$ mutants and PEI and PEII subunits separated by HPLC. Absorption spectra only differed between WT and mutants for MpeA (Fig. 2 and *SI Appendix*, Fig. S2). Indeed, compared to the WT, MpeA from the *mpeW*$^-$ mutant had a lower PEB absorption peak at 550 nm in GL (Fig. 2*A*), whereas MpeA from the *mpeQ*$^-$ mutant exhibited a PEB peak at 550 nm in BL not seen in WT cells (Fig. 2*D*). This confirmed that both MpeW and MpeQ are involved in MpeA chromophorylation, with MpeW being responsible for PEB attachment and MpeQ for PEB attachment and simultaneous isomerization to PUB. MS/MS of CpeA and MpeA, the two PE subunits that change chromophorylation during CA4 (see above), further showed that the *mpeW*$^-$ mutant mainly differed from the WT by having a PUB instead of a PEB at MpeA-C83 in GL, whereas the *mpeQ*$^-$ mutant differed from the WT by having a PEB instead of a PUB at MpeA-C83 in BL (Table 1 and *SI Appendix*, Figs. S8 and S9). Surprisingly, both WT and mutants displayed a mixed chromophorylation at MpeA-C140 in GL. The slightly higher proportion of PUB in mutants compared to the WT (35 and 15%, respectively; Table 1) could suggest that MpeQ and MpeW might also have a role in the chromophorylation changes occurring during CA4 at this position. If true, this effect is likely non-physiological since no chromophorylation of MpeA-C140 could be detected in our recombinant assays (see below).

Recombinant MpeW and MpeQ Are Both Acting on MpeA-C83. Activities of MpeW and MpeQ were tested using a heterologous plasmid coexpression system in *Escherichia coli*. Four different hexahistidine-tagged (HT-) versions of recombinant MpeA were used as substrates in the coexpressions: A15-62 and RS9916 HT-MpeA; a mutant RS9916 MpeA with C83 replaced by alanine, RS9916 HT-MpeA(C83A); and a mutant RS9916 MpeA with C75 and C140 each replaced with alanine, RS9916 HT-MpeA(C75A, C140A). RS9916 HT-MpeB and RS9916 HT-CpeA were also used to further test the substrate preference. These phycobiliprotein subunits were coexpressed with PEB synthesis enzymes and with either MpeW or MpeQ and purified, and their spectral properties were analyzed. The negative control (A15-62 HT-MpeA expressed with PEB synthesis enzymes but without any lyase) showed no detectable fluorescence (Fig. 4 *A* and *C*). The coexpressions of MpeA (either from A15-62 or RS9916) and either MpeW or MpeQ confirmed that the former attached PEB while the latter attached PUB to both versions of MpeA (Fig. 4 *A*–*D*), while coexpressions of MpeW or MpeQ and either MpeB or CpeA demonstrated that neither had lyase activity on these two proteins. Coexpressions of MpeW and mutants RS9916 MpeA, in which cysteine residues are replaced by alanine [RS9916 MpeA (C83A) or (C75A and C140A)], further

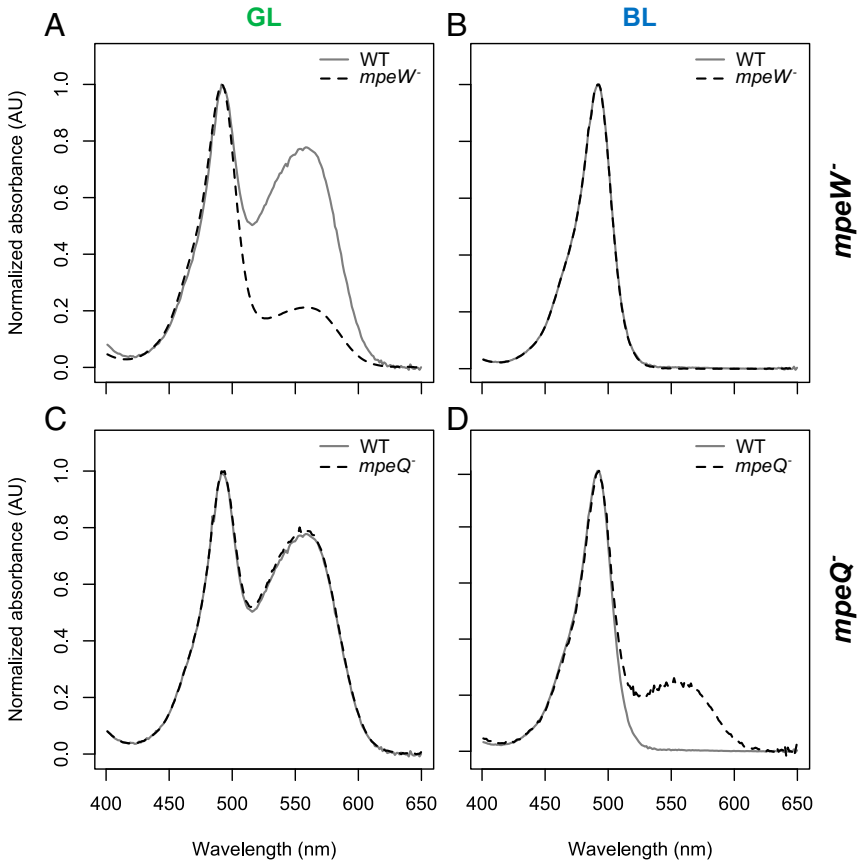

Fig. 2. Absorbance spectra of HPLC-purified phycoerythrin II α-subunit (MpeA) for WT *Synechococcus* sp. A15-62, *mpeW* inactivation mutant (*mpeW*$^-$), and *mpeQ* inactivation mutant (*mpeQ*$^-$) grown under GL or BL. (*A* and *C*) WT or mutant cells grown in GL. (*B* and *D*) WT or mutant cells grown in BL. See *SI Appendix*, Fig. S2, for absorbance spectra of the other PEI and PEII subunits.

showed that PEB was covalently bound to MpeA-C83 (Fig. 4 *A* and *B*). The same system was used to demonstrate that MpeQ also acted specifically on MpeA-C83 by ligating PEB and isomerizing it to PUB (Fig. 4 *C* and *D*). The minor fluorescence emission peak at ∼600 nm in the *mpeW* system (Fig. 4*A*) is likely coming from PEB that is not held in a stretched conformation.

Collectively, these results indicate that in the CA4-B process, MpeW is the lyase responsible for the attachment of PEB to MpeA-C83 in GL while MpeQ is the lyase-isomerase responsible for the attachment of PEB and its isomerization to PUB at MpeA-C83 in BL.

Discussion

With six phycobilin binding sites per α−β monomer, PEII is the most pigmented phycobiliprotein known so far in the *Cyanobacteria* phylum (7, 19), and the ecological success of marine *Synechococcus* spp. is likely tied with this biological innovation, as evidenced by the large predominance of PEII-containing cells (so-called PT 3) in open waters of the world ocean (9). Gain of PEII, which is thought to have occurred by duplication and divergence from the corresponding PEI encoding genes (20), also implied the concomitant acquisition of enzymes necessary for its proper chromophorylation (5). In this context, the diversification of the MpeQWYZ enzyme family, which belongs to the E/F structural clan (16) and likely derived by duplication and divergence from a *cpeY*-like gene ancestor (Fig. 1) (21, 22), has been pivotal for the development of CA4, the only example among the six chromatic acclimation forms known so far in *Cyanobacteria* to involve color-induced changes only in chromophore content (23). The fact that CA4 seemingly appeared twice during the evolution of marine *Synechococcus*, with CA4-A and CA4-B occurring in most cases in phylogenetically distant lineages and colonizing different light niches (Fig. 1) (9, 12), is particularly intriguing. Although the molecular mechanism underpinning CA4-A is starting to be understood (13, 17, 18), CA4-B was thus far biochemically uncharacterized, and the differences between the two CA4 systems remained unclear. Here we show that CA4-B is mechanistically similar to CA4-A since it also involves two enzymes that vie for the same binding site on α-PEII subunit (MpeA) and whose gene expressions are differentially regulated by the ambient light color (*SI Appendix*, Fig. S10). We demonstrate that MpeW is a phycobilin lyase attaching PEB at MpeA-C83 in GL, whereas MpeQ is a phycobilin lyase-isomerase binding PUB at the same position in BL. Intriguingly, the CA4-B-specific lyase/lyase-isomerase MpeW/Q pair exhibits a reversed activity compared to its counterpart pair MpeZ/Y in the CA4-A strain RS9916 (*SI Appendix*, Fig. S10). Indeed, the lyase-isomerase *mpeZ* was found to be up-regulated under BL, while in the CA4-B process the lyase *mpeW* is up-regulated under GL. In both cases, expression of the gene coding for the second enzyme of the couple is not differentially expressed between GL and BL (12, 13, 17). Our data therefore shed light on a fascinating dual-enzyme coevolution process that explains the cooccurrence of two mutually exclusive CA4 types in the marine *Synechococcus* radiation. Our data provide strong evidence for the previously proposed hypothesis (23, 24) that the basal state for CA4-A strains (i.e., in absence of CA4) corresponds to a GL specialist phenotype (low PUB:PEB ratio) and that CA4 confers

Table 1. Percentage of PUB chromophores, expressed as a PUB/(PEB+PUB) ratio, found at the different cysteinyl sites of the PEI and PEII α- (CpeA and MpeA) and β-subunits (CpeB and MpeB) in *Synechococcus* sp. A15-62 WT cells and *mpeW*⁻ and *mpeQ*⁻ mutants grown under GL and BL

			%PUB attached in GL			%PUB attached in BL		
PBP	Subunit	Cysteine position	WT	*mpeW*⁻	*mpeQ*⁻	WT	*mpeW*⁻	*mpeQ*⁻
PEII	MpeA	75	100	100	100	100	100	100
		83	**9**	**99**	**2**	**100**	**99**	**14**
		140	**15**	**35**	**36**	**93**	**99**	**99**
PEII	MpeB	50, 61	100	ND	ND	100	ND	ND
		82	0	ND	ND	0	ND	ND
		165	0	ND	ND	0	ND	ND
PEI	CpeA	82	0	0	0	0	0	0
		139	**0**	**100**	**21**	**100**	**98**	**43**
PEI	CpeB	50, 61	100	ND	ND	100	ND	ND
		82	0	ND	ND	0	ND	ND
		159	0	ND	ND	0	ND	ND

Underlined values indicate that the chromophorylation was confirmed by LC-MS/MS. Bold indicates that the chromophore is expected to change during the CA4 process. At some sites, the mass spectrometry analysis detected a mix of PUB and PEB, and the percentage of PUB (%PUB) is indicated. ND, not determined; PBP, phycobiliprotein.

these cells the ability to change their spectral properties upon BL illumination, by inducing the expression of the phycobilin lyase-isomerase MpeZ. Conversely, the basal state for CA4-B strains would correspond to a BL specialist phenotype (high PUB:PEB ratio), CA4 providing these cells the ability to acclimate to GL by inducing the synthesis of the PEB lyase MpeW. Thus, in both cases, CA4 appears to be a plug-and-play mechanism that adds on top of the existing basal chromophorylation. This view is further supported by the gathering into a small dedicated genomic island of genes necessary for CA4, including the regulators *fciA* and *fciB* and one phycobilin lyase (either *mpeZ* or *mpeW*; Fig. 1*A*). Furthermore, the absence of any allelic difference between PBS genes of PT 3c and 3dB strains (Fig. 1*C*; see also ref. 9) might suggest that the CA4-B island is more readily transferred between *Synechococcus* strains through horizontal gene transfer than is the CA4-A island. Surprisingly, however, the latter displays several typical genomic island features, including a biased GC content compared to the surrounding genomic context and presence in the vicinity of the island of hotspots for DNA recombination such as tRNA or *psbA* genes, which are absent in the case of the CA4-B island (12, 17). So, the mechanism by which a CA4-B island could be transferred from a PT 3dB strain to a BL specialist (PT 3c) and the frequency of such transfers remain unclear.

It is also interesting to note that as previously observed for *mpeZ*⁻ and *mpeY*$^{3dA-}$ mutants in the CA4-A strain RS9916 (17, 18), both *mpeW*⁻ and *mpeQ*⁻ CA4-B mutants still showed some degree of chromatic acclimation, variations of the $Exc_{495:545}$ ratio observed in the *mpeW*⁻ and *mpeQ*⁻ mutants representing about 51 and 18% of that occurring in the WT, respectively (Fig. 2 *C* and *D*). In both lyase-isomerase mutants (*mpeZ*⁻ and *mpeQ*⁻), $Exc_{495:545}$ is strongly decreased in BL (about 0.8) compared to the WT (1.5 to 1.6) but higher than in GL (0.6 to 0.7 for both mutants and WT; Fig. 2 *C* and *D* and ref. 17). Similarly, both lyase mutants (*mpeY*⁻ and *mpeW*⁻) had an increased $Exc_{495:545}$ in GL relative to the WT (about 1.1 vs. 0.6) but still lower than in BL (1.5 to 1.6 for both; Fig. 2 *A* and *B* and ref. 18). This suggests that other genes are involved in CA4. In the absence of any obvious other CA4-specific phycobilin lyase, an interesting candidate could be the conserved hypothetical gene *unk10*, which is present in both versions of the CA4 genomic island and strongly overexpressed in BL in the CA4-A strain RS9916 (12, 13, 17, 23). Future characterization of this unknown gene as well as the putative regulator *fciC*, present in CA4-A but not CA4-B islands (Fig. 1*A* and *SI Appendix*, Fig. S10), will help unravel the different regulatory mechanisms behind this remarkable dual light color acclimation process, which strongly impact the ecology and photophysiology of a key component of marine phytoplankton communities.

Materials and Methods

Phylogenetic Analyses. Sequences used in this study (MpeQ, W, Y, and Z) can be found in Dataset S1. Briefly, MpeQ, W, Y, and Z protein sequences were aligned using the multiple sequence alignment program MAFFT 7.299b L-INS-I (25). Pairwise identity between sequences was computed from this alignment using BioPython. Best model for phylogenetic reconstruction corresponded to LG aa substitution model with empirical base frequencies (F), gamma model of rate heterogeneity (G), and invariable sites (I) as selected with ProtTest 3.4.1 (26). Tree search was conducted using RAxML (Randomized Axelerated Maximum Likelihood) 8.2.9 (27) without the rapid hill-climbing heuristic (-f o). One hundred tree searches were conducted starting from randomized maximum parsimony tree (default), and 100 searches were conducted starting from fully random trees (-d). Best tree was selected, and 250 bootstraps performed. Tree was plotted using ETE3 (28). The same procedure was used to generate a tree with CpeY sequences to root the tree represented in Fig. 1*C*.

Strains and Growth Conditions. *Synechococcus* strain A15-62 was obtained from the Roscoff Culture Collection (http://roscoff-culture-collection.org; RCC strain 2374). It was originally isolated near Cape Verde (Atlantic Ocean) from 30 m depth (29). Both WT and mutant strains were routinely grown at 22 °C in PCR-S11 (30) in polystyrene flasks (CytoOne, StarLab) at ca. 25 μmol quanta m^{-2} s^{-1} white light. A15-62 mutants were maintained with 50 μg mL^{-1} kanamycin. Prior to whole-cell absorption and fluorescence measurements as well as to PBS isolation and separation of phycobiliproteins, WT and mutant strains were acclimated for at least two weeks at 20 μE m^{-2} s^{-1} BL or GL provided by LED ramps (Luxeon Rebel LED LXML-PB01-0040 and LXML-PM01-0100, respectively; Alpheus).

Fluorescence Measurements. Spectral properties of WT and mutant strains were measured using a LS-50B spectrofluorimeter (Perkin-Elmer). Fluorescence excitation was monitored from 420 to 560 nm (0.5-nm steps) with emission monitored at 580 nm.

Quantitative PCR Analysis of *mpeW* and *mpeQ* Gene Expression. The expression levels of *mpeQ* and *mpeW* genes were monitored by real-time qPCR, as previously described (12). Briefly, triplicate cultures of *Synechococcus* sp. strain A15-62 were acclimated at 20 μmol photons m^{-2} s^{-1} BL or GL, then cells were harvested and RNA was extracted. For *mpeW* and the control *rnpB* gene, encoding the RNA component of ribonuclease P, we used previously designed primers (12), while for *mpeQ*, gene-specific primers were designed using Primer Blast (National Center for Biotechnology Information; Dataset S3). After optimization steps, reverse transcription and qPCR analyses were performed according to ref. 12. The $2^{-\Delta\Delta CT}$ method (31) was finally used to quantify the relative fold change in mRNA levels using *rnpB* as a reference gene to normalize the relative transcript levels.

Plasmid Construction for *mpeW* and *mpeQ* Disruption and Complementation and Construction of Expression Plasmids. Primers are listed in Dataset S3, and plasmids are listed in Dataset S4. The pMUT100-A15-62-mpeW and pMUT100-A15-62-mpeQ constructs were made by PCR amplification of an 820 to 885 bp internal fragment of A15-62 *mpeW* and A15-62 *mpeQ* using the primers pairs Syn_A15-62_mpeW_126F_BamHI/999R_SphI and Syn_A15-62_mpeQ_123F_EcoRI/983R_SphI (*SI Appendix*, Fig. S11). The pMUT100 backbone was PCR-amplified using the primer pair pMUT100_1291F_SphI/5535R_BamHI (pMUT100-A15-62-mpeW) or pMUT100_1291F_SphI/5535R_EcoRI (pMUT100-A15-62-mpeQ). Vector and inserts were digested with SphI and either EcoRI or BamHI (NEB) and ligated. Conjugation between *E. coli* MC1061 containing either pMUT100-A15-62-mpeW or pMUT100-A15-62-mpeQ and *Synechococcus* A15-62 was performed as previously described (17, 32). Individual colonies were picked and tested for *mpeW* or *mpeQ* disruption by PCR amplification. For complementation, a derivative of the plasmid pRL153 (autonomously replicating in marine *Synechococcus*) was first constructed by cloning a lacZ cassette (pBClacZ) into the BamHI/EagI cloning site of pJS1 for convenient screening of *E. coli* colonies (*SI Appendix*, Fig. S11).

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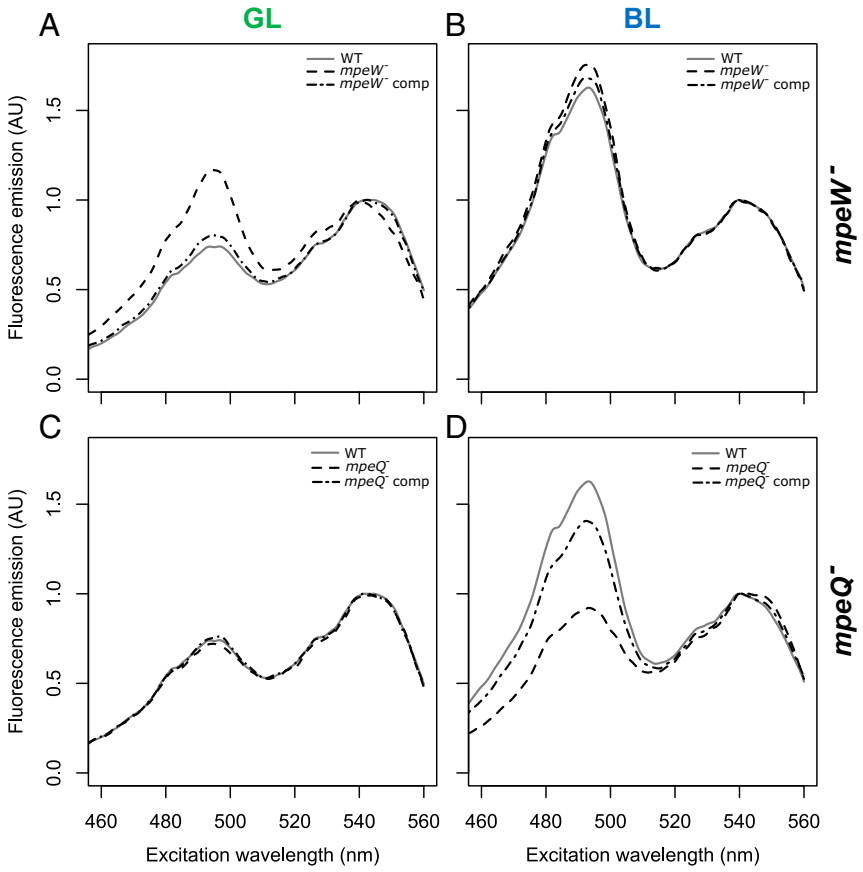

Fig. 3. Whole-cell fluorescence excitation spectra for WT *Synechococcus* sp. A15-62 and mutant strains. WT, *mpeW*⁻ mutant, and *mpeW*⁻ complemented strains grown either in (*A*) GL or (*B*) BL. WT, *mpeQ*⁻, and *mpeQ*⁻ complemented strains grown either in (*C*) GL or (*D*) BL. Fluorescence emission spectra are normalized at 545 nm. Emission was set at 580 nm. Measurements shown here were repeated twice (biological replicates). See *SI Appendix*, Fig. S6, for complementation control.

The pBClacZ-A15-62-mpeW and pBClacZ-A15-62-mpeQ constructs were made by PCR amplification of the promoter and ribosome-binding site of *mpeW* and *mpeQ*, using the primer pairs Syn_A15-62_unk10_38R_BamHI/Syn_A15-62_fciB_892F_EagI and Syn_A15-62_unk9_13R_ApaI/Syn_A15-62_mpeB_56R, respectively (*SI Appendix*, Fig. S11). PCR fragments were digested with EagI and either BamHI (*mpeW*) or ApaI (*mpeQ*) and ligated into similarly digested pBClacZ. Conjugation between *E. coli* MC1061 containing either pBClacZ-A15-62-mpeW or pBClacZ-A15-62-mpeQ and *Synechococcus* A15-62 *mpeW*⁻ or *mpeQ*⁻ was performed as previously described (17, 32). Individual colonies were picked and tested for *mpeW* or *mpeQ* complementation by PCR amplification (*SI Appendix*, Fig. S11).

PBS Isolation and Separation of Phycobiliproteins. PBS were purified as previously described (33), starting from 10 L of cultures. All steps were performed at room temperature unless specified. Briefly, cells were harvested by centrifugation, washed twice and resuspended in 0.65M phosphate buffer, and lysed twice using a French press system. Membranes and hydrophobic pigments were removed by 1 h incubation with 5% wt/vol Triton X-100 and centrifugation. The red aqueous layer was loaded onto 0.25 to 1.0 M discontinuous sucrose gradient in phosphate buffer and centrifuged overnight at 22,500 rpm (range: 42,500 to 91,300 × *g*) in a SW28 rotor (Beckman Coulter) at 22 °C, or 2 to 3 h at 49,000 rpm (198,000 × *g*) in a VTi 50 rotor (Beckman Coulter). Colored bands were collected and frozen at −20 °C until analysis (see figure 4 in ref. 34 for illustration).

Purified PBS were dialyzed in 5 mM sodium phosphate buffer, pH 7.0, with 5 mM 2-mercaptoethanol (β-Me) and then concentrated by ultrafiltration using Amicon Ultra-15 centrifugal filters (Millipore). A sample containing 500 μL of a 1:3 ratio of sample to 9 M urea (pH brought to 2.0 with HCl) was prepared prior to HPLC separation. A Waters E2695 pump (Waters Corporation) was used in conjunction with a Waters 2996 photodiode array and a Thermo-scientific BioBasic-4 HPLC column (Thermo-Scientific; 250 mm × 4.6 mm, 5 μm particle size) to separate each phycobiliprotein. The program used a flow rate of 1.5 mL min^{-1} with a gradient program starting at a 65:35 ratio of Buffer A (0.1% trifluoroacetic acid solution with 0.001 M sodium azide) to Buffer B (2:1 acetonitrile:isopropanol in 0.1% TFA with 0.001 M sodium azide). The starting conditions are 65% A:35% B. After the sample was injected, after holding for 2 min, buffer conditions were ramped to 100% B over 45 min. The samples were monitored at 280, 490, and 550 nm. Separated samples were concentrated using a vacuum centrifuge and stored in the freezer at −20 °C until ready for digestion by trypsin.

Heterologous Expression and Purification of Recombinant Proteins. For recombinant protein expression, each gene was amplified by PCR from *Synechococcus* RS9916 or A15-62 chromosomal DNA using the primers listed in Dataset S3 and cloned into plasmids as described in Dataset S4. Recombinant proteins were expressed and purified from *E. coli* BL21 (DE3) competent cells (Novagen/EMD Millipore Corp.) as previously described (35). Once at OD_{600nm}= 0.6, cultures were induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG), after which the cells were allowed to grow at 18 °C for an additional 24 h before being harvested by centrifugation. HT proteins were purified as described (36).

Protein and Bilin Analysis by Spectroscopy and Gel-Electrophoresis. Fluorescence emission and absorbance spectra were acquired on a Perkin-Elmer LS55 fluorescence spectrometer and a Lambda 35, dual-beam UV/Vis spectrometer (Perkin-Elmer) as previously described (37). Polyacrylamide gel electrophoresis (PAGE, 15% wt/vol) with sodium dodecyl sulfate (SDS) was used to analyze polypeptide samples acquired after purification of proteins as previously described (17). Zn-enhanced fluorescence of covalently attached bilins was visualized using an imaging system (Bio-Rad MP) with excitation at 460 nm (PUB detection) or 540 nm (PEB detection). Proteins in the gels were then stained by incubation in Coomassie brilliant blue G-250 overnight and destained in 10% methanol and 10% acetic acid and visualized with trans-white light (Bio-Rad MP).

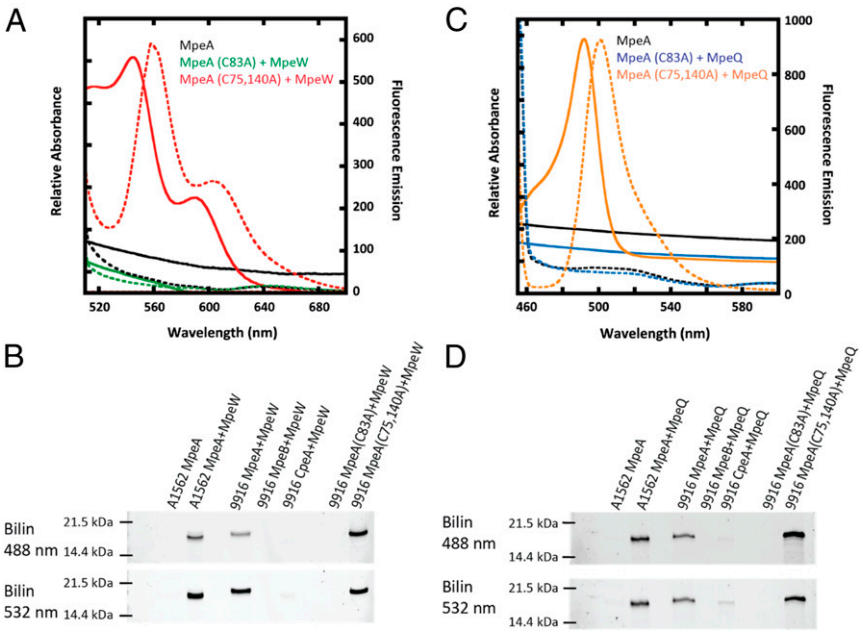

Fig. 4. Determination of the phycobiliprotein subunit and binding site specificities of the MpeW and MpeQ enzymes. Recombinant PEII α-subunit (MpeA) from *Synechococcus* sp. A15-62, PEII α- and β-subunits (MpeA and MpeB), and PEI α-subunit (CpeA) from RS9916, as well as site-mutated RS9916 MpeA in which either Cys-83 only or both Cys-75 and Cys-140 were replaced by an alanine [so-called MpeA (C83A) and MpeA (C75A and C140A), respectively], were coexpressed with either MpeQ (*A, B*) or MpeW (*C, D*) from A15-62 along with genes necessary for PEB synthesis. (*A* and *C*) Absorbance (solid lines) and fluorescence emission (dotted lines) spectra of PEB (*A*; excitation set at 490 nm) or PUB (*C*; excitation set at 440 nm) of purified recombinant WT and site-mutated MpeA from RS9916. The secondary emission peak at 610 nm in *A* likely corresponds to PEB at C83 (see text). (*B* and *D*) Zinc-enhanced fluorescence of SDS-PAGE gel showing relative PEB or PUB covalently bound to different recombinant phycoerythrin subunits under various coexpression conditions (indicated on top) for MpeW (*B*) or MpeQ (*D*). Data shown here are representative of three technical replicates.

Tryptic Digestion and Mass Spectrometry. Tryptic digestion of proteins was conducted as previously described (38). Tryptic-digested samples were analyzed using liquid chromatography (LC)-MS/MS on an Orbitrap Lumos Fusion mass spectrometer (Thermo Fisher) with an Agilent 1100 Capillary HPLC as its inlet. LC-MS/MS analysis was performed as previously described with a few modifications (13, 17). Alternatively, some samples were reanalyzed using a Thermo Easy-nLC 1000 capillary LC with the following conditions: Buffer A = 0.1% vol/vol aqueous formic acid; Buffer B = 80% vol/vol acetonitrile, 0.1% vol/vol formic acid in water. Load 2 μL with 0% B onto a 5 mm long, 0.1 mm i.d. C18 trapping column. A 0.075 mm i.d. × 150 mm long C18 analytical column led directly into the nanospray needle. The gradient was 2% B at 0 min, ramp to 7% B at 0.5 min, ramp to 50% B at 20 min, ramp to 100% B at 21 min, hold 100% B for 9 min. The trap column was reequilibrated with 12 μL of Buffer A and the analytical column with 10 μL of 2% B between each injection. Mass spectra were recorded continuously at 120,000 resolving power. Data-dependent tandem mass spectra were recorded at 30,000 resolving power with 3 s between MS1 spectra. The tandem mass spectra were processed using Thermo Proteome Discover 2.1 software, a simplified protein database consisting of only the 40 proteins expected to be part of the PBS was used to speed up the analysis. Bilin-containing peptides were confirmed by manual inspection of their associated MS1, MS2, and UV-VIS spectra. Extracted ion chromatograms for each of the bilin-containing peptides were generated using XCalibur 4.0 software (Thermo Fisher). Briefly, intensities for a 20-ppm window around the masses indicated were extracted, and the resulting chromatograms were smoothed with a five-point boxcar algorithm. Areas were computed using the default Genesis algorithm in XCalibur; a combination of UV-VIS spectra confirmation, retention time, and tandem MS/MS data were used to differentiate between the PUB and PEB isomers. The ionizability of PEB and PUB modified versions of the same peptide were considered to be identical for comparisons. The areas of multiple peaks corresponding to the same bilin isomer were added together (oxidized versions of PEB-containing peptides sometimes split into more than one peak with nearly identical tandem mass spectra). The PUB fraction was computed by summing the area of all PUB-containing features for a single modified cysteine and dividing it by the total of both PUB and PEB modified versions of features containing the same cysteine.

Data Availability. All study data are included in the article and *SI Appendix*.

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