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Abstract

Marine *Synechococcus* play a key role in global oceanic primary productivity. Their wide latitudinal distribution has been attributed to the occurrence of lineages adapted to distinct thermal niches, but the physiological and molecular bases of this ecotypic differentiation remain largely unknown. By comparing six strains isolated from different latitudes, we showed that the thermostability of their light-harvesting complexes, called phycobilisomes, varied according to the average sea surface temperature at strain isolation site. Comparative analyses of thermal unfolding curves of the three phycobiliproteins constituting phycobilisome rods suggested that the differences in thermostability observed on whole phycobilisomes relied on the distinct molecular flexibility and stability of their individual components. Phycocyanin was the least thermostable of all rod phycobiliproteins, constituting a fragility point of the phycobilisome under heat stress. Amino acid composition analyses and structural homology modeling notably revealed the occurrence of two amino acid substitutions, which might play a role in the observed differential thermotolerance of this phycobiliprotein among temperature ecotypes. We hypothesize that marine *Synechococcus* ancestors occurred first in warm niches and that during the colonization of cold, high latitude thermal niches, their descendants have increased the molecular flexibility of phycobiliproteins to maintain optimal light absorption capacities, this phenomenon likely resulting in a decreased stability of these proteins. This apparent thermoadaptability of marine *Synechococcus* has most probably contributed to the remarkable ubiquity of these picocyanobacteria in the ocean.

Introduction

Temperature is an environmental factor that greatly impacts the distribution of living forms on our planet. Temperature varies widely over the course of the day, seasons as well as across latitudes and therefore constitutes a major ecological constraint on the physiology of organisms and hence on the functioning of ecosystems. In particular, temperature is one of the main factors controlling inorganic carbon fixation, a process which in the oceans is prevalently ensured by phytoplanktonic cells (Behrenfeld *et al.,* 2006; Falkowski, 1994). Among these, *Prochlorococcus* and *Synechococcus*, two highly abundant picocyanobacteria (< 2 µm), are thought to be responsible for up to 25% of the global net oceanic primary production (Flombaum *et al.,* 2013; Partensky *et al.,* 1999). While *Prochlorococcus* is restricted to the 40 °S – 45 °N latitudinal band, *Synechococcus* occurs from the equator to polar circles (Huang *et al.,* 2012; Neuer, 1992; Zwirglmaier *et al.,* 2008), suggesting that this ubiquitous picocyanobacterium has developed efficient adaptive strategies to cope with natural temperature variations (Mackey *et al.,* 2013; Pittera *et al.,* 2014).

Phylogenetic studies using various markers have evidenced the large genetic microdiversity occurring within the *Synechococcus* genus (Ahlgren & Rocap, 2012; Fuller *et al.,* 2003). For instance, based on the high resolution *petB* marker, about 15 clades and 28 subclades (Mazard *et al.,* 2012) have been delineated within the main radiation, called subcluster 5.1 (Herdman *et al.,* 2001). Basin-scale phylogeographical studies have shown that the most prevalent marine *Synechococcus* lineages, *i.e.* clades I to IV, occupy distinct ecological niches (Sohm *et al.,* 2015; Zwirglmaier *et al.,* 2008). Clades I and IV are confined to nutrient-rich, cold or temperate waters at high latitude (> 30°N/S), whereas clades II and III preferentially thrive in warm waters, with the former being prevalent in subtropical and tropical open ocean and the latter dominating in the eastern Mediterranean Sea (Farrant *et al.,* 2016; Mella-Flores *et al.,* 2011; Sohm *et al.,* 2015).

Pittera *et al.* (2014) have evidenced a correspondence between the thermophysiology of *Synechococcus* clades I and II and their respective thermal niches. Indeed, members of these lineages were shown to exhibit thermal *preferenda* (i.e., temperature growth ranges and growth maxima)

consistent with the seawater temperature at their isolation site, as well as a differential sensitivity to thermal stress. These genetically defined lineages, physiologically adapted to specific thermal niches, therefore correspond to different 'temperature ecotypes' (or 'thermotypes'), a concept previously defined for *Prochlorococcus* clades HLI and HLII, which preferentially thrive in cool temperate waters and warm subtropical waters, respectively, a discrepancy also explained by the distinct growth temperature characteristics of representative isolates (Johnson *et al.,* 2006; Zinser *et al.,* 2007). Although other factors such as the macronutrients can be important sources of diversification within the marine *Synechococcus* radiation, recent field studies have demonstrated that temperature is one of the main factors explaining the variability of the genotypic composition of marine *Synechococcus* assemblages, with different thermotypes forming well-defined populations in distinct latitudinal bands at oceanic basin scales (Farrant *et al.,* 2016; Sohm *et al.,* 2015).

Pittera *et al.* (2014) also showed that during thermal stress experiments the capacity of the temperature ecotypes to acclimate and endure temperature variations notably relies on their ability to optimize the functionality of their photosystem II (PSII) at different temperatures. This macromolecular complex is indeed known to be a particularly temperature responsive component of the photosynthetic machinery (Murata *et al.,* 2007). Like in red algae, the major PSII light-harvesting antenna of *Synechococcus* is a giant, water soluble pigment-protein complex, the phycobilisome (PBS). This macrocomplex, composed of a central core surrounded by six rods, is made of phycobiliproteins (PBP), themselves composed of two subunits (α and β) aggregated as hexameric discs $(\alpha\beta)_6$. Different open-chain tetrapyrrolic chromophores, the phycobilins, are bound to the apoproteins by thioether bonds on cystein residues, and absorb at specific wavelength bands (Glazer, 1985; Glazer, 1989; Sidler, 1994; Six *et al.,* 2007b). Based on their amino acid sequence and absorption properties, the PBPs have been assigned to distinct classes. The PBS core is always made of allophycocyanin (APC), which binds the blue chromophore phycocyanobilin (PCB, $A_{max} \sim 620$ nm). In marine *Synechococcus*, the basal part of PBS rods is composed of phycocyanin (PC) that most often binds PCB and phycoerythrobilin (PEB, $A_{max} \sim 550$ nm), usually at a molar ratio of 1:2 (Ong & Glazer

1987; Six *et al.,* 2007b; but see also Blot *et al.,* 2009). The distal part of the rods is generally made of two types of phycoerythrins, PEI and PEII, which display different combinations of PEB and phycourobilin (PUB, A_{max} ~ 495 nm), depending on the strain (Humily *et al.,* 2013; Ong & Glazer, 1991; Sidler, 1994). The whole PBS structure is further stabilized by a set of linker polypeptides (Glazer, 1984). The design of the complex induces a directional transfer of excitation energy from the rod periphery to the PBS core and *in fine* to reaction centers (Glazer, 1989). The efficiency of the energy transfer in the PBS may depend on environmental factors, such as incident photon flux or osmotic pressure, and can be monitored by measuring energy leaks, emitted as fluorescence, for each PBP (see *e.g.* Ke, 2001; Kupper *et al.,* 2009; Six *et al.,* 2007a).

As the PBS constitutes the main entrance gate of light energy into the photosynthetic machinery, the sustainability of the PBP function is of crucial importance for the cell. In all PBPs, the native protein molecular environment constrains the phycobilins to a planar conformation that allows maximal light absorption (Glazer, 1985; MacColl *et al.,* 1980; Scheer & Kufer, 1977). In disordered proteins, the bilin conformation is no more constrained by the protein environment and tends to adopt a more cyclic conformation, inducing lower visible light but higher UV absorbance. Temperature is well known to influence protein conformation and is therefore likely to affect the function of cofactors such as phycobilins (Bowen *et al.,* 2000). Consequently, during evolution, different variants of a protein have evolved, with cold environment selecting for more flexible proteins at the cost of thermostability, whereas warm environments favour stability at the cost of psychroflexibility (Jaenicke & Böhm, 1998; Szilagyi & Zavodszky, 2000).

Here, we combined different approaches to compare the functional thermostability and molecular flexibility of the photosynthetic antennae of six marine *Synechococcus* strains representative of different thermotypes and unveiled some of the molecular bases of this adaptation to temperature.

Materials and methods

Biological material and growth conditions

We selected six marine strains isolated at a similar distance from the coast (mesotrophic waters), along a latitudinal gradient of temperature (Fig. 1, Table 1). The six strains belong to the same PEB-rich pigment type (3a *sensu* Six *et al.,* 2007b) and have therefore a similar PBS structure and composition. Clonal *Synechococcus* strains M16.1, RS9907, WH7803, ROS8604, MVIR-16-2 and MVIR-18-1 (http://roscoff-culture-collection.org/) were grown in PCR-S11 culture medium (Rippka *et al.,* 2000) supplemented with 1 mM sodium nitrate, under 80 µmol photons m^{-2} s^{-1} white light irradiance provided by fluorescent tubes (Sylvania Daylight F18W/54-765 T8). Cultures were acclimated for at least four weeks to a range of temperature (9-35°C) using temperature controlled growth chambers.

In vivo *fluorescence emission in response to increasing temperature*

Synechococcus cultures in mid exponential growth phase were placed in a temperature-controlled water bath at growth temperature in the dark. Temperature was then progressively increased by 3°C steps of 10 min, up to 46°C. At the end of each temperature level, an *in vivo* fluorescence emission spectrum was recorded upon excitation at 530 nm using a LS-50B spectrofluorimeter (Perkin Elmer). The energy transfer between the PBPs was assessed by measuring their relative fluorescence emission levels, since increases in the relative heights of the PC or PE emission peaks are indicative of energy leaks. The PE to PC fluorescence emission ratio was then calculated, log-transformed and plotted against instantaneous temperature by homology with the well-known Arrhenius breaking temperature plots, allowing us to determine the PBS breaking temperature (T_{PBS}; Dahlhoff *et al.,* 1991; Stillman & Somero, 1996); Fig. S1). For further analyses, the fluorescence emission spectra were decomposed using the a|e – UV-Vis-IR Spectral software 2.0 (www.fluortools.com).

Phycobiliprotein denaturation curves

Phycobiliprotein purification was carried out using sucrose gradients and isoelectric focusing, as previously described (Six *et al.,* 2005). PBP structural thermostability was assessed by monitoring

150 phycobilin absorbance and fluorescence along protein thermal unfolding curves (Grimsley *et al.,*
151 2013), from 30°C to 85°C, with PBP solutions at about 30 nM for PEs (2.15 10^6 M^{-1} cm^{-1} and 2.41 10^6
152 M^{-1} cm^{-1} for at 545 nm PEII and PEI respectively; Glazer & Hixson, 1977; Wyman 1992) and 100 nM
153 for PC (2 10^5 M^{-1} cm^{-1} at 620 nm as estimated from Glazer *et al.,* 1973 and Glazer & Hixson, 1975).
154 Absorption and fluorescence emission spectra were recorded during a progressive temperature
155 increase, performed by 5°C steps of 5 min each. The fluorescence emission spectra were recorded
156 upon excitation at 545 nm for PE and 620 nm for PC and the absorbance was monitored at the
157 wavelength of the acceptor phycobilins, *i.e.* PEB in PEs (~ 545 nm) and PCB in PCs (~ 620 nm; Sidler,
158 1994). For each PBP, the absorbance values were plotted against instantaneous temperature and
159 fitted with a sigmoid curve using the following equation:

$$y = \mathrm{b} + \frac{a - \mathrm{b}}{1 + 10^{(\log \mathrm{T}_{50\%})\mathrm{s}}}$$

160 where *a* is the initial maximal absorption, *b* the absorption minimum at 85°C and *s* the Hill slope of
161 the function. The mid-unfolding temperature $T_{50\%}$, *i.e.* the temperature at which the protein has lost
162 half of its absorption capacities, which is related to the molecular flexibility of the protein, was
163 determined using the Sigma Plot v10 software (Figs. S2 & S3).
164
165 ***Analysis of phycobiliprotein sequences and homology modeling***
166 Using GenBank and the Cyanorak interface (http://application.sb-roscoff.fr/cyanorak/), we
167 compiled a database encompassing the sequences of the two subunits of 21 PC, 21 PE-I and 20 PE-II.
168 Unpublished sequences were deposited in GenBank nucleotide sequence database under accession
169 numbers mentioned in Table S1. All sequences were aligned using Bioedit 7.2.3 (Hall, 1999) and the
170 distributions and amino acid frequencies of PC (RpcA and RpcB), PEI (CpeA and CpeB) and PEII (MpeA
171 and MpeB) subunits were computed using ProtParam Tools (Expasy, Gasteiger *et al.,* 2005).
172 Differences in the biochemical parameters of the α- and β-subunit sequences were tested using the
173 non-parametric statistical test of Wilcoxon, and differences among clades were tested using Kruskal-
174 Wallis test. Whether the observed substitutions were conservative or not was assessed using the

MAFFT v7 software (Katoh & Standley, 2013). Physicochemical parameters of the PBP were retrieved using the ProtParam tool and the molecular flexibility was calculated with ProtScale (Gasteiger *et al.,* 2005).

The crystal structure of *Spirulina* platensis PC (Padyana *et al.,* 2001; PDB ID: 1HA7) was retrieved from the Protein Data Bank (Berman *et al.,* 2003) and used as a template for modeling *Synechococcus* PC. Structural protein models were generated using Phyre^2 (Kelley & Sternberg 2009). The PCB and PEB structures were retrieved from *Polysiphonia urceolata* (Jiang *et al.,* 2001; PDB ID: 1F99). Models were then visualized and aligned using PyMOL v 1.7.4. *Synechococcus* PC models were superimposed and the PC structural differences related to amino acid substitutions were compared between cold- and warm-environment strains.

Results and discussion

Marine Synechococcus *phycobilisome function optimally in specific thermal ranges*

The six *Synechococcus* strains were isolated from different latitudes and thermal niches (Fig. 1). The temperature range for growth and *preferenda* of five of them were characterized in a previous study, while those of a sixth one isolated from the Red Sea, RS9907, is reported here (Table 1). Their markedly distinct temperature tolerance ranges as well as the 10°C difference in optimal growth temperature between tropical (RS9907 and M16.1) and subpolar strains (MVIR-16-2 and MVIR-18-1), clearly indicate that these strains correspond to distinct thermotypes (Pittera *et al.,* 2014).

All strains were submitted to a stepwise temperature increase and their *in vivo* fluorescence emission spectra were recorded at each step. During the light-harvesting process, the distal PBP discs transfer the light excitation energy to the proximal ones, which in turn transfer it to the PBS core. A low efficiency of energy transfer between two PBPs directly results in an increase in the fluorescence emitted by the donor PBP, at a specific wavelength. Thus, *in vivo* fluorescence emission spectra reflect the energy transfer efficiency along the PBS rod down to the reaction center II, and therefore the overall coupling of the PBS components. As expected from a number of previous studies (Bailey

& Grossman, 2008; Six *et al.,* 2004; Six *et al.,* 2005; Six *et al.,* 2007b), the fluorescence emission spectra typically exhibited three maxima, attributable to PE (565-575 nm), PC (650 nm) and the combined signal from APC and RC chlorophylls, altogether called the PBS terminal acceptor (TA; 680 nm; Fig. 2A, B). We used the PE:PC emission fluorescence ratio as a parameter integrating the variations of the fluorescence emitted by the rod PBPs. This therefore allowed us to indirectly monitor the excitation energy transfer within the PBS rods (Ke, 2001; Six *et al.,* 2007a).

PBS performance was strongly affected by the temperature increase and the progressive alteration followed the same succession of steps in all *Synechococcus* temperature ecotypes. The first temperature increments (from 22 to 28-31°C, depending on strains) induced no significant change in the PE:PC emission ratio, showing that the energy was still efficiently transferred along the rod (Fig. 2). As temperature kept increasing, fluorescence first increased in the red region (650-680 nm), leading to a decrease of the PE:PC ratio (Fig. 2A, B, C). To identify which component between PC and TA first underwent this energy leak, we carried out a spectral decomposition of the fluorescence emission spectra (Fig. 2D). Results showed that the first alterations occurred at the TA level (680 nm) and this energy leak likely mostly originated from chlorophylls bound to the reaction center II, consistently with a previous report that showed that heat induces a strong chl *a* fluorescence increase in *Synechocystis* sp. PCC 6803 (Inoue *et al.,* 2001). The reaction center II is indeed known to be highly sensitive to increases in temperature, which notably causes the oxygen evolving complex to lose two out of the four Mn atoms of the Mn_4CaO_5 cluster, a dissociation resulting in the breakdown of oxygen evolution (Kimura *et al.,* 2002).

Spectral decompositions further showed that the PC fluorescence component (650 nm) also increased but at a higher temperature than the reaction center one (Fig. 2D), leading the PE:PC ratio to keep on decreasing as temperature increased (Fig 2C). This indicates that the reaction center heat-induced disturbance then propagated to the base of the PBS rod, likely through APC. This energetic jamming between the reaction center and the base of the PBS, which at this stage was still reversible, eventually destabilized the whole complex and ultimately led to the dismantling of the PBS. The

latter process occurred abruptly and was recognizable by the sudden and large increase in fluorescence at ~570 nm that indicated the release of free, highly fluorescing PEs in the cytosol (Fig. 2A-B). At this stage, the PBS was irreversibly broken down and light harvesting was no longer possible. This PBS dismantling phenomenon has been characterized in detail at the transcriptomic, proteomic, and biophysical levels in several previous studies (Lao & Glazer, 1996; Pittera *et al.,* 2014; Sah *et al.,* 1998; Six *et al.,* 2007a).

By applying an Arrhenius-type analysis (Fig. S1), it was possible to determine accurately the temperature at which the PBS dismantling occurred, T_{PBS}, which we used as an indicator of the functional PBS thermostability in *in vivo* conditions. This parameter may thus provide information on the thermal plasticity of the light harvesting function among strains. The comparison of the six strains grown at different temperatures showed that, in most cases, T_{PBS} increased with acclimation temperature until reaching a plateau (Fig. 3). The apparent enhanced stability of PBS observed for strains grown at high temperature is most likely due to an increase in the thermotolerance of the reaction center itself, from where the disturbance originates (Inoue *et al.,* 2001; Nishiyama *et al.,* 2006). Temperature-induced variations of reaction center thermostability are thought to result from the capability of PsbU and PsbV proteins to stabilize the oxygen evolving complex (Nishiyama *et al.,* 1997; Yamasaki *et al.,* 2002) and/or from adjustments of the fluidity of thylakoid membranes where the PS II is embedded (Inoue *et al.,* 2001; Loll *et al.,* 2007). The reaction center thermoacclimation could also be modulated by changes in the D1 protein isoform from D1.1 to the more stable D1.2 (Kós *et al.,* 2008). The difference in the amplitude of the increase of T_{PBS} in strains M16.1 and ROS8604 (Fig. 3A & D) could thus originate in a different PS II composition or in different capacities to induce such processes.

The plateau reached by the T_{PBS} parameter was variable and appeared to be related to their thermal niche of the marine *Synechococcus* strains (Fig. 3). Indeed, strains isolated from subtropical (M16.1 and RS9907) or warm temperate waters (WH7803) displayed T_{PBS} values ranging from 35°C to 43°C whereas the clade I strains isolated from cold temperate and subpolar waters showed

significantly lower values between 29°C and 39°C (Wilcoxson test, W = 81, p-value < 0.01). These results show that the PBSs of high latitude strains are functionally less thermostable than the PBSs of low latitude ones, and therefore suggest that these light-harvesting complexes exhibit different levels of molecular flexibility. The different thermotypes of marine *Synechococcus* thus use PBSs that are functionally constrained by temperature.

Differences in phycobilisome molecular flexibility among strains can be explained by differences in molecular flexibility of individual phycobiliproteins

In the six marine *Synechococcus* strains studied here, PBS rods are made of three classes of hexameric PBPs, namely PC, PE-I and PE-II, which bind different sets of chromophores and are maintained together thanks to specific linker polypeptides (Ong & Glazer, 1991; Six *et al.,* 2005; Six *et al.,* 2007b). To understand whether the differences in PBS thermostability among strains are related to differences in the thermostability of individual PBPs, we first purified all rod PBPs in their native state, devoid of linker polypeptides. The six strains possess PBPs with identical optical properties, typical of *Synechococcus* pigment type 3a (Six *et al.,* 2007b), i.e. a PC binding two PEB and one PCB molecule (so-called R-PC-II; Ong & Glazer, 1987), a PE-I bearing only PEB, and a PE-II binding one PUB and five PEB molecules (Fig. S2; Ong & Glazer, 1991). The PC, PE-I and PE-II, displayed fluorescence emission maxima at 635, 569 and 562 nm, respectively. We then performed thermal unfolding curves for each of these 18 purified PBPs, in order to determine their mid-unfolding temperatures, $T_{50\%}$, a commonly used proxy for assessing and comparing the thermostability and molecular flexibility of proteins (Fig. S2 & S3; Grimsley *et al.,* 2013; Jaenicke, 1991).

Upon temperature increase, all PBPs underwent a progressive drop of absorbance following a sigmoid curve (Fig. S3), down to levels lower than 10% of the initial value, and at 85°C proteins were completely unfolded and denatured. Fluorescence also decreased until complete quenching, following a quasi-linear function (Fig. S4). The comparative analysis of mid-unfolding temperatures for the three rod PBP types revealed two remarkable trends (Fig. 4). Firstly, the different classes of

PBPs showed significantly distinct thermostability (Kruskal-Wallis, $X^2 = 37.11$, df = 2, *p-value* < 0.01). Indeed, on average, PEII lost half of its absorption capacity at 60.9 ± 2.6°C, whereas the mean mid-unfolding temperature was 56.0 ± 2.5°C and 51.8 ± 2.0°C for PE-I and PC, respectively. These results indicate that PBP thermostability decreases towards the core of the PBS rod, with the most distal PE-II being the most stable PBP and PC the least. The latter finding is quite consistent with our observation that when cells undergo a moderate temperature shock, PC is the first rod PBP to show energetic disturbance in the PBS (Fig. 2). As concerns PEII, it is known to be released in the cytosol under excess light, UV or temperature stress conditions (Pittera *et al.,* 2014; Six *et al.,* 2004; Six *et al.,* 2007a), during which it may constitute a soluble screen dissipating excess excitation energy as fluorescence, a role that likely requires molecular stability. Secondly, subtropical strains use PBPs that are significantly more thermostable than strains isolated from cold waters (Fig. 4). While the southernmost strain M16.1 and RS9907 displayed PEII, PEI and PC $T_{50\%}$ of 63.3 ± 1.2°C, 58.4 ± 0.7°C and 54.0 ± 1.0°C, respectively, this parameter was 5°C lower for the northernmost strains MVIR-16-2 and MVIR-18-1, with corresponding values of 57.8 ± 0.4°C, 53.4 ± 1.5°C and 49.6 ± 0.4°C (Student t-test: t = 9.68, 7.49 and 10.15 for PC, PE-I and PE-II, respectively; all *p-values* < 0.01). Consistently, mid-unfolding temperatures were strongly correlated to the latitude of strain isolation sites (Fig. S5). This indicates that the observed differences in overall PBS functional stability among strains isolated from distinct thermal niches rely at least in part on the differential thermostability of their constituting PBPs. The distinct thermal unfolding properties of PBPs strongly suggest that the molecular flexibility of these proteins differs. It is worth noting that the latter experiments were carried out on PBPs devoid of linker polypeptides, a lack that may somewhat modulate the thermotolerance of these molecules. Yet, the mid-unfolding temperature values reported here are quite in the range of previously reported values for other PBPs (Chaiklahan *et al.,* 2012; Edwards *et al.,* 1997; Patel *et al.,* 2004; Pumas *et al.,* 2011; Pumas *et al.,* 2012).

To our knowledge, the high latitude *Synechococcus* ecotypes studied here possess the least thermostable rod PBPs described so far. Indeed, most previous studies have focused on PBPs

extracted from thermophilic organisms isolated from hot springs, an environment in which these complexes require a particularly high thermostability (Adir *et al.,* 2001; Edwards *et al.,* 1997; Munier *et al.,* 2014). Our study therefore shows that the diversification of PBP variants among PBP-containing organisms has occurred not only in specific extreme thermal niches, such as hot springs, but also along the large latitudinal temperature gradients of the Earth.

Marine* Synechococcus *phycocyanins exhibit molecular characteristics specific of the thermotypes.

In order to unveil possible differences in the molecular conformation of PBSs resulting from adaption to the low temperature niche, as described for other proteins (Croce & van Amerongen, 2014; Závodszky et al., 1998), we analyzed 62 PBP subunit sequences from 21 *Synechococcus* strains belonging to clades adapted either to cold (I and IV) or warm environments (II and III; Table S1). Amino acid sequence identity was higher than 80% among MpeA, MpeB, CpeA, RpcA and RpcB, and 70% among CpeB. Analyses of the PBP α- and β-subunits sequences showed that they exhibit a number of conserved characteristics. First, the aliphatic index of PBP α-subunits (RpcA, CpeA and MpeA) is always lower than that of their β-subunit counterparts (Table 2). This proxy for the relative volume occupied by aliphatic side chains was shown to be significantly higher in thermostable proteins (Jollivet *et al.,* 2012; Závodszky *et al.,* 1998). The grand average hydropathy index (GRAVY) is also different between the two PBP subunits, with α- being less hydrophobic than β-subunits (Table 2). Consistently, α-subunits always contain a lower percentage of hydrophobic amino acids than their β counterparts. Thus, even though α-subunits exhibit a higher content of charged residues than β-subunits (Dill, 1990; Ladbury *et al.,* 1995), α-subunits globally appear as the least stable of the two subunits and the most vulnerable to unfolding factors, such as high temperature, and it thus may constitute a fragility point within PBP aggregates.

Protein molecular adaptations to temperature are known to be in part based on specific substitutions of amino acids, with glycine, serine, lysine and asparagine in mesophiles, generally replaced in thermophiles by alanine, threonine, arginine and glutamate, respectively (Argos *et al.,*

1979; Fields, 2001; Szilagyi & Zavodszky, 2000). Extensive comparisons of the amino acid content of each PBP polypeptide revealed that RpcA sequences from members of the warm-environment clade II had a significantly higher content in alanine than their counterparts from cold clade I strains, *i.e.* 16.73 ± 0.36 % and 15.06 ± 0.55 %, respectively (Kruskal-Wallis, $X^2 = 9.83$,df = 3, *p-value* < 0.05). This suggests that the observed differential PC thermal unfolding properties between the high and low latitude strains might partly arise from differences in alanine content. Indeed, alanine accumulation in proteins is thought to increase hydrophobicity and therefore to decrease molecular flexibility, notably because this amino acid is an excellent helix former (see *e.g.* Dalhus *et al.,* 2002; Kumwenda *et al.,* 2013).

In contrast to PC, comparative analyses of PE sequences (CpeA, CpeB, MpeA and MpeB) did not point out any clear evidence of differential amino acid composition that could be related to the differential thermostability of PEs from subtropical and subpolar strains, possibly due to the fact that PE genes have been subjected to recurrent lateral transfers during *Synechococcus* evolution (Everroad & Wood, 2012; Humily *et al.,* 2014; Six *et al.,* 2007b).

In order to further understand the possible conformational differences among PCs from different *Synechococcus* thermotypes, we compared structural homology models of these proteins. Despite a high overall conservation of the 3D structure of PCs among strains (root mean square deviation < 0.5), we identified two semi-conservative substitutions that are specific of the cold-environment clades I and IV (Fig. S6). Both of them are located on an exposed domain of helix A (amino acids 35 – 45), which ensures the contact between α and β subunits (Adir *et al.,* 2006). The first substitution, located on the α-43 residue, involves the permutation of alanine found in clades II, III and V to glycine in clades I and IV. Such a substitution was previously observed when comparing orthologous proteins of thermophilic *vs.* mesophilic organisms (Szilagyi & Zavodszky, 2000). Indeed, glycine is thought to provide increased backbone conformational flexibility (Matthews *et al.,* 1987). The prediction of protein flexibility allowed us to detect a clear difference in this specific region of the RpcA chains between cold and warm-environment strains (Fig. 5).

Furthermore, the decrease in stability resulting from the substitution from alanine to glycine is thought to be stronger when it takes place within a helix (Menéndez-Arias & Argosf, 1989; Serrano *et al.,* 1992). Alanine indeed consistently stabilizes helical conformations relatively to glycine, because it buries more polar areas upon folding and because its backbone entropy is lower. This substitution in RpcA might thus contribute to the lower stability of PC in the cold-environment *Synechococcus* clades I and IV compared to warm-environment clades II and III.

The second substitution is located on the β-42 residue and involves the replacement of a serine in cold-environment clades by an asparagine in warm-environment clades (Fig. 6). As this residue is located on helix A, it likely also participates to the stability of the aggregation between the PC α- and β-subunits in the whole αβ hexameric aggregate. Furthermore, the structural modeling showed that this residue is located in the close vicinity of the D cycle of the PEB chromophore bound to the cysteinyl residue β-152. It is likely that the side chain difference between the asparagine in cold clades and the serine in warm ones influences the molecular stability of the planar conformation of the chromophore and therefore its absorption properties. In particular, this substitution is expected to decrease the steric hindrance and might lead to an increase in flexibility of the polypeptide chain. It is also important to note that, in such complex protein assemblages, the two abovementioned substitutions are repeated six times each, resulting in a summation effect that may greatly impact the overall protein properties.

Conclusion

Comparative analyses of the photosynthetic antenna complexes of six different marine *Synechococcus* strains isolated from different latitudes revealed clade-specific adaptations to temperature that are likely critical to maintain the biophysical properties of these pigment-protein complexes in the specific thermal niches of these strains. This result nicely complements previous work (Pittera *et al.,* 2014), which showed that the sensitivity of the light-harvesting process to temperature variations was thermotype-dependent and that the thermo-resistance of PBS

383 complexes was directly associated with the growth capacities of the considered strain. Altogether,
384 these complementary studies suggest that the thermoadaptability of light-harvesting complexes
385 likely plays a key role in the wide latitudinal distribution of thermotypes (Farrant *et al.,* 2016; Sohm
386 *et al.,* 2015), although other factors are certainly also involved, such as different membrane
387 composition adjustments or of heat shock proteins and/or antifreeze metabolites.

388 The functional differences between PBSs of cold- and warm-environment strains are seemingly
389 due to large differences in the molecular flexibility of the three PBPs constituting the rods. *In vivo*
390 temperature response curves indicated that PC is the first rod PBP to undergo energetic decoupling,
391 which then propagates through the entire rod, until complete dismantling of the PBS complex.
392 Consistently, we show that among the three rod PBPs, PC is the least thermostable and probably
393 constitutes a fragility point of the PBS rod under heat stress. Our results suggest that the
394 thermotype-specific differences in the stability or flexibility of PC originate at least in part from the
395 modulation of the alanine content as well as from two amino acid substitutions. We hypothesize that
396 temperature has exerted significant adaptive pressure on PBP evolution in marine *Synechococcus,*
397 conferring a fitness advantage to cold-adapted strains at high latitude, as shown by their specific
398 ability to grow and photosynthesize at low temperature (Pittera *et al.,* 2014). Further evidence for
399 the central role of the trade-off between PBP flexibility and stability for adaptation to cold and warm
400 niches, respectively, could be obtained from directed mutagenesis targeting the amino acids that we
401 found to differ between the PCs of cold and warm thermotypes. Yet, such genetic manipulation
402 remains a challenging task in marine picocyanobacteria.

403 In many organisms, including marine picocyanobacteria (Johnson *et al.,* 2006; Moore *et al.,* 1995;
404 Pittera *et al.,* 2014), maximal growth-rate temperature occurs very close to the cell death
405 temperature and physiological models suggest that the latter temperature coincides with a
406 denaturation catastrophe of the proteome (Dill *et al.,* 2011). The high sensitivity of protein structure
407 and function to temperature variations indicates that the ongoing global climate change is likely to
408 affect cells through perturbation of enzymatic and structural proteins. The evolutionary process

described here, which is a component of the thermotypic diversification occurring in several major *Synechococcus* lineages, is probably one of the key factors explaining the global ecological success of these cyanobacteria. Elucidating such processes is important in the context of global climate change as the latter will undoubtedly induce alterations of the composition of the phytoplanktonic communities over large oceanic spatial scales and thus impact top levels of food webs.

Supplementary information is available at ISMEJ's website.

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Figure legends

Figure 1: Isolation sites of the six marine *Synechococcus* strains used in this study. Squares, diamonds and circles correspond to subtropical, temperate and subpolar strains, respectively. Colors indicate the mean average annual sea surface temperature (MODIS) over the 2004-2014 period (QGIS 2.10.1).

Figure 2: Examples of variations of the fluorescence emission spectra (with excitation at 530 nm) for the tropical *Synechococcus* strain M16.1 and the subpolar strain MVIR-16-2 acclimated at 22°C and submitted to a progressive temperature increase. Fluorescence emission spectra of M16.1 **(A)** and MVIR-16-2 **(B)** strains at different temperatures. **(C)** Variations of the PE:PC (570:650nm) fluorescence emission ratio during the course of temperature increase in both strains, expressed as percentage of the initial value (at 22°C). **(D)** Relative variations of fluorescence intensity expressed as percentage of the initial fluorescence value, after spectral decomposition of the two red components: phycocyanin at 650 nm (dashed line) and terminal acceptor at 680 nm (solid line). Note that the APC fluorescence component of the 680 nm peak cannot be easily separated from that of RC chlorophylls in decomposed spectra but it is expected to be much lower.

Figure 3: Phycobilisome breaking temperature as a function of growth temperature for the six tested marine *Synechococcus* strains: M16.1 **(A)**, RS9907 **(B)**, WH7803 **(C)**, ROS8604 **(D)**, MVIR-16-2 **(E)**, MVIR-18-1 **(F)**. Three replicates are shown. Tropical strains are represented by squares, temperate ones by diamonds, and subpolar strains by circles.

Figure 4: Mid-unfolding temperature of the three PBP of the six marine *Synechococcus* strains, derived from the thermal unfolding curves (see Fig. S2 & S3). Error bars are standard deviation from the mean based on at least three replicates.

Figure 5: Predicted average flexibility along the amino acid sequence of phycocyanin α-subunit (RpcA) for *Synechococcus* strains of the cold-environment clades I and IV (dark blue, light blue, respectively), and the warm-environment clades II and III (orange and red, respectively). Error bars

778 are calculated from the mean of 5, 9, 5 and 2 sequences for clades I, II, III and IV, respectively (see
779 Table S1). The amino acid chain flexibility level was calculated using the Expasy tool ProtScale
780 (Gasteiger *et al.,* 2005). The insert shows the only major flexibility difference at the clade specific
781 substitution.

782 **Figure 6**: Computational homology model of an R-phycocyanin-II (R-PC-II) trimer structure of the
783 subpolar strain *Synechococcus* sp. MVIR-18-1 and structural differences with the subtropical strain
784 M16.1 R-PC-II. (**A**) Upper view of the R-PC-II trimer with α-subunits (RpcA) in blue and β-subunits
785 (RpcB) in pink. On each subunit, the amino acid substitutions specific to cold-environment clades,
786 located at α-43 and β-42, are shown in red. Phycoerythrobilins (PEB) are shown in dark pink and
787 phycocyanobilins (PCB) in blue. (**B)** Side view of a PC monomer, showing the location of the
788 substitution sites on helix A, involved in the α-β subunit interaction in *Synechococcus* sp. MVIR-18-1.
789 (**C**) Zoom in the β-42 substitution with the subpolar strain *Synechococcus* sp. MVIR-18-1 displaying a
790 serine and the subtropical *Synechococcus* sp. M16.1 displaying an asparagine (**D**).

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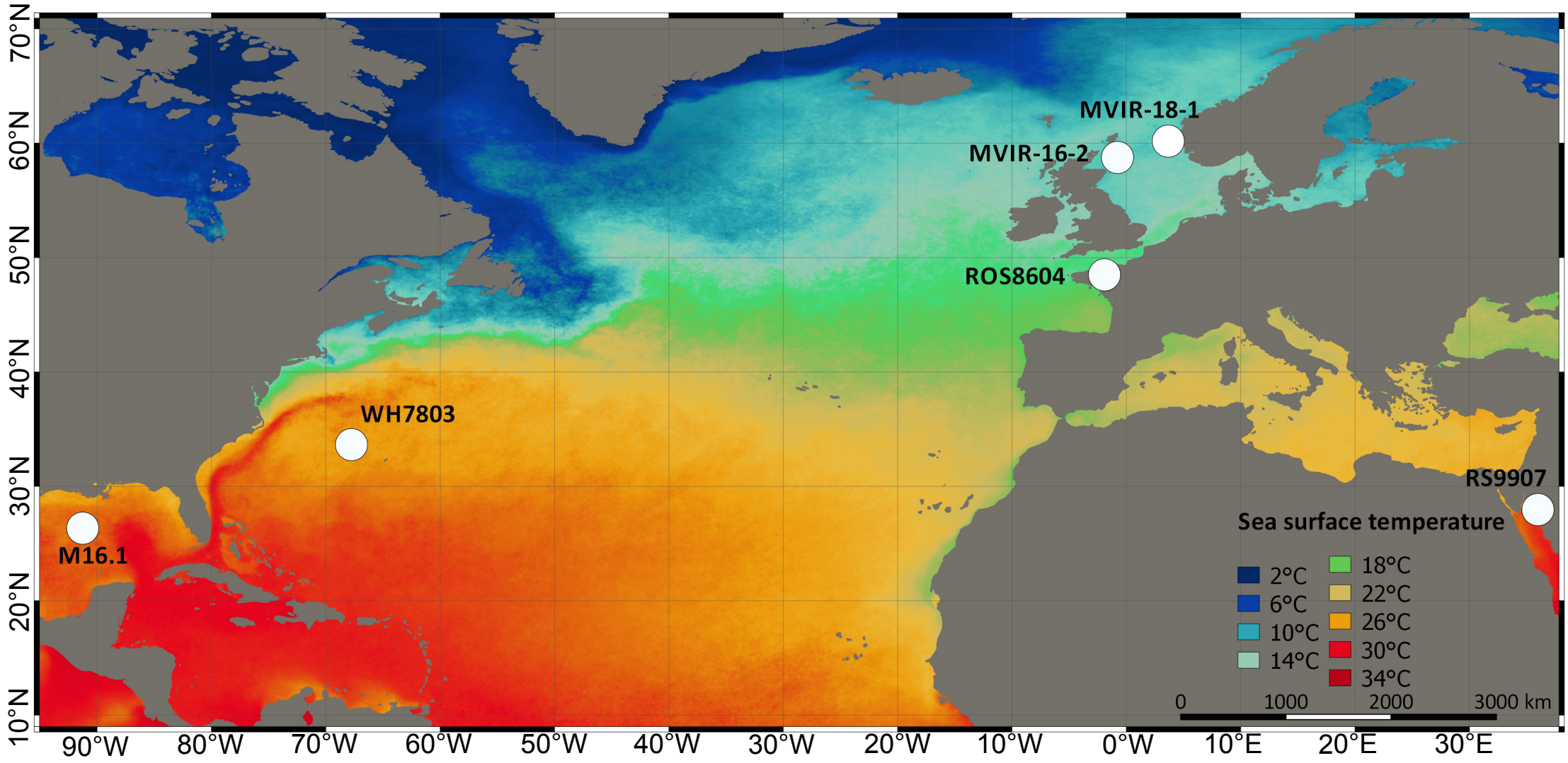
MVIR-18-1
MVIR-16-2
ROS8604
WH7803
M16.1
RS9907
Sea surface temperature
2°C
6°C
10°C
14°C
18°C
22°C
26°C
30°C
34°C
0
1000
2000
3000 km
70°N
60°N
50°N
40°N
30°N
20°N
10°N
90°W
80°W
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20°W
10°W
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20°E
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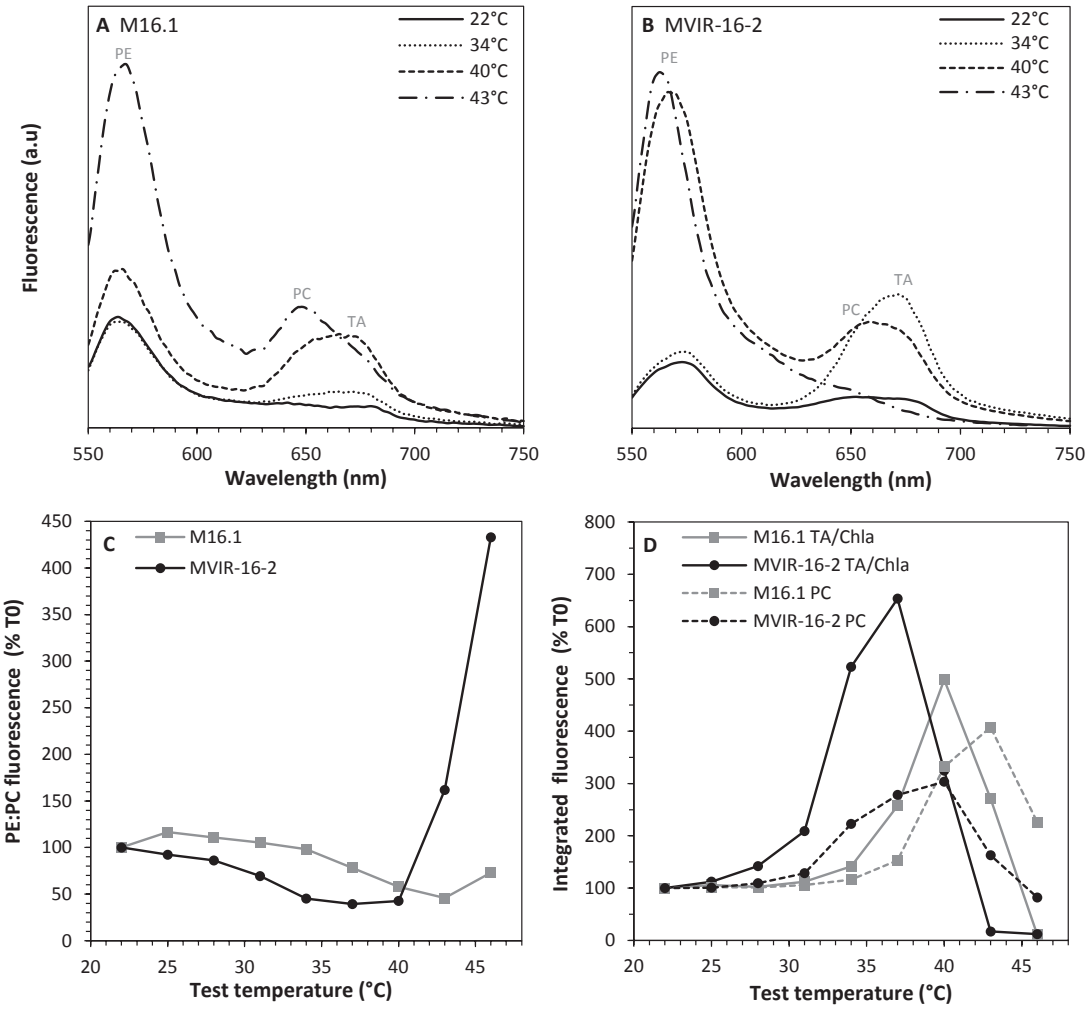
A M16.1
22°C
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B MVIR-16-2
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43°C
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PC
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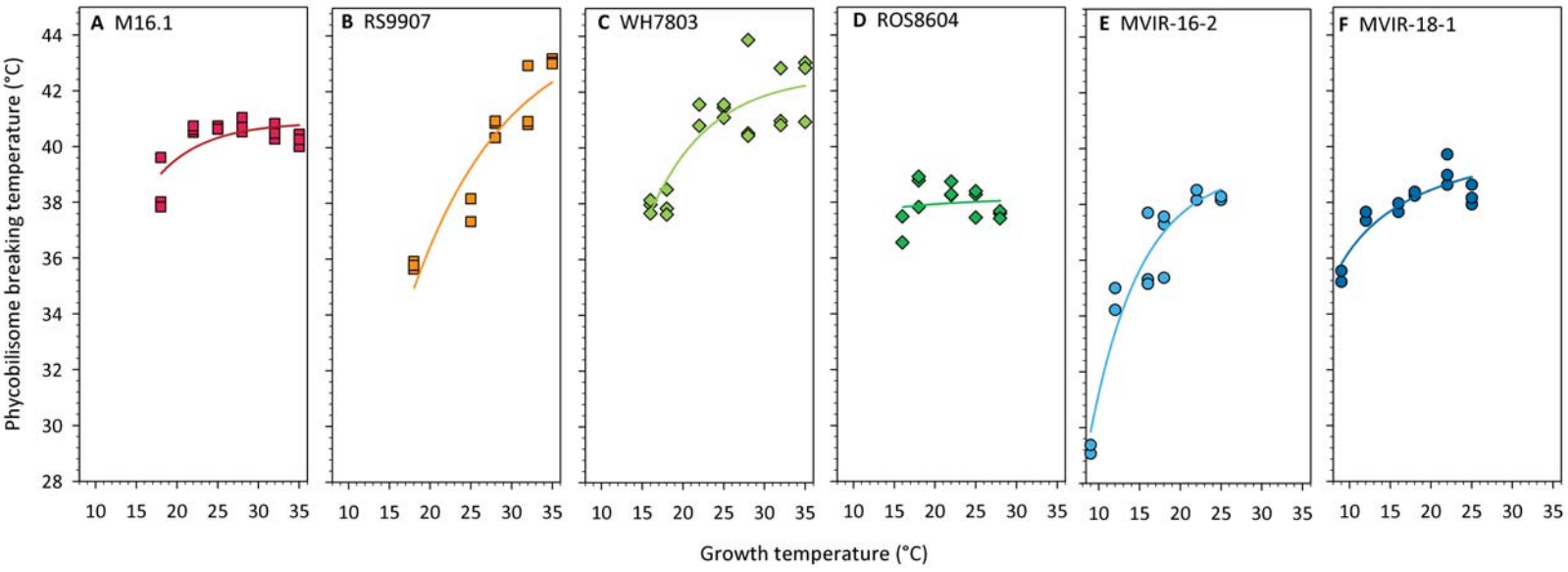
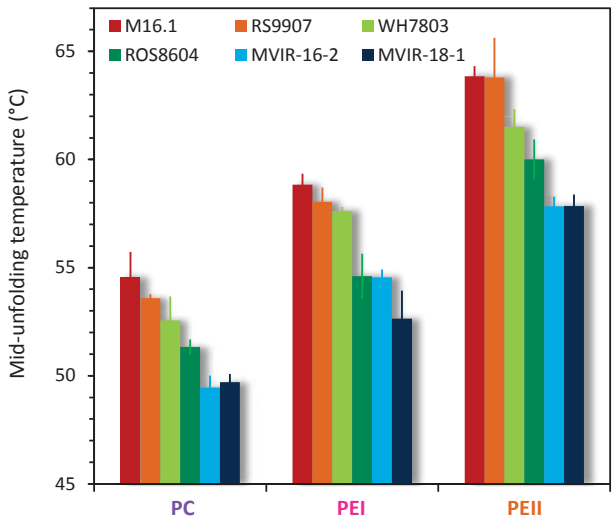
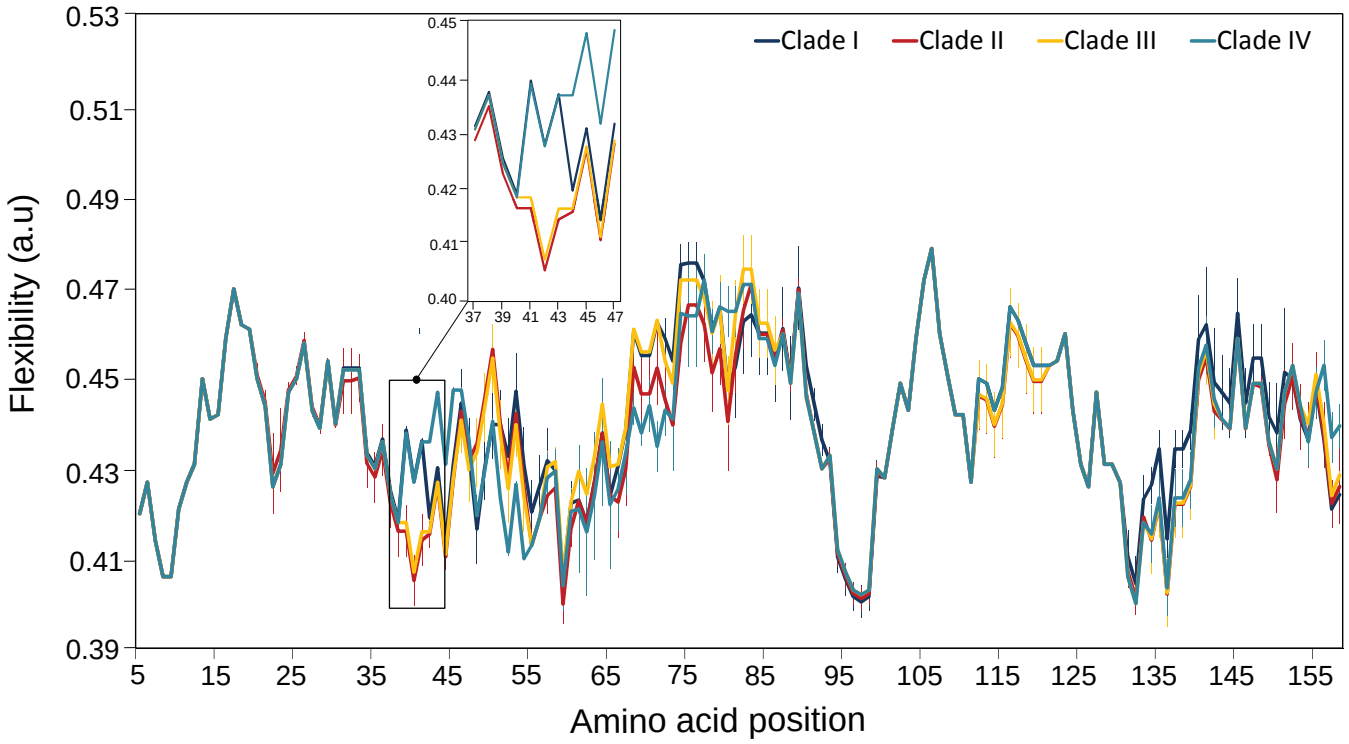
Wavelength (nm)
C
M16.1
MVIR-16-2
PE:PC fluorescence (% T0)
Test temperature (°C)
D
M16.1 TA/Chla
MVIR-16-2 TA/Chla
M16.1 PC
MVIR-16-2 PC
Integrated fluorescence (% T0)
Test temperature (°C)


A M16.1
B RS9907
C WH7803
D ROS8604
E MVIR-16-2
F MVIR-18-1
Phycobilisome breaking temperature (°C)
Growth temperature (°C)
28
30
32
34
36
38
40
42
44
10
15
20
25
30
35


M16.1
RS9907
WH7803
ROS8604
MVIR-16-2
MVIR-18-1
Mid-unfolding temperature (°C)
65
60
55
50
45
PC
PEI
PEII


Clade I
Clade II
Clade III
Clade IV
Flexibility (a.u)
Amino acid position
0.53
0.51
0.49
0.47
0.45
0.43
0.41
0.39
5
15
25
35
45
55
65
75
85
95
105
115
125
135
145
155
0.45
0.44
0.43
0.42
0.41
0.40
37
39
41
43
45
47

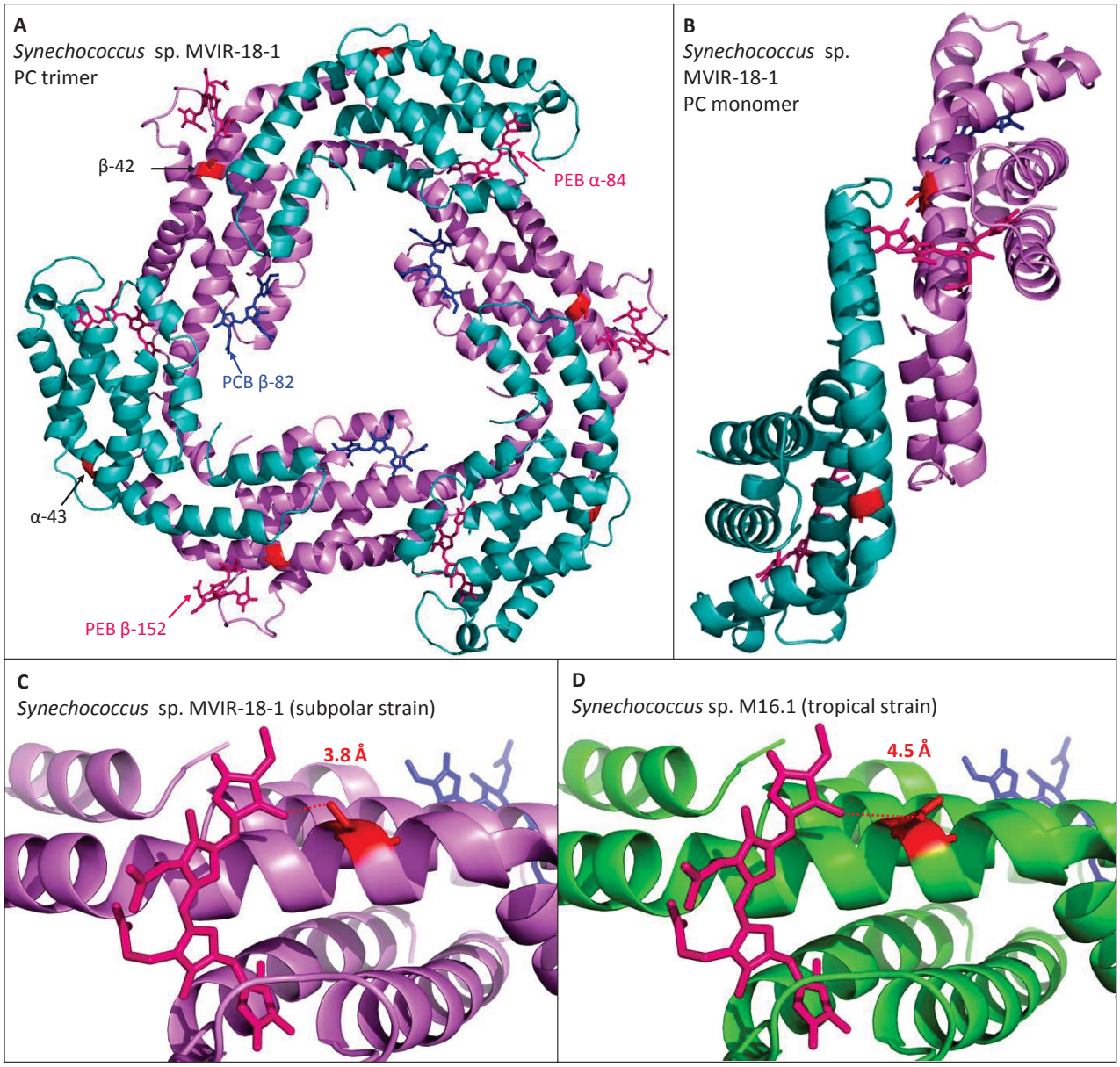

A
Synechococcus sp. MVIR-18-1
PC trimer
β-42
PEB α-84
PCB β-82
α-43
PEB β-152
B
Synechococcus sp.
MVIR-18-1
PC monomer
C
Synechococcus sp. MVIR-18-1 (subpolar strain)
3.8 Å
D
Synechococcus sp. M16.1 (tropical strain)
4.5 Å

Table 1: Isolation characteristics and growth temperature properties of the six marine *Synechococcus* strains used in this study. For more details, see the Roscoff Culture Collection (RCC) website.

Strain name	RS9907	M16.1	WH7803	ROS8604	MVIR-16-2	MVIR-18-1
RCC strain number	2382	791	752	2380	1594	1682
Phylogenetic clade [1]	II	II	V	I	I	I
Isolation area	Red Sea	Gulf of Mexico	Sargasso Sea	English Channel	Southern Norway Sea	Southern Norway Sea
Isolation latitude	29°28' N	27°42' N	33°45' N	48°43' N	60°19' N	61°00' N
Isolation longitude	34°55' E	91°18' W	67°30' W	3°59' W	3°29' W	1°59' E
Mean annual water temperature (°C)[2]	24.1 ± 0.6	25.8 ± 0.3	22.7 ± 0.2	13.6 ± 0.3	10.3 ± 0.2	10.2 ± 0.2
Annual water temperature amplitude (°C)[2]	5.5 ± 0.7	6.9 ± 0.9	5.9 ± 0.5	5.3 ± 0.6	3.1 ± 0.4	4.4 ± 0.4
Temperature range for growth (°C)	18 - >35[3]	18 - >35[4]	16 - 34[4]	16 - 30[4]	<12 - 25[4]	<9 - 25[4]
Optimal growth temperature (°C)	30 – 32[3]	32[4]	33[4]	26[4]	22[4]	22[4]

[1] According to Fuller *et al.* (2003), Mazard *et al.* (2012) and Pittera *et al.* (2014) [2] Average temperature and temperature amplitude at the strain isolation sites (resolution: 5x5° squares) over the 2004-2014 period, as derived from satellite data from the National Oceanic and Atmospheric Administration (NOAA) [3] This study [4] According to Pittera *et al.* (2014).