Discrimination of Two Phycoerythrin-Pigment Types of *Synechococcus* and Their Seasonal Succession in the Uwa Sea

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We identified phycoerythrin-pigment phenotypes of *Synechococcus* strains in samples obtained from the Uwa Sea, and investigated the seasonal changes in the pigment-type composition of the *Synechococcus* community in this sea from January 2001 to March 2002. Two *Synechococcus* strains (UT01 and UT02) with different flow cytometric signatures were isolated by sorting and subsequent culturing. Excitation spectra showed that these two strains possessed different ratios of phycourobilin (PUB) to phycoerythrobilin (PEB). Flow cytometric signatures of the two types were also different; UT01 showed a higher green to red fluorescence ratio than UT02. The ranges of green to red fluorescence intensity of the strains did not overlap, though the strains were cultured under various light intensities (7–160 $\mu\text{E m}^{-2} \text{ s}^{-1}$). In seawater samples, we could divide *Synechococcus* into lower- and higher-PUB types based on the ratio. There were two peaks of total *Synechococcus* abundance in June ($2.8 \times 10^5 \text{ cells ml}^{-1}$) and August ($1.6 \times 10^5 \text{ cells ml}^{-1}$) in the upper layer at a depth of 0 to 15 m. Major types during these two peaks were different; the higher-PUB type contributed 75.6% to the total *Synechococcus* community in June, and the lower-PUB type, 58.6% in August. This is the first report which describes the seasonal succession of two phycoerythrin-pigment types of *Synechococcus* in a coastal sea.

Key words: *Synechococcus*, chromophore, seasonal succession, flow cytometry

Synechococcus distributed ubiquitously in oceans and coastal seas is one of the most important components of primary producers especially in oligotrophic marine environments^{12,17}. These bacteria possess phycobiliproteins (phycoerythrin, phycoerythrocyanin, phycocyanin and allophycocyanin) as light-harvesting pigments. The peak wavelength of absorbance for each phycobiliprotein depends on the composition of binding chromophores such as phycourobilin (PUB), phycoerythrobilin (PEB) and phycocyanobilin (PCB)¹⁰. Many phycoerythrin-rich *Synechococcus* strains have so far been isolated from various marine environments^{1,15,16}. They are divided into at

least three types using the ratio of PUB to PEB: a no-PUB type, low-PUB type and high-PUB type^{2,3,7}.

PUB and PEB respectively have absorption maxima at ca. 495 and 545 nm¹⁰. Glover *et al.* (1986) reported that *Synechococcus* strain WH7803, containing PUB, was able to use a low flux of blue light more efficiently for both photosynthesis and growth than could *Synechococcus* WH7805, which lacks PUB. The ratio of PUB to PEB in *Synechococcus* cells is an important phenotype which affects their photosynthesis and growth rates in coastal seas.

In previous studies, the high-PUB type dominated in the North Pacific Ocean² and the North Atlantic Ocean⁷, while the low-PUB type dominated only in coastal waters of the Arabian Sea³, North Atlantic Ocean and Pacific Ocean⁸ where the high-PUB type was also found. To understand the

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ecological role of *Synechococcus* communities in coastal seas, evaluations of pigment-type composition are important. However, information about the composition of *Synechococcus* populations in the Western Pacific Ocean is still limited. The succession of each phenotype in the *Synechococcus* community should be followed.

The aim of the present study is to clarify types of *Synechococcus* in the Uwa Sea that facing the West Pacific Ocean. First, we isolated two *Synechococcus* strains with different pigment types. Then, we separately determined cell densities of each type with a flow cytometer in the sea from January 2001 to March 2002.

Materials and Methods

The present study was carried out at a station (St. Ub) in the Uwa Sea located southwest of Ehime Prefecture, Japan (Fig. 1). The station is 500 m offshore where water depth is ca. 50 m. The water samples were collected monthly from January 2001 to March 2002 with a 6 liter Van-Dorn water sampler. Samples were taken from water depths of 0, 2, 5, 10, 15, 20, 30, 40 and 50 m. Water temperature was measured vertically with a CTD profiler (Arec Electronics, Japan). The samples were stored in a 20-liter polyethylene tank and brought to the laboratory.

From the sample collected at 10 m on 19 December, cells of *Synechococcus* were sorted with a flow cytometer (FACSVantage SE, Becton Dickinson, USA) equipped with a water-cooled Argon laser (488 nm, 1 W, Coherent, USA) using the relative intensity of green and red fluorescence under blue-light excitation and incubated under a white fluorescent lamp ($20 \mu\text{E m}^{-2} \text{s}^{-1}$) at 20°C in aged seawater enriched with NaNO_3 and Na_2HPO_4 at final concentrations of $40 \mu\text{mol N l}^{-1}$ and $2 \mu\text{mol P l}^{-1}$, respectively. We isolated

two *Synechococcus* strains by serial dilution. The strains were analyzed for their chromophore composition with a fluorescence spectrophotometer (F-2500, Hitachi, Japan) at 570 nm as a function of excitation wavelength.

The sea water samples were filtered through $2 \mu\text{m}$ pore-size polycarbonate filters to remove larger particles. The filtered seawater samples were used to make counts of each pigment type with a flow cytometer. Another portion of the water sample was filtered through a $0.1 \mu\text{m}$ pore-size membrane filter (Millipore, USA), and the filtrate was used as sheath fluid. Forward scatter, side scatter, and three fluorescence intensities (red, 675–715 nm; orange, 564–586 nm; green, 515–545 nm) were recorded for each *Synechococcus* cell. All parameters were normalized with $2 \mu\text{m}$ fluorescent beads (Polyscience Inc., USA). The beads were also used as an internal standard for the enumeration of *Synechococcus* cells⁹⁾.

Dual-beam flow cytometry has been used to discriminate between types of *Synechococcus* based on PUB and PEB fluorescence^{7,8)}. In the present study, we used a single-beam flow cytometer to distinguish two types of *Synechococcus* in a seawater sample (Fig. 3). The rationale for the discrimination is described in following paragraphs.

First, *Synechococcus* cells with higher levels of PUB emit more orange light under blue excitation. Olson *et al.* (1988) pointed out that the orange fluorescence intensity excited by 488 nm reflects relative PUB content. They also stated that phycoerythrin fluorescence intensity per cell increased with depth up to a factor of 30, apparently as a result of photoadaptation. Thus, it is difficult to discriminate between two types based only on the fluorescence intensity of the cell.

Second, the ratio of green to orange fluorescence under blue light excitation should indicate the ratio of PUB to PEB^{2,3)}. Some of the energy of excited PUB is emitted as

Fig. 1. Map of the Uwa Sea.

green fluorescence, although most is transferred to PEB and PCB^{7,16}). Toledo and Palenik (1997) showed that low- and high-PUB types could be easily identified in a mixed laboratory sample when grown under low light. However under high light, distinguishing between these types was difficult¹⁴). Thus, the difference in the ratio between the two was not great enough for the discrimination in seawater samples.

Third, the most powerful index for the discrimination of subpopulations in seawater samples was the ratio of green to red fluorescence. Types were easily distinguished based on the ratio of green to red fluorescence intensity under blue-light excitation. However, this index indicates the ratio of PUB to chl. *a* fluorescence intensity.

For reasons mentioned above, we distinguished between phycoerythrin-pigment phenotypes by gating two subpopulations with green and red fluorescence, confirming that the subpopulations are similar in size with side and/or forward scatter, and finally confirming that the subpopulation with the higher green to red ratio corresponds to that with the higher green to orange ratio.

To evaluate the ranges of green and red fluorescence intensities of the two pigment types, we cultured the strains (UT01 and UT02) under various light intensities (7, 15, 24, 40, 86 and 160 $\mu\text{E m}^{-2} \text{s}^{-1}$). After 8 days of incubation, these cultures were analyzed with a flow cytometer. We also calculated the ratio of the two strains. The ratios of each type in all seawater samples were also calculated using the results obtained from the flow cytometric analysis.

We divided the water column into two layers: the upper layer at a depth of 0 to 15 m and the deeper layer at a depth of 20 to 50 m. All the data shown in this paper are mean values of 0, 2, 5, 10 and 15 m depths for the upper layer and 20, 30, 40 and 50 m depths for the deeper layer.

Results

We cultured two strains of *Synechococcus* (UT01 and UT02) from the same water sample. Phycoerythrin-pigment type was examined using excitation spectra (Fig. 2). Peaks were obtained at 545 nm and 495 nm corresponding to PUB and PEB, respectively. Thus, both these strains possessed PUB and PEB chromophores. The peak height of PUB was lower than that of PEB in UT01, while the two were similar in UT02. The ratio of PUB to PEB in strain UT01 was similar to that in *Synechococcus* strain WH7803⁷). The ratio was lower in UT02 than high-PUB types such as *Synechococcus* strains CC9317 and CC9318 where it ranged between 1.7 and 1.9, but similar between UT02 and *Synechococcus*

Fig. 2. Fluorescence emission of cultured *Synechococcus* at 570 nm as a function of excitation wavelength.

Fig. 3. Flow cytometric signatures of *Synechococcus* cells under blue light excitation (488 nm): strain UT02 (a, b, c); strain UT01 (d, e, f); *Synechococcus* community collected at a depth of 20 m on 10 September 2001 (g, h, i). Vertical axes show orange fluorescence (a, d, g) and green fluorescence (b, c, e, f, h, i). Horizontal axes show forward scatter (a, d, g), red fluorescence (c, f, i), and orange fluorescence (b, e, h). Fluorescence beads (2 μm) were used as an internal standard.

strain WH8103 (1.1)¹¹).

The flow-cytometric signatures of the two *Synechococcus* strains in the present study were different (Fig. 3b, c, e, and f) though cell sizes of the two strains were similar (Fig. 3a, d). The orange and red fluorescence intensities of UT01 and UT02 were roughly similar (Fig. 3b, c, e, and f). However, the green fluorescence of UT02 was obviously stronger than

Fig. 4. Changes in the ratio of green to red fluorescence intensity analyzed with a flow cytometer (a), and green and red fluorescence intensities (b) of strains UT01 and UT02 incubated under different light intensities.

Fig. 5. Distribution of the ratio of green to red fluorescence intensity of the *Synechococcus* cells in natural samples analyzed with a flow cytometer. Bars under the panel show the ranges of the ratio of strains UT01 and UT02.

that of UT01. In a natural sample, we found two types of *Synechococcus* (Fig. 3h, i). The two types were more easily discriminated based on green and red fluorescence. Thus, the ratio of green to red fluorescence was considered to be a good indicator of phycoerythrin-pigment phenotypes.

Fig. 6. Seasonal changes in water temperature (a), cell density of the *Synechococcus* community (b) and the contribution of the higher-PUB type to the *Synechococcus* community (c).

The green and red fluorescence intensities increased in the cells cultured under low light (7 and $15 \mu\text{E m}^{-2} \text{s}^{-1}$, Fig. 4a). However, the ratio of green to red fluorescence intensity within each strain was relatively stable, and the range of values did not overlap (Fig. 4b, Fig. 5). The ratios of these two types in the seawater samples collected from depths with various relative-light intensities again did not overlap during the investigation period (Fig. 5). From these results, we conclude that the *Synechococcus* community in the Uwa Sea consists of two types: one with a higher PUB to PEB ratio (higher-PUB type), and the other with a lower PUB to PEB ratio (lower-PUB type). We followed seasonal changes in the cell density of each type.

Water temperature at the surface was lowest in February (16°C) and highest in August (29°C), and thermal stratification developed from April to September (Fig. 6a). Cell densities of *Synechococcus* in the upper layer of 0 to 15 m remained high ($>1 \times 10^5 \text{ cells ml}^{-1}$) during the stratified period (Fig. 6b). There were two distinct peaks of abundance in June and August. The highest density was recorded in June ($2.8 \times 10^5 \text{ cells ml}^{-1}$). Cell densities of *Synechococcus* in the

deeper layer of 20 to 50 m were also relatively high during the stratified period, but the increases were not remarkable relative to those of the upper layer (Fig. 6b). Low cell densities of *Synechococcus* were detected during the destratified period, and no difference in cell density could be found between the upper and deeper layers (Fig. 6b).

The relative contribution of the higher-PUB type to total *Synechococcus* abundance from January to June remained high (75 to 95%) except in February and March in the upper layer, and remarkably decreased from July to September with a minimum of 40% in August (Fig. 6c). It increased again from October onwards. The relative contribution in the deeper layer showed a similar seasonal pattern without the decrease from July to September. When *Synechococcus* abundance exceeded 10^5 cells ml^{-1} in June (Fig. 6b), the higher-PUB type contributed 75% to the total *Synechococcus* community (Fig. 6c). In contrast, the higher-PUB type contributed only 40% when the cyanobacterial density exceeded 10^5 cells ml^{-1} again in August (Fig. 6b, c). The highest cell density of the higher-PUB type was recorded in June (2.09×10^5 cells ml^{-1}), while the cell density of the lower-PUB type was highest in August (0.94×10^5 cells ml^{-1}).

Discussion

In coastal seas, the importance of picophytoplankton has been neglected because of the dominance of larger phytoplankton. The study of picophytoplankton in coastal waters of the Western Pacific Ocean has also been limited. In the Uwa Sea, the fraction of chl. *a* less than 2 μm in size contributes up to 80% of the total (Hirose, pers. comn.). Thus, an ecological study of *Synechococcus* is important for understanding the community structure of phytoplankton in the Uwa Sea.

We used the ratio of green to red fluorescence intensity to distinguish between the phycoerythrin-pigment phenotypes. The ratio that reflects relative intensities of PUB and chlorophyll fluorescence was relatively stable (Fig. 4). The ratio is a useful index for studying phycoerythrin-pigment phenotypes in marine environments, because most flow cytometers are equipped with a single laser (488 nm).

Both the lower- and higher-PUB types were detected throughout the study period (Fig. 6c). Olson *et al.* (1990) reported that the low-PUB type was detected at 40 out of 81 stations in coastal seas, and that 34 out of these 40 stations had both low- and high-PUB types. In the Arabian Sea, low-PUB types were more abundant at coastal stations³). In the open ocean, only the high-PUB type has been found. The dominant *Synechococcus* in the North Atlantic and East

Pacific Oceans was the high-PUB type^{7,8}). Campbell and Vaulot (1993) showed that only the high-PUB type was detected at a station off Hawaii. Thus, the coexistence of two types of *Synechococcus* might be characteristic of coastal seas. Coastal seas are probably affected by river inflow, human activities and other factors all of which would have negligible effects on the open ocean. Thus, the environmental conditions in coastal seas would be variable, and this is a possible reason for the detection of the two types.

The present study first shows that the seasonal changes in the abundance of the two types differed. The highest cell density of the lower-PUB type was found in June, while that of the higher-PUB type occurred in August. As a result, the major type during the two peaks of *Synechococcus* cell densities in June and August was different (Fig. 6b, c). In June, more than half of all *Synechococcus* abundance was accounted for by the higher-PUB type, while the predominant pigment type in August was the lower-PUB type (Fig. 6b, c). The results suggest that the optimal temperature and/or other environmental factors for growth differ for each type of *Synechococcus*.

The light environment may be one of the most important factors affecting pigment-type composition in *Synechococcus*. There is plenty of light available in the upper layer, suggesting that underwater light quality is less important in determining the dominant type of *Synechococcus*. In August and September, the lower-PUB type dominated in the upper layer, while the higher-PUB type dominated in the deeper layer (Fig. 6b, c). Similar results were obtained by Olson *et al.* (1990), who found that vertical distributions of low-PUB types were restricted to shallower depths. These results suggest that a high light intensity favors the low-PUB type. The low-PUB strain WH7803 showed a high rate of growth even under a light intensity of $2000 \mu\text{E m}^{-2} \text{s}^{-1}$ ⁵), though it is known that phytoplankton at the surface often exhibit photoinhibition under high light intensities.

In the deeper layer, the quality of light may strongly affect the pigment-type composition of the *Synechococcus* community. The high-PUB type efficiently uses blue light because of its high content of PUB, while the low-PUB type uses green light⁶). The dominant light below 15 m in the open ocean is blue light with a wavelength of 440–490 nm⁶). Wood (1985) demonstrated a high rate of photosynthesis by PUB-containing *Synechococcus* strain in the deeper layer of the Pacific Ocean. Thus, the dominance of the high-PUB type in the deeper layer may be due to the dominance of blue light in the Uwa Sea though we did not examine the light environment in the sea.

Before May 2002 and after October 2002, water tempera-

ture, total *Synechococcus* cell density and pigment-type composition of *Synechococcus* were almost the same between the upper and deeper layers (Fig. 6). For more than half the year, seawater might be vertically mixed at the station. Takeoka *et al.* (1997) reported that thermal stratification was much weaker in our sampling area than offshore probably due to stagnant tidal currents. For elucidation of the difference in the vertical distribution of each pigment type, the depth of the mixing layer and duration of stratification would be important. *In situ* incubation experiments and field observations should clarify the relationship between the light environment (light intensity and quality) and the pigment-type composition of *Synechococcus* in the sea.

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