REVIEW ARTICLE

Molecular ecology studies of marine *Synechococcus* *

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Abstract Cyanobacteria of the genus *Synechococcus* is a dominant component of microbial community in the world's oceans, and is a major contributor to marine primary productivity and thus plays an important role in carbon cycling in the oceans. Besides the ecological importance, the cultivability also made *Synechococcus* a very special group of marine microorganisms, which has attracted great attention from oceanographers and biologists. Great progress in the physiology, biochemistry and phylogeny of *Synechococcus* has been made since its discovery. We here review the current status of molecular ecology of marine *Synechococcus* and give a perspective into the future based on our understanding of the literature and our own work.

Keywords: *Synechococcus*, molecular ecology, 16S rRNA.

1 The importance of molecular ecology studies of *Synechococcus*

About 71% of the global surface is covered with the oceans, where live about up to 80% organisms in the world. Among these organisms, the oxygenic photoautotroph of the genus *Synechococcus*, discovered in 1979, is one of the most representative genera of marine cyanobacteria[1,2]. This tiny (0.5~2 μm), unicellular, and rod-shaped to coccoid microorganism, can be easily identified by its intense orange phycoerythrin (PE) fluorescence. *Synechococcus* spp. are one of the predominant components of the picophytoplankton and are ubiquitous in all marine environments, especially in inshore or coastal ecosystem, where their abundance ranging from 10^3 to 10^5 cells/mL[3]. They are also one of the major contributors to the carbon fixation and to the primary production on a global scale. Scientists estimate that *Synechococcus* and *Prochlorococcus* (the closest relatives of *Synechococcus*) remove about 10 billion tons of carbon from the air each year as much as two-thirds of the total carbon fixation that occurs in the oceans. Besides, the biomass of *Synechococcus* cells circulates rapidly and the energy transition efficiency is high in the marine micro-food webs, thus they are one of the most important food sources for microzooplankton[4]. Recently, the genome of one representative *Synechococcus* strain has been decoded[5], which is one of the first declared marine bacterial genomes. The comparative genomic analysis between *Synechococcus* and *Prochlorococcus* indicated that *Synechococcus* can fulfill more chemical reaction and is more nutritionally versatile, and thus has a broader distribution compared with *Prochlorococcus*. One of the surprises from the analyses of the genome of *Synechococcus* is the prediction that *Synechococcus* can use some new organic compounds as nitrogen and phosphorus sources, this probably means that we need resurvey the metabolizing manners of these compounds in oceans.

Since *Synechococcus* plays an important role in the marine ecosystems, the investigation of their community structure, species composition, phylogenetic development, and their relationship to the natural environments and other ecological issues will not only help us have a better understanding of their roles in the ecosystem, but also facilitate us studying the global climate change, exploring recyclable energy sources and understanding conservation of biodiversity. Furthermore, as *Synechococcus* cells are relatively simple in structure, easier to culture than *Archaea* and *Prochlorococcus*, and have a great diversity of

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genotypes, ecotypes, and physiological varieties, they become an excellent model organism for studying the ecology of picophytoplankton.

The traditional methods in microbial ecology studies rely basically upon pure cultivation and microscopic observation, and are confined to morphological, physiological and biochemical characterization. These methods are difficult to get reliable results for the community structure and phylogenic analysis since many natural microbes are uncultivable[6] and many ecologically important ecotypes or genotypes may be missed during the isolation and cultivation processes, leading to the underestimation of the biodiversity of marine microbes[7]. Furthermore, the previous work[8] has demonstrated that the cultivated species were different from naturally occurring species, so cultivated bacteria are less valuable in understanding the ecology of bacterial communities.

The development of molecular biology techniques has led our studies of microbial ecology to a new era. The application of these techniques allows us to identify microorganisms directly from the environmental samples without the need of prior cultivation and isolation, and enables us to explain the mechanism of the species diversion at a molecular level.

2 Current status of molecular ecology study on *Synechococcus*

2.1 The approaches to molecular ecology studies of *Synechococcus*

The community structure of *Synechococcus* is one of the important issues of molecular ecology of *Synechococcus*. The routine procedure for studying community structure is as follows: the total DNA or RNA is extracted from isolates or directly from the environmental samples, then a given gene sequence is amplified by PCR. PCR products are used for library construction or for denaturing gradient gel electrophoresis (DGGE) analysis. After sequencing of the positive clones or the DGGE bands, a comparative analysis of the retrieved sequences is performed, and a phylogenetic tree is established. Two main approaches have been used for this purpose. One is culture-based, this is a method of cultivating *Synechococcus* by the traditional method and then manipulating them by molecular techniques. The advantages of this method is that we can acquire physiological data of the isolates, and the molecular manipulation and the results analysis are relatively easier; and by this method, we can design some specific probes, primers and markers with particular physiological information for molecular ecological analysis[9]; also the pure cultured isolates can be used for genomic sequencing. But, some strains are difficult to culture and the cultured strains cannot fully reflect the biodiversity of the environmental samples. The second approach is *in situ* studies. The DNA is extracted directly from collected environmental samples and used for subsequent studies. This approach may avoid the disadvantages of the first approach, but it requires high universal and specific primers, otherwise some undesired sequences may be amplified whereas those target strains escaped the detection during PCR amplification, making the results very complicated to be intepreted. Besides, PCR methods are sometimes inefficient in amplifying those minor components in natural environments while these strains are not always unimportant in ecosystems.

2.2 The genetic markers in phylogenetic studies of *Synechococcus*

At present, the genetic marker for *Synechococcus* community structure and phylogeny analysis is mainly small subunit ribosomal RNA (16S rRNA) gene of prokaryotes. Although there are not many kinds of rRNAs, they account for about 80% of the total RNA in bacteria, and their length (about 1.5 kb) is suitable for sequencing. In addition, they are universal and well-conserved in all prokaryotes. Now large data sets of 16S rDNA are available in GenBank, and they have been used widely to establish evolutionary relations among various species. However, 16S rRNA genes vary very little in their length as well as in their sequences, and they always have up to 99.6% sequence identity at the species level, so it is only appropriate for identifying the relationship between or above the genus levels but not at the species levels. On the other hand, sequence heterogeneities due to multiple copies of rrn operons within single genomes of bacteria may complicate the interpretation of sequence data or DGGE band patterns, particularly when samples were retrieved from natural microbial communities.

2.3 Genetic diversity of *Synechococcus*

Synechococcus spp. were clustered together in a general group by traditional classification according to their morphological observations (unicellular, rod-

shaped to coccoid organisms), but the physiology and molecular ecology data indicated that they are polyphyletic but not a natural taxon, they likely comprise organisms originating from several evolutionarily distinct and deeply branching groups. At present, according to their 16S rDNA sequences combined with their major light harvesting accessory pigment profiles, growth requirements and their ability to carry out swimming motility, *Synechococcus* can be classified as the following groups[10~14]: The MC-A (marine cluster A) group, containing phycoerythrin (PE) and having an elevated salt (Na^+, Cl^-, Mg^{2+}, and Ca^{2+}) requirement for growth, is abundant within the euphotic zone of both open-ocean and coastal waters, and its G+C content is about 55%~62%. The MC-B (marine cluster B) group, possessing phycocyanin (PC) but lacking PE, often appears in coastal waters, and the members in this group have a relatively higher G+C content of about 63%~69.5%. The third group, MC-C (marine cluster C), also possessing PC but lacking PE, is often distributed in brackish or coastal marine waters, and has a distinguished low G+C content of 47.5%~49.5%, this group has been poorly studied so far and the number of the *Synechococcus* isolates obtained is small.

In studies of Honda et al.[10] five *Synechococcus* strains belonging to "MC-B" and "MC-C" were further divided into three lineages based on their 16S rDNA sequence, and some strains were independent and loose in the phylogenetic tree. Robertson et al.[15] analyzed 16S rDNA sequences from 14 *Synechococcus* isolates, and the *cpc* (phycocyanin-encoding) gene sequence from 38 *Synechococcus* isolates, including the intergenic spacer (IGS) between *cpcB* and *cpcA* and the corresponding flanking regions (*cpcBA*-IGS), their results indicated that the members of the genus *Synechococcus* were affiliated to three of seven deeply branching cyanobacterial lineages, but some strains do not appear to be associated with any of these lineages, they were only loosely affiliated to a cyanobacterial lineage in which no other *Synechococcus* strains were found, thus *Synechococcus* should be reclassified into several independent genera. The previous studies[16,17] based on *rpoC1* gene (encoding the γ subunit of RNA polymerase) and 16S rRNA gene sequences and the RFLP patterns also indicated that although the *Synechococcus* are morphologically similar, they are not a natural but a polyphyletic taxon. The high genetic diversity also reflects that *Synechococcus* is an old genus.

So far, studies on genetic diversity of *Synechococcus* are basically focused on a few strains isolated from a few oceanic regions, data directly from environmental samples are very limited. Therefore, classification of *Synechococcus* species is unclear, and we are still far from obtaining a complete understanding of the genetic diversity within marine cyanobacterial (*Synechococcus*) populations.

3 Prospects on molecular ecology of marine *Synechococcus*

3.1 Flow cytometry combined with molecular biology techniques for ecological studies on *Synechococcus*

Flow cytometry (FCM) is one of the most advanced techniques used in marine microbial ecological studies. *Synechococcus* can be easily sorted from phytoplankton using FCM according to its small size and unique intense orange phycoerythrin fluorescence so that the environmental samples can be analyzed directly. Recently, we sorted axenic *Synechococcus* cells from natural ocean waters, and then enriched the cells with culture solution for pure culturing. By using DNA extracted from the cultures or using cells directly sorted from natural samples as PCR templates, we acquired target PCR products (Fig. 1). Compared with above mentioned approaches, this method has many advantages. Firstly, the sampling process is simplified (seawater filtrating is unnecessary) and a small amount of cells (sorted from several milliliters of sea water) is enough for PCR amplification, and the sorting is very fast (up to 10^2~10^3 cells/s). Secondly, by FCM sorting, we can acquire some pure strains and those very minor component strains in natural environments that are hard to be obtained by a routine isolating route. This method also simplifies the analysis of the results from complicated samples and decreases the unwanted amplification. For physiologically and ecotypically different strains, such as those containing different pigment compo-

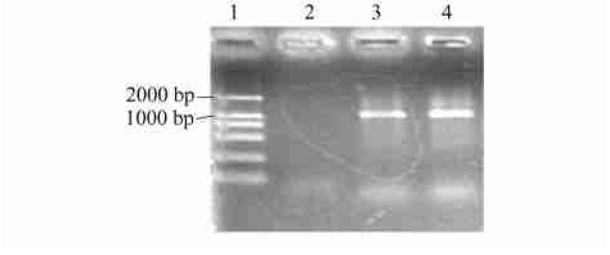

Fig. 1. PCR amplification products from *Synechococcus* cells sorted by FCM. Lane 1, DL-2000 Marker; lane 2, H_2O (negative control); lanes 3 and 4, FCM-sorted *Synechococcus* cells.

nents and pigment contents, they can be discriminated roughly by FCM. Therefore, it is a promising and an alternative method for current culture studies and *in situ* studies.

3.2 Multiple genetic markers for studies on *Synechococcus* genetic diversity

Compared with the mostly used 16S rRNA gene, the internal transcribed spacer (ITS) located between the genes encoding mature 16S and 23S rRNAs is more variable in length as well as in sequence, and thus is better for resolving the relationship between closely related species. For example, PCR amplicons of the ITS of the rrn operon of three axenic strains of *Synechococcus* differ greatly in length from that of one axenic *Prochlorococcus* marinus subsp. although these four cyanobacterial clusters are closely related in phylogenetic trees inferred from 16S rRNA gene sequences. Thus, size, sequence data and RFLP of the ITS amplicons will therefore be valuable markers for identification of different *Synechococcus* (or *Prochlorococcus*) genotypes and for their discrimination from other cyanobacterial relatives with which they often co-exist in oceanic ecosystems[18]. Rocap et al.[19] did a similar study in 2002, and also found dramatic variation in the length and G+C content of the ITS among the *Synechococcus* strains, based on which they differentiated the genetically closely related (show greater than 96% identity in their 16S rDNA sequences) but physiologically widely diverse strains (different pigment components or growth responses to light and nutrients). We amplified both the 16S rDNA and 16S-23S ITS sequences of some physiologically diverse *Synechococcus* strains using cyanobacteria-specific primers, our results indicated that all the two green inshore strains and one red open-ocean strain have the same length of PCR products of 16S rDNA, but not all the strains have the same ITS length (Fig. 2), which also indicated that the 16S-23S rDNA is better than 16S rDNA in species classification. The fact that two green inshore strains have the same ITS length, and they both are different from the red open-ocean strain reflected that, to a certain extent, there existed congruence between genotypes and ecotypes or physiological characteristics.

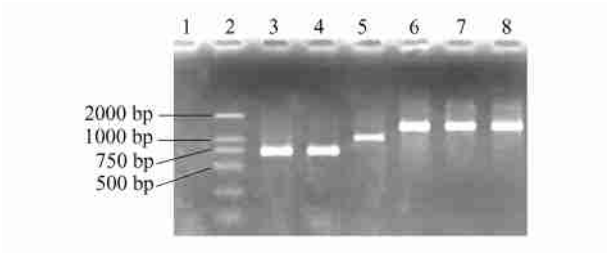

Fig. 2. PCR amplification of 16S rDNA and ITS region of different *Synechococcus* strains. Lanes 3~5, PCR amplicons of the ITS regions; lanes 6~8, amplicons of the 16S rDNAs. Lane 1, H_2O (negative control); lane 2, DL-2000 Marker; lanes 3 and 6, *Synechococcus* sp. CCMP 1379 (green inshore strain); lanes 4 and 7, *Synechococcus* sp. CCMP1630 (green inshore strain); lanes 5 and 8, *Synechococcus* sp. CCMP839 (red open-ocean strain).

Some less conserved protein-coding genes show more advantages than 16S rDNA and the ITS region in discriminating closely related groups or species. In general, functional genes are more variable in function and structure than rRNA genes, and they can provide more genetic information in species determination within genus. For example, the 16S rRNA gene differs by 1.4% between *Synechococcus* isolates WH7805 and WH8103, while the *rpoC1* fragment differs by 17%[20]. In addition, functional genes are thought to exist in single copy in prokaryotic genomes, and thus can avoid an overestimation of the biodiversity within natural community resulting from the sequence microheterogeneity of 16S rRNA genes. Furthermore, using protein-coding genes to study genetic diversity in environmental samples may decrease some nonspecific amplification, simplify the complexity of environmental samples, and thus make the results more reliable. For example, by using *cpc* gene, we can avoid many non-cyanobacterial products, and by using *rbcL* (ribulose bisphosphate carboxylase/oxygenase form I) gene, we can obviate the disturbance of heterotrophic bacteria. Recent work in our laboratory attempted to amplify *rpoC1* gene fragments from the environmental samples collected from the South China Sea using cyanobacteria-specific primers, and more than 90% of the acquired PCR products were *rpoC1* gene fragments of *Prochlorococcus* and *Synechococcus* (unpublished data). But, we should note that the available sequences for functional genes are rather limited, which will make it difficult for specific primer design and the subsequent sequence comparison. However, as functional genes are being more and more used in the phylogenetic analysis, more and more strains are being sequenced, and database of functional genes are being enriched, func-

tional genes will surely be more and more used as a powerful tool in phylogenetic analysis in the near future.

More attention in the future should be paid to the application of the high variable ITS region of 16S-23S rRNA and functional genes to the phylogeny of different species within *Synechococcus* genus. Besides, since application of different gene loci to the study of same batch of *Synechococcus* isolates or samples from the same environment may cause difficulties in direct comparison of different data, the 16S rRNA, ITS region, and various functional genes should be combined in studying the same samples, which will not only allow the different data to support each other, but also offset the limitations of single genetic marker, and obtain a more reliable conclusion, and thus is a comprehensive approach and a trend direction for future studies.

3.3 Relationship between the genetic diversity and the physiological and ecotypical adaptation

Does the genetic diversity of marine *Synechococcus* correlate with specific physiological adaptation or with different ecotypes? Can these different physiological characteristics or ecotypes be used as classification markers? These concerns are important issues to be addressed in molecular ecology studies. As *Synechococcus* cells are relatively easier to culture than other picoplankters, and they possess rich physiological traits and ecotypes, and differ from each other physiologically in a number of ways, they are perfect materials for understanding these questions.

It was reported[16] that a total of 15 *Synechococcus* strains isolated from different depths of the California Current could be divided into two major genetic lineages by *rpoC1* gene fragment sequences, the California Current low-phycourobilin (CCLPUB) and the California Current high-phycourobilin (CCHPUB) groups. The six isolates of the CCLPUB group were closely related, while the latter group was more diverse and was subdivided into three relatively divergent lineages. It should be noted that, both the CCHPUB and the CCLPUB groups cover strains obtained from surface (5 m) and deep (95 m) samples. Thus, although the pigment contents had some correlation to the genetic diversity, there was no clear correlation between sampling depth and isolation of genetic groups, and the genetically divergent *Synechococcus* groups can coexist in the same seawater sample. Whereas using a new spectrofluorometric assay method, Lantoine et al.[21] quantified PEs and found that, in open oceans, the phycourobilin/phycoerythrobilin (PUB/PEB) ratio increased with the depth and with the distance from the coast, indicating that the PUB content was correlated with depth.

In 1999, Toledo et al.[14] studied the *rpoC1* gene fragment sequences of a set of marine *Synechococcus* isolates that were able to swim. Their results showed that these isolates were closely related and formed a monophyletic group, indicating an example of correspondence between a physiological trait and a phylogenetic group in marine *Synechococcus*, but the PUB/PEB pigment ratios of members of the motile clade varied considerably. An isolate (strain CC9703) displayed a pigment signature identical to that of nonmotile strain WH7803, which is considered a model for low PUB/PEB ratio strains, whereas several motile strains had higher PUB/PEB ratios than strain WH8103, which is considered a model for high PUB/PEB ratio strains. This indicated that the PUB/PEB pigment ratio is inconsistent with the phylogenetic position and is not a useful marker for phylogenetic analysis at least in motile group. At present, the genome of one motile strain WH8102 has been annotated[5], which will further hasten our understanding of the relationship between genetic properties and physiological adaptations.

The *in situ* analysis of phycobiliprotein-associated fluorescence characteristics of environmental samples using flow cytometry by Olson et al.[22] have shown that populations with low PUB/PEB ratios tend to dominate in mesotrophic or coastal green waters, whereas high PUB/PEB ratios are common in oligotrophic areas, which is consistent with our observation. But similar to the above results inferred from the pure culturing, the pigment components have no certain correlation to the phylogenetic positions of strains. Generally, we know that strains do not alter their PUB/PEB ratio in response to light quality, but one study[23] showed that indeed some strains are capable of chromatic adaptation, increasing their PUB/PEB chromophore ratio when growing under blue light. The latter trait seems to present phylogenetic coherence amongst those non-motile strains so far studied but not within motile strains where both adapters and non-adapters exist. These results suggested that different pigment compositions in *Synechococcus* strains may result from their adaptation to

different niches, and the difference may be exhibited only by ecotypic diversity but not by genetic diversity, or sometimes, in some strains, a long period physiological variation may result in corresponding genotypes.

It has been revealed[24] that *Synechococcus* populations inhabiting at different depths of the Pacific Ocean could be divided into eight or more clades, thereinto clades 2 and 8 might represent surface-adapted organisms, which correspond to their surface oceanic niches. In another study[19], 25 MC-A *Synechococcus* isolates from around the globe were partitioned into six clades according to their 16S-23S rDNA ITS sequence variations, three of which were associated with a particular phenotype (chromatic adaptation, low content or lack of phycourobilin, and motility). The studies also indicated that many of the clades consist of strains isolated from disparate regions of the world's oceans, implying that the *Synechococcus* are geographically widely distributed, and that genetically related natural communities consist of multiple coexisting ecotypes.

Other physiological differences among genetically related strains include differences in the responses to nitrogen depletion[25], in preferences for nitrate or urea for growth[26], in cell size and growth rate[11], and in cell cycle behavior[27] and so on. For example, the *Synechococcus* strains WH7803 and WH7805 show contrasting abilities to utilize urea and differences in their cell cycle behavior but are phylogenetically closely related. Although only a few strains have been analyzed with regard to these physiological properties, these studies suggested that there is no inevitable phylogenetic correspondency with physiological properties. Perhaps this is not so surprising, it may be just the physiological function variation endowed different *Synechococcus* strains the adaptive capacity to different environments (light, temperature, nutrients), or different ecotypes of *Synechococcus* developed in order to adapt to different environments during the natural selection processes (despite that their heredities did not change much).

So far, we are still far away from a complete understanding of the relationship between genetically and physiologically diversity of *Synechococcus*, and existing studies have only focused on some strains of limited oceanic areas, with the results being not very consistent with each other. However, these questions are very attractive and also are in urgent need of elucidation in *Synechococcus* molecular ecological studies. Answers to these questions will no doubt give a better understanding of the function of *Synechococcus* in oceanic environments. In order to better elucidate the relationship between genetically and physiologically divergence within *Synechococcus* genus in the future, new isolation strategies are required for obtaining more isolates and the physiological and genetic characteristics of the model strains should be well investigated. Meanwhile, with more and more decoded genomic information, more physiologically related specific probes should be designed for studying the natural distribution of different physiological and ecotypical adaptation and their relationship to genetic diversity. Moreover, multiple genetic markers should be used for corroborating each other.

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