Pigment Compositions and Phylogenetic Positions of Filamentous Bacteria Coexisting in Marine Microalgal Cultures

TAKESHI YOSHIKAWA^{1*}, KOZO IWAMOTO¹ and TAIZO SAKATA¹

¹ Laboratory of Microbiology, Department of Applied Biochemistry and Technology of Aquatic Resources, Faculty of Fisheries, Kagoshima University, Shimoarata 4-50-20, Kagoshima, 890-0056, Japan

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The sequences of small subunit ribosomal RNA gene (SSU rDNA) and pigment composition were examined for the five filamentous bacteria isolated from the unialgal cultures of marine microalgae *Chattonella marina*, *Heterosigma akashiwo*, or *Tetraselmis* sp. The SSU rDNA data suggested that the filamentous bacteria were clustered into the same species, which belonged to the *Roseobacter* group in α -proteobacteria. Sequence similarities of SSU rDNA to each of the other strains were more than 99%. The strains possessed bacteriochlorophyll *a*, but were different in carotenoid composition: four of the strains contained a carotenoid that was similar to spheroidenone but the other one did not.

Key words: filamentous bacteria, marine microalgae, bacteriochlorophyll *a*, SSU rDNA, aerobic photosynthetic bacteria

We have previously reported that some bacteria in multicellular filament form were predominant in the unialgal cultures of raphidophytes *Heterosigma akashiwo*, *Chattonella marina*, and a prasinophyte *Tetraselmis* sp.^{12,14} The bacteria formed rhizoidal colonies that were whitish pink or greenish yellow on agar plate media. The whitish pink filamentous bacteria enhanced the growth of *C. marina* cells¹³. Although the phenotypic characteristics of the filamentous bacteria were similar to those of the genus *Flexibacter*, DNA/DNA homologies of the bacteria to the species of *Flexibacter* were very low¹². In this report, we examined the sequences of the small subunit ribosomal RNA gene (SSU rDNA) and the pigment composition of the filamentous bacteria to elucidate the phylogenetic positions of the filamentous bacteria.

The whitish pink filamentous bacterial strains, FI90-32, FI93-3, FI95-1, and FI96-1, were isolated from the unialgal cultures of raphidophytes, *C. marina*, *H. akashiwo*, and a prasinophyte *Tetraselmis* sp., and the greenish yellow bac-

terial strain, FI95-5R (formerly termed FI95-5), was isolated from *H. akashiwo*^{12,14}. Carotenoids were extracted from the bacterial cell pellets with ethanol for 24 h at 4°C. The extract was shaken with a twofold volume of a mixture of acetone/*n*-hexane (2 : 1). The hexane phase was washed 10 times with water, and evaporated on dried anhydrous sodium sulfate. The pigment was re-dissolved in petroleum ether. Bacteriochlorophyll (bchl) was extracted according to the method described by Shiba *et al.*⁹. The absorption spectra of the pigments were determined using a spectrophotometer, MPS-2000 (Shimadzu, Japan).

DNA was extracted according to the method of Sukoso *et al.*¹⁴. Polymerase chain reaction (PCR) amplification of SSU rDNA was carried out based on the method previously reported³, with a pair of the oligonucleotides³. PCR-amplified DNA fragments were subjected to the restriction fragment length polymorphism (RFLP) analysis with *AluI*, *BsuRI*, *MspI*, and *SmaI*. The nucleotide sequences were also determined in the products cloned into a plasmid vector, pT7Blue (R) (Novagen, USA), with the genetic analyzer ABI PRISM 310 (PE Biosystems, USA) using the BigDye Terminator Cycle Sequencing Kit (PE Biosystems, USA).

The accession number of FI90-32 sequence in DDBJ is AB046591. Multiple sequence alignment, genetic distance

*Corresponding author; E-mail: yoshi@fish.kagoshima-u.ac.jp, Tel: +81-99-286-4192, Fax: +81-99-286-4192

Abbreviations: bchl, bacteriochlorophyll; RFLP, restriction fragment length polymorphism; PCR, polymerase chain reaction; SSU rDNA, small subunit ribosomal RNA gene.

Fig. 1. Phylogenetic relationship inferred from the SSU rDNA sequence similarities. The phylogenetic tree was constructed using the neighbor-joining method⁸⁹. The bootstrap values are indicated at the branching points. The phylogenetic groups of the α -proteobacteria are also shown.

Fig. 2. Absorption spectra of methanol extract (a) and carotenoid fraction (b) of the cells of the filamentous bacterial strains FI90-32 and FI95-5R. The carotenoid fractions were dissolved in petroleum ether. The wavelengths of the absorption maxima are indicated in the figures.

calculations with Kimura's 2-parameter method⁵⁾, bootstrap resamplings²⁾, and phylogenetic tree constructions by Nei and Saitou's neighbor-joining method⁸⁾ were performed by the program Clustal W version 1.8¹⁵⁾.

The PCR-amplified SSU rDNA of the five strains gave indistinctive RFLP patterns (data not shown). The SSU rDNA nucleotide sequences of FI90-32, FI95-5R, and FI96-1, whose lengths were 1274 bp, corresponding to the *Escherichia coli* numbers 49–1400, also showed almost the identical sequences and their similarity values were more than 99%. Therefore, we speculated that the five strains were part of the same species.

The phylogenetic tree in Fig. 1 showed that the filamen-

tous bacterial strains were closely related to the *Roseobacter* group containing the genera *Roseobacter*¹¹⁾ and *Roseovarius*⁶⁾, which indicates the close phylogenetic relationship between the filamentous bacteria and the bchl *a*-containing aerobic photosynthetic bacteria¹⁰⁾.

Figure 2a shows absorption spectra of the crude methanol extract of the filamentous bacterial cells. The spectra of FI90-32 and the other three strains show three absorption peaks at 768 nm, around 500 nm, and 364 nm. The peaks at 768 and 364 nm are characteristics of bchl *a*⁷⁾, and the peak around 500 nm seems to indicate the presence of carotenoid. The spectrum of FI95-5R is similar to that of FI90-32 in the peaks at 768 and 364 nm but different in the peak at around 500 nm. Hence, we speculated that all of the filamentous bacteria contained bchl *a*.

Possible carotenoid fraction was purified by the partition between ethanol and the mixture of acetone/*n*-hexane (2:1). The absorption spectra obtained for the hexane fraction indicated only the peaks around 500 nm but not the peak at 364 nm or 768 nm (Fig. 2b). The absorption spectra of the major pigment of the whitish pink bacteria in petroleum ether indicated three peaks at 455, 478, and 509 nm. These absorption maxima coincided with the major carotenoid of *Rhodobacter sphaeroides* IFO12203 (455, 478, and 509 nm) and were similar to spheroidenone (460, 483, and 515 nm in light petroleum¹⁾ or petroleum ether⁴⁾). On the other hand, the greenish yellow strain FI95-5R showed no similarity to the others: the spectrum of the pigment from FI95-5R had the absorption maxima at 410, 432, and 463 nm (Fig. 2b).

In conclusion, all of the whitish pink and greenish yellow filamentous bacteria, which were predominant in the cultures of *H. akashiwo*, *C. marina*, or *Tetraselmis* sp., belonged to a species that was included in the *Roseobacter* group in α -proteobacteria. The sequence similarities of SSU rDNA to each of the other strains were more than 99%. Although the carotenoid spectra of the whitish pink and the greenish yellow bacteria were different from each other, the phylogenetic meaning of the difference cannot be elucidated without information on the chemical structure. We have not yet obtained any data on the photosynthetic activity of the filamentous bacteria, although it has been confirmed that the bacteria contained bchl *a*.

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