Physiological and Molecular Biological Studies on Co-existent Filamentous Bacteria Isolated from the Culture of Red Tide Microalgae

(赤潮藻類培養液から分離した糸状細菌の生理学的および分子生物学的研究)

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General Introduction

1.1 General introduction

Red tides of noxious microalgae such as *Chattonella marina*, *Heterosigma akashiwo*, *Gymnodinium mikimotoi* have frequently occurred on a large scale in coastal waters of Japan, causing damages of aquacultured and wild fishes and creating big social and environmental problems. The large blooms of *C. marina* and *H. akashiwo* especially occurred in Kagoshima Bay in 1985 and 1995, respectively.

Although the studies on blooming plankton in Japan have been started in 1899, when Tokichi Nishikawa reported the bloom of *Noctiluca miliaris* and *Gonyaulax polygramma* outbreak in Shizuoka and Mie Prefectures, respectively, but intensive studies to solve red tide problems all over the country started only after the great bloom of *G. mikimotoi* occurred in Ohmura Bay, Nagasaki Prefecture in 1966.

To date, various species of red tide microalgae have been isolated and identified from around coastal waters of Japan, and a great deal of studies have centered on the physical and chemical factors that influence the growth of red tide microalgae, such as temperature, light intensity, salinity and nutrients. On the other hand, less information is available on the relationship between algae and bacteria, that can utilize algal extracellular products, stimulate or inhibit algal growth.

The important roles were suggested by co-existing bacteria, when they had shown their capability to enhance the algal growth in mixed-cultures. Studies indicated that the addition of the co-existent bacteria to the culture of *Chattonella marina* axenic strain resulted in the increase of algal cell yield and extension of algal growth period as compared with those in the algal axenic culture. However, it is suggested that the co-existent bacteria provide some nutrients for algal growth.

In the present study, needle-like filamentous bacteria were chosen among co-existent bacteria to clarify the role of bacteria on microalgal growth. The nutritional inter-dependence among bacteria and microalgae was clarified by detecting vitamin B_{12} produced by co-existent bacteria. Moreover, the characteristics of filamentous bacteria were investigated by conventional and molecular identification methods.

1.2 Review of literature

1.2.1 Microalgae-bacteria interactions

Generally, all living organisms are dependent upon the surrounding organisms, and rarely found as pure cultures. Most natural environments are characterized by a great diversity of microbial species interacting in complex ways and taking highly varied forms of mutual relationship.

Interactions between bacteria and algae in aquatic ecosystems have been reviewed by Cole (1982). Furthermore, the "interactions" remain to be an interesting point in aquatic ecology, since the phenomenon of interactions have the specific characteristic for each species, especially in the term of food web.

The algal blooms usually occur in the surface of water layer, which is characterized by the abundance of sunlight and dissolved oxygen. On the other hand, the positive-phototaxis of *Chattonella marina* should be as one of the important points to motivate the bloom process, especially after algal cells reach the surface layer and contact with other factors which are able to support the algal cells to grow. Tamori, *et al.*, (1991) reported that the initial appearance of *G. nagasakiense* occurred in the western and southern coastal waters of Suo-nada of which environmental conditions were characterized by high water temperature, low salinity and distribution of oxygen-deficient water in the bottom layer.

Concerning marine bacteria, Imai (1989) reported that the majority of bacteria distributing in the ocean ecosystems is heterotrophic and using dissolved organic matters as nutrition and energy sources. In the same time the increased use of these dissolved organic matters will result in their decomposition to inorganic matters. Furthermore, Ietswaart *et al.*, (1994) explained that the major trophic levels in marine pelagic food webs are due to the phytoplankton and heterotrophic bacteria, and in this relationship, phytoplankton or algae are the primary producers of organic matters, while the bacteria are the main consumers.

Heterotrophic bacteria tend to be associated with algal surfaces or detrital particles, which offer a nutritional advantage, compared to the extremely low concentrations of dissolved organic nutrients in the pelagic sea water (Atlas and Bartha, 1986). Red tide microalgae such as *Chattonella marina* produce various kinds of extracellular substances including toxic substances causing mass mortality of cultured and feral fishes (Imai and Itoh, 1987; Yamaguchi, Imai, and Honjo, 1991) and some organic compounds that were used by microorganisms as nutrients. This relationship was described for phycosphere as the zone surrounding algal cells within which microorganisms are influenced by extracellular organic compounds (EOC) from algal cells (Cole, 1982). The term of phycosphere has been used to focus attention on the possible responses of bacteria to an algal-mediated environment (Bell and Mitchell, 1972).

Interactions within microbial populations can be divided as positive and negative interactions. The positive interactions within a population are called cooperation and negative interactions are called competition (Atlas and Bartha, 1987). The positive interactions increase the growth rate of a population whereas the negative interactions have the opposite effect. Moreover, interactions between diverse microbial populations can be divided as neutralism, commensalism, synergism (protocooperation),

mutualism (symbiosis), competition, amensalism, predation, and parasitism.

Bacteria and microalgae are numerically dominant organisms in the lakes and oceans. Microalgae have a role as primary producers, while the role of bacteria may stimulate or inhibit algal growth through decomposition of organic compounds, endosymbiosis and production of stimulative or inhibitory substances. Furthermore Atlas and Bartha (1987), remarked that the cooperative interaction within populations can be particularly important when the population is utilizing insoluble substrates such as lignin and cellulose and acting as a protective mechanism against hostile environmental factors.

The relationship between bacteria and algae can occur temporarily or permanently, within or in the surface of algal cells. This relationship can be caused by the physical support or nutritional necessity, or both of them. On the basis of nutritional necessity, the possible interactions between microbial population can be recognized as negative interactions (competition and amensalism); positive interactions (commensalism, synergism, and mutualism); and interaction that is positive for one but negative for the other population (parasitism and predation).

As a part of components in marine microbial food webs, the activity of algae is always followed by that of other microorganisms. Bratbak, *et al*., (1990) reported, that the culmination of the diatom bloom was followed by a peak in the concentration of bacteria and an increase in the concentration of heterotrophic flagellates. This pattern was consistent with the concept of a food chain from photosynthetically produced organic materials, through bacteria, to bacterivorous flagellates. In this concept, the kinds of bacteria that affect the growth of algae depend on the location where the algal cells exist. Since the blooms take place on water

surfaces, the bacteria that affect the growth of algae are able to adapt to water surfaces (Berman and Zohary, 1989).

Usually the bacteria associated with algae exist in the slime of capsule of algae, and apparently the slime serves as a nutrient for them. However, in other cases, the bacteria only exist in populations of algae which are stagnating or declining, whereas the growing algal cells are free from bacterial growth. It is indicated that the growing algal cells release the bacteriostatic or bactericidal substances in their cell surfaces, whereas the stagnating and declining cells are in the contrary condition. In a general way, the bacteria utilize their host cells as the physical support, and they derive their nutrients from the water milieu.

Although the bacterial effects on algal growth in the culture conditions have been documented by some workers (Sukoso and Sakata, 1996; Requelme, Fukami, and Ishida,1988; Paerl and Gallucci,1985), the algae-bacteria interactions in the fields are poorly understood due to the complexity of aquatic ecosystems. The addition of co-existent bacteria to the culture of red tide algae resulted in a large increase in the biomass and extended the growth period of microalgae, while microalgae failed to grow longer in axenic culture (Sukoso and Sakata, 1996). This relationship may be based on the capability of algae to produce organic compounds and oxygen, which are utilized by bacteria. In turn, bacteria supply nutrients such as vitamins, amino acids or other metabolites that are required for algal growth.

Many investigators reported that some bacteria showed the high vitamin B_{12} production capability in the freshwater and marine environments. The vitamin production and utilization capability by phytoplankton were also observed by Carlucci and Bowes (1970), but not much information is available on the interaction between bacteria and microalgae based on vitamin production and utilization.

The internal factors such as stimulatory and inhibitory substances as well as the external factors such as light, temperature, carbon dioxide and other sea water elements affect bacterial-algal interaction within such ecosystems. Based on the above review by combining the internal and external factors, the principle feature of algae-bacteria interaction in general is illustrated in Fig. 1.1.

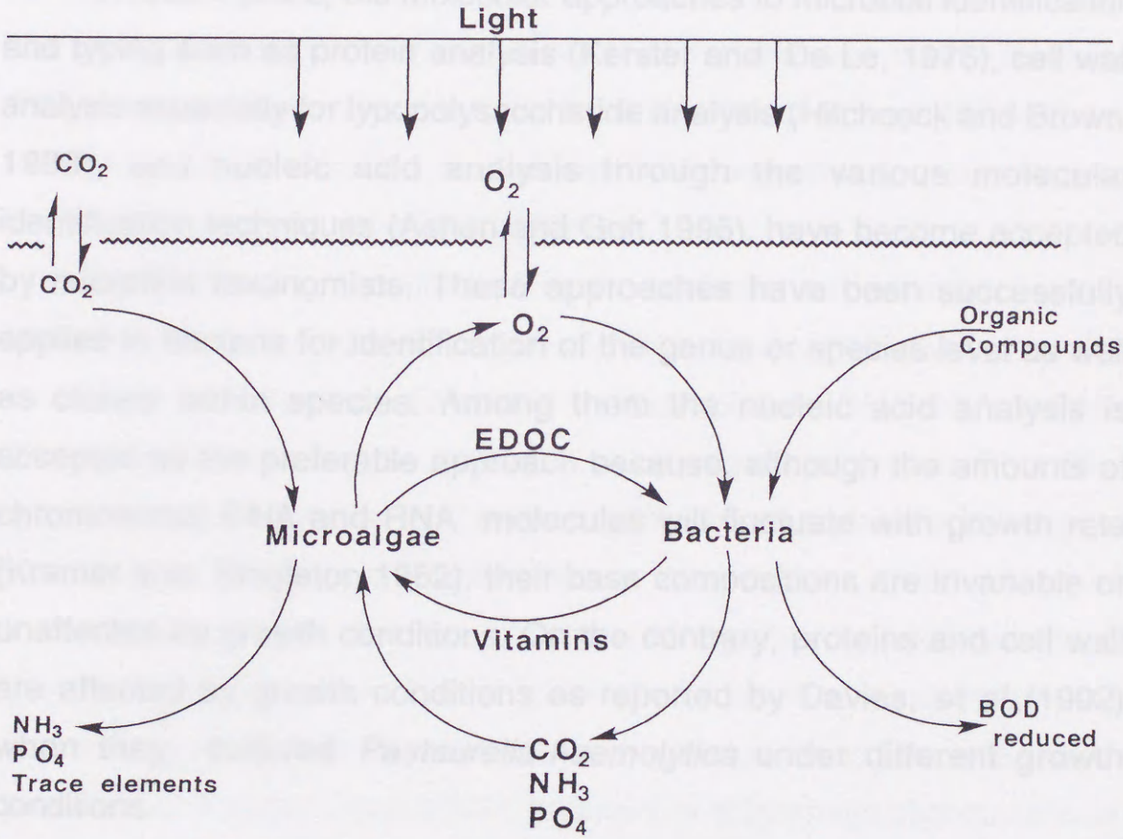

Fig.1.1 Biotic and abiotic environmental factors affecting alga-bacteria growth interactions

1.2.2 Identification of bacteria

To identify new isolated bacteria, the conventional taxonomic characterization is usually used. Unfortunately, the conventional methods can not always offer an ideal approach to discrimination of bacterial strains, because of time-consuming to carry out. Moreover, misidentifications are also more common as described by Boivin-jahns, *et al.*,(1995), when they compared the phenotypical and molecular genetical methods for identification of bacterial strains isolated from environments.

In recent years, the molecular approaches to microbial identification and typing such as protein analysis (Kerster and De Le, 1975), cell wall analysis especially for lypopolysaccharide analysis (Hitchcock and Brown, 1983), and nucleic acid analysis through the various molecular identification techniques (Ashen and Goft,1996), have become accepted by microbial taxonomists. These approaches have been successfully applied to bacteria for identification of the genus or species level as well as clones within species. Among them the nucleic acid analysis is accepted as the preferable approach because, although the amounts of chromosomal DNA and RNA molecules will fluctuate with growth rate (Kramer and Singleton 1982), their base compositions are invariable or unaffected by growth conditions. On the contrary, proteins and cell wall are affected by growth conditions as reported by Davies, *et al.*,(1992) when they cultured *Pasteurella haemolytica* under different growth conditions.

To date, analysis based on the nucleic acids has been developed broadly to examine the size and structure of individual nucleic acid molecules extracted from test organisms such as mol% G+C, restriction fragment length polymorphism (RFLP) and sequence analysis. The hybridization techniques clarify the degree of relatedness among test organisms using a chemilluminescent or radioactive compounds for

labeling single strand DNA probes that is complementary to chromosomal DNA of the target organisms. DNA probes have been used widely for detection of the presence of pathogenic bacteria in food and water including *E. coli* and *Salmonella* sp. (Fitts, *et al.*, 1983; Escheverria, *et al.*, 1982), detection of catabolic genotypes in environmental bacterial communities (Sayler *et al.*, 1985), and distinguishing strains of *Aspergillus flavus* (McAlpin and Mannarelli, 1995). The use of universal DNA probe is able to hybridize with different DNA restriction fragment from a wide variety of bacterial species, while the specific DNA probe is vice versa. The presence of new techniques such as Polymerase Chain Reaction (PCR) with the capability to amplify the DNA strand at least 10^6 fold, has made a great contribution to develop DNA sequencing and molecular identification systems for microorganisms.

By sequencing specific pairing of complementary strands of one or two polynucleotides and an oligonucleotide, the hybridization of nucleic acid results in formation of homo or heteroduplexes. DNA-DNA or RNA-RNA hybridizations are the formation of homoduplexes, while RNA-DNA hybridization is heteroduplex. Hybridization reactions take advantage of the ability of nucleic acid molecules to form double strands in which nucleotides and opposing strands are held together by hydrogen bond. The hydrogen bonds are broken normally by alkali or heat treatments, then the opposing strands separate and hybridize to appropriate probes derived from a specific clone DNA fragment or a synthetic oligonucleotide. Along with the increasing attention to the development of hybridization techniques, the simple and rapid methods to detect the presence of specific microorganism have been investigated, such as the use of in situ hybridization with 16S rDNA oligonucleotide probes for detection and enumeration of marine sulfate-reducing bacteria as reported by Park Young-Tae, *et. al.*, (1977). The use of simple hybridization method

including dot hybridization provides only the quantitative result. Base on this regard, the higher homology of chromosomal DNA target with DNA probe outcomes the more positive reaction. On the contrary, the simple hybridization method can not provide qualitative informations such as the degree of difference among tested organisms.

The number of DNA fragment band represented in restriction fragment length polymorphism (RFLP) depends on the kind of restriction endonucleases or restriction enzymes. Each enzyme differs in nucleotide sequence and size recognizing specifically. These are endonucleases that recognize a specific base sequence in a DNA molecule and make two fragments generating 3'-OH and 5'-P termini. By increasing the use of restriction enzymes for numerous purposes, now thousands of different restriction enzymes have been purified from hundreds of different microorganisms.

Maloy, *et. al.*, (1994) stated that sequence recognized by restriction enzymes is an important determinant of how many positions the enzyme will cut a particular piece of DNA. Thus, the longer the recognition sequence, the lower the probability that the sequence will be found in a particular DNA molecule. In general, there are two types of DNA fragment termini cut by the restriction enzyme. Blunt ends are found in DNA fragments cut at the center of symmetry, while the cohesive ends are in place around of the center of symmetry. By conducting restriction fragment length polymorphism (RFLP), the differences among tested organism can be detected specifically based on the restriction pattern on gel electrophoresis. By using RFLP data, Moyer, *et. al.*,(1996) reported that the data, though providing less direct information on the evolution of DNA sequences, are easier to obtain and more economical than complete SSU rRNA gene sequences.

Many informations are needed before deciding the taxonomic position of unclassified microorganisms. By summarizing and cataloguing information about an organism, the classification will be able to be done. Since molecular approach has been done in this experiment, information from DNA sequence is a vital point to devote the microbial systematic and ultimately, the taxonomic position based on the information which is encoded in the nucleotides sequences of the chromosome. A great deal of informations about the structure and function of genes have come from direct determination of the nucleotide sequence of DNA. The nucleotide sequence of DNA can be detected according to the procedures proposed by Maxam and Gilbert (1977) and Sanger (1977) that were described in all biochemistry and molecular biology books. Recently, these procedures have been automated in order to make differences among the nucleotide sequence obvious by use of fluorescent dyes for four nucleotides. In the sequencer, the migration of the bands in the electrophoresis gel is detected by a laser beam and the sequence is compiled by computer.

Among the ribosomal DNA (rDNA), 16S rDNA has been used extensively for comparative sequencing studies, because 16S rDNA is somewhat easier to handle than the 23S rDNA (Woese, 1987) and a much larger data base is available for 16S rRNA. More than 3,000 sequences of small subunit ribosomal RNA (SSU rRNA) are now available (Van de Peer, *et. al.*, 1994) and over 1,500 prokaryotes have been characterized by small-subunit RNA sequencing (Olsen, *et. al.*, 1994). The success for phylogenetic studies based on the sequence of 16S rDNA have been done by some workers (Schmidt, *et. al.*, 1991; Weisburg, *et. al.*, 1991). Based on the DNA sequencing data, the phylogentic trees can be constructed. Saitou and Nei (1987) proposed a new method called the neighbor-joining method for reconstructing phylogenetic trees from evolutionary distance data. The principle of this

method is to find pairs of operational taxonomic units (OTUs [= neighbors]) that minimize the total branch lengths at each stage of clustering of OTUs starting with a starlike tree. For 16S rRNA sequence data analysis, the use of CLUSTAL W program promoted by Thomson, *et. al.*, (1994) is also popular.

A phylogenetic tree is a graph composed of nodes and branches in which only one branch connects the two adjacent nodes. Cladograms are displayed as branch diagrams or trees. The complex procedures for constructing phylogenetic trees, now can be simplified by using computer programs connected with the DNA data bank. The DNA data bank is collecting the DNA sequence and playing a role in providing the data to researchers world widely. With the advent of the computer age, a large amount of data can be computerized, calculated, estimated, simulated rapidly with minimum errors. This development has revolutionized the approach of bacterial taxonomy.

Chapter 2

Effect of Co-existent Bacteria on the Growth of *Chattonella marina*

2.1. Introduction

Microalgae and bacteria are numerically dominant organisms in aquatic ecosystems and they play important roles as primary producers and decomposers, respectively. Microalgae produce various organic substances photosynthetically and excrete a part of them, which is utilized by bacteria as carbon and energy sources (Cole, Liken and Strayer, 1982; Fogg, 1983; Brock and Clyne, 1984). On the other hand, the roles of bacteria are to stimulate or inhibit algal growth through the decomposition of organic compounds, endosymbiosis, and production of stimulative or inhibitory substances for algal cells. The relationship between microalgae and bacteria can occur temporarily or permanently, within or in the vicinity of algal cells. This relationship can be caused by physical support or chemical factors, or both. On the basis of nutritional necessity, the possible interactions between microalgae and bacteria can be recognized as competition, commensalism or parasitism (Fenchel and Jorgensen, 1977; Baker and Herson, 1978; Delucca and McCracken, 1978).

Many attempts to elucidate the relationship between microalgae and bacteria in aquatic environments have been done by various investigators. For examples, Riquelm, *et al.*,(1988) reported that the growth of a marine diatom, *Asterionell glacialis* was strongly stimulated by a *Pseudomonas* strain through some bacterial metabolites. Fukami, *et al.*,(1992) demonstrated that natural communities of bacteria give stimulating or inhibitory effect on *Skeletonema costatum* and *Gymnodinium nagasakiense*, and cause the succession of these algal species. As cyanobacterial-bacterial association, Paerl and Gallucci (1985) observed that *Anabaena oscillarioides* excreted products responsible for positive chemotaxis in pseudomonads.

Red tide by *Chattonella marina* happened in Kagoshima Bay in 1985 and algal strains were isolated by some workers. A strain of *C. marina* was given and have been maintained in the Microbiology Laboratory. The stock culture of *C. marina* strain harbors several species of bacteria as co-existent partners. Non-axenic cultures of this alga are found to maintain viable algal cells for longer time than the axenic culture.

In this chapter, the author attempted to clarify the effect of co-existent bacteria on the growth of red tide microalga, *Chattonella marina* isolated from Kagoshima Bay and maintained in the laboratory.

2.2 Materials and Methods

2.2.1 Algal culture

The author is indebted to Mr. Aramaki, Fisheries Station of Kagoshima Prefecture, for the *Chattonella marina* strain, who isolated it when red tide occurred in Kagoshima Bay in 1985 and the alga was cultured in 10 ml of Provasoli's enrichment sea water medium (ESS)(Provasoli, McLaughlin, and Droop, 1957) under illumination (7,000 lx in the light cycle of 12L : 12D, 23°C). ESS medium consisted of 5 ml of Na_2SiO_3 solution (1 mg/l), 40 ml of Provasoli's enrichment solution (ESP) containing $NaNO_3$ 350 mg, α-glycerophosphate 50 mg, Fe-EDTA 2.5 mg, vitamin B12 10 μg, biotin 5 μg, vitamin B_1 500 μg, tris-aminomethane 500 mg, PII metal solution 25 ml/l, 900 ml of filtered natural sea water, and 100 ml of distilled water (pH 7.8). The compositions of ESS medium and PII- metal solution are given in Table 2.1.

2.2.2 Axenic algal culture

An axenic algal strain was isolated from the original non-axenic stock culture by using the long tube technique (Imai, Yamaguchi, 1994). By exploiting the positive-phototaxis of algal cells and antibiotics including

streptomycin sulfate, kanamycin sulfate and penicillin G (each, 50 mg/l) in ESS medium, the algal cell isolation was carried out under illumination.

Table 2.1a. Composition of ESS medium

Ingredients	Total
ESP:	40 ml
$NaNO_3$	350.0 mg
α glycerophosphate	50.0 mg
Fe-EDTA	2.5 mg
Tris-aminomethan	500.0 mg
Vitamin B_1	0.5 mg
Biotin	5.0 μg
Vitamin B_{12}	10.0 μg
Na_2SiO_3 (1mg/l)	5 ml
PII metal solution	25 ml
Sea water	900 ml
D.W	100 ml
pH	7.8

Table 2.1b. Composition of PII-metal solution

Ingredients	Total
$Na_2EDTA.2H_2O$	110.7 mg
$FeCl_3.6H_2O$	4.8 mg
H_3BO_3	114.3 mg
$MnCl_2.4H_2O$	14.4 mg
$ZnCl_2$	1.0 mg
$CoCl_2.6H_2O$	0.4 mg
D.W.	100.0 ml
pH	7.8

The first step was done by preparing a sterile ESS medium with antibiotics. The medium was filled in sterilized glass tubes (400 mm length and 8 mm inner diameter) with a silicon tube and a silicon cap at the upper and lower ends. Then, about three drops (0.15-0.30 ml) of original algal culture at 10-15 days of incubation (late logarithmic or early

stationary phase) were dropped in the glass tube containing medium. The glass tube was closed tightly and avoided the air bubble in the tube, then the glass tube was turned upside down smoothly until the algal cells turn down at the bottom end of a glass tube. The glass tube was stood and left in a clean bench at 22°C and illuminated under a 100-300 V sun-lamp for about 20-30 min., or until the algal cells swam up to the surface of glass tubes. Immediately after the algal cells swam up to the surface of glass tube, algal cells in 0.05 ml of medium were aseptically pipetted with a Pasteur pipette into 10 ml of sterile ESS culture media, then incubated at 23°C under illumination 7,000 lx (12D : 12L) for about 20-30 days. In each test tube in which algal cells grew, further confirmation was needed to examine bacterial contamination. By using a smear plate method a 0.1 ml portion from each test tube (algal cells grow) was poured and smeared on a ZE-CI agar plate (5 g, polypepton : 1 g, yeast extract : 40 ml, ESP : 750 ml, sea water : 250 ml, distilled water).

2.2.3 Algal cell count

Algal cells in axenic or mixed cultures were counted by a direct count method (Thoma cytometer chamber) under a light microscope. Algal motility was stopped by keeping algal cells that were placed on Thoma glass in petri-dish with an acetic acid solution for 5 min. Algal cell counts (log No. / ml) were expressed as an average of 5 samples from an algal culture.

2.2.4 Growth of algal cells in axenic and mixed cultures

The growth of alga and co-existent bacteria were determined after transferring 0.5 ml of a non-axenic algal culture into 9.5 ml of ESS liquid medium and incubated 23°C under illumination of 7,000 lx (12L:12D). On the other hand, axenic algal cells and bacterial cells were mixed as

follows; 0.5 ml of a bacterial dilution solution prepared from the rotating cultures in L-shape test tubes containing 10 ml of a ZE-CI medium and 0.5 ml of axenic cultures were transferred into 9.5 ml ESS media in large test tubes.

2.2.5 Effect of enrichment solution and polypepton on algal growth

In order to examine the effect of enrichment solution (ESP) on axenic algal growth, 0.4 ml of ESP was added to algal cultures (10 ml) after algal cells reached the maximum number. The effect of polypepton on non-axenic culture of the alga was examined by adding polypepton at 50, 75, 100, 150, 250 mg/l concentrations into ESS medium (10 ml). The cultures were incubated at 23°C under illumination of 7,000 lx, and algal growth was monitored.

2.2.6 Bacterial strains

The bacterial strains were isolated from a stock culture of *C. marina* by using a dilution-plate method. Bacterial colonies were picked up from an agar plate and purified on an YES agar. The YES agar medium was prepared by the addition of yeast extract (Nihon Seiyaku) 1 g and agar 15 g to 1,000 ml of ESS medium.

2.2.7 Bacterial colony count

Bacterial viable cells were detected by a dilution spread-plate technique. Samples were transferred by pipette into a serial dilution solution in 3/4 artificial sea water (Herbst's formula artificial sea water, ASW) and then 0.1 ml of the respective dilution solution was smeared on a ZE-CI agar medium and incubated at 25°C. The apparent colonies were counted after 6 days of incubation at 25°C. The composition of artificial sea water (Herbst's formula) is given in Table 2.2.

Table 2.2. Composition of artificial sea water (A.S.W)-Herbst's formula

Ingredients	ASW	2 ASW
NaCl	30.0 g	600.0 g
KCl	0.7 g	14.0 g
$MgCl_2.6H_2O$	10.8 g	216.0 g
$MgSO_4.7H_2O$	5.4 g	108.0 g
$CaCl_2.2H_2O$	1.0 g	20.0 g
D.W.	1.0 L	10.0 L

2.2.8 Characterization of bacterial strains

For the identification of co-existent bacterial strains isolated from non-axenic algal cultures, the basal characterization tests were carried out based on morphological characters (colony, cell form, gram stain and motility), biochemical reaction (pigmentation, catalase, oxidase and Hugh -Leifson test), and macromolecular degradation (casein, starch and DNA) according to general procedures. Bacterial genera were identified according to the identification scheme presented by Simidu (1985).

A loopful of test strains was picked up from stock culture by a platinum wire and cultured at 2-3 days of incubation in 10-ml ZE-CI broth in the orbital L-test tube at 25°C before bacteriological characterization test.

The observation of cell form and motility was made by picking up one colony grown on the ZE-CI agar plate and suspending in one drop of ASW on a slide glass. The bacterial cells were observed by a microscope under 100 x 10 magnifications, after covering the slide glass with a cover glass.

The gram stain was conducted according to Hucker's modified method. Bacterial cells grown on the ZE-CI agar medium was mixed with one drop of ASW on the slide. Then, the bacterial smear was stained with the crystal violet for 1 minute and then with a Lugol solution for 1 min.

After the slide glass was washed with tap water and cleaned with filter papers. It was quickly rinsed with alcohol for 3 minutes. Finally, the slide was stained with safranin (counter stain) for 1 minute and washed gently with water and dried.

DNA-hydrolysis was conducted by using a DNA-agar medium (EIKEN), dissolved in ASW with on ESS solution. Test strains were cultivated on the agar plate and incubated at 25°C for 6 days. DNA-hydrolyzing activity was confirmed by detecting red color of media surrounding bacterial colony.

Casein-hydrolysis was observed on casein-agar media containing 2 g skim milk, 15 g agar, and 1,000 ml of a ZE-CI liquid medium. Tested bacteria were cultivated on the agar plate and incubated at 25°C for 6 days. The activity of bacteria to hydrolyze casein was confirmed by the clear zone surrounding bacterial colony.

A starch hydrolysis test was conducted on starch-agar media containing 0.5 g soluble starch, 15 g agar in a ZE-CI medium. The tested bacteria was cultivated on the agar plate and incubated at 25°C for 3-6 days. The activity of bacteria to hydrolyze starch was observed by dropping a lugol solution on bacterial colony. The violet color surrounding the colony indicated the negative result and the positive result was shown by yellow color.

Hugh & Leifson test was done by using a stab medium shown in Table 2.3. Test bacteria were incubated at 25°C for 3-7 days after stabbing them into medium, and incubated at 25°C for 3-7 days. The yellow color indicated glucose fermentation, whereas no altering color indicated negative result.

Catalase activity was done by culturing test bacteria on the ZE-CI agar medium and incubated at 25°C, 3-4 days. Bacterial cells picked up with a glass rod were dipped in a 3% H_2O_2 solution and a catalase

positive reaction was observed by the bubbles produced from the bacterial cells.

Oxidase activity was done by culturing test bacteria on the ZE-CI agar medium and incubated at 25°C for 3-4 days. Cytochrome disks (EIKEN) were sticked on colonies. After approximately 30 seconds, the inoculated area of the disk caused color change. A blue or violet color indicated a positive oxidase reaction.

Table 2.3. Composition of Hugh and Leifson Media

Ingredients	Total
Solution A	
Polypepton	2.0g
K_2HPO_4	0.3g
B.T.B	0.03g
Agar	3.0g
N.S.W	500.0 ml
D.W	400.0ml
pH	7.6
Solution B	
Glucose (Dextrose)	10.0g
D.W	100 ml
Solution C	
E.S.P solution	10.0 ml

2.2.9 Effect of bacterial culture filtrate on algal growth

A loopful of bacterial strains was inoculated into ZE-CI liquid media in L-shape test tubes and incubated rotationally at 25°C for 2-3 days. After two centrifugations of bacterial cultures at 8,000 rpm for 20 minutes, pH of the obtained supernatants was adjusted to 7.6-7.8 and the supernatants were filtered by Millipore filter (0.22 μm). Then, they were

transferred at 1, 3, 5, 8, and 10% (v/v) into axenic algal cultures in 10 ml of an ESS medium, respectively.

2.3 Results

2.3.1 Characterization of bacterial strains

The representative bacterial strains isolated from a stock culture of *C. marina* were characterized according to bacteriological tests as shown in Table 2.4. The results indicated that all co-existent bacteria are gram-negative, non fermentative in Hugh & Leifson test and positive in catalase test. All of *Flexibacter* strains (tentatively identified) were negative in motility and hydrolysis of starch, casein and DNA, whereas weak in oxidase test. On the other hand, *Alteromonas* strains were positive in motility, oxidase test and hydrolysis of starch, casein and DNA, except a strain A93-1. The identification test pointed out that the strains of *Pseudomonas* were positive in motility, oxidase and catalase test, and non fermentative in Hugh & Leifson test.

2.3.2 Growth of alga in original culture and mixed culture of axenic alga and bacterial strains.

Growth curves of *C. marina* and co-existent bacteria in a non-axenic algal cultures are shown in Fig. 2.1. Algal growth reached the maximum cell number at 5.6 log No./ml level in 15 days of incubation. During 6 days of incubation, total colony of co-existent bacterial groups including *Alteromonas, Pseudomonas,* and *Flexibacter* strains (tentatively identified) also reached maximum cell numbers at levels 7.3, 7.3 and 6.8 log No./ml, respectively and then followed by stationary phase in viable cell numbers. In this experiment, each bacterial group was distinguished on the basis of the colony morphology on a ZE-CI agar plates; *Alteromonas*, *Pseudomonas* and *Flexibacter* strains formed smooth whitish brown,

smooth whitish gray and rhizoidal whitish pink colonies, respectively. Fig. 2.2 shows colonies of bacteria grown on ZE-CI agar after the algal sample was smeared on the ZE-CI agar plate and incubated at 25°C.

For clarifying the effect of co-existent bacteria on algal growth, a set of mixed cultures was carried out by culturing an axenic algal strain with isolated co-existent bacterial strains. After commenced by 3.9 log No./ml as shown in Fig. 2.3, the algal cell numbers in mixed cultures for all combinations with co-existent bacteria gradually increased to about 5.7 log No./ml for 30 days of incubation. On the other hand, the algal cell count in the axenic culture reached a maximum level of 5.2 log No./ml in 10 days, then decreased and was not detected at 30 days of incubation. Algal growth in mixed-cultures indicated no significant difference for all combinations with bacteria. Apart from the growth of alga in mixed-cultures, the viable counts of bacterial groups reached the cell number of 6.4 to 7.7 log No./ml as shown in Fig. 2.4.

Table 2.4. Biochemical and physiological characteristics of bacterial strains

Srains	Gram	Cell	Motility	Catalase	Oxidase	H&L	Casein	Starch	DNA	Colony	Genus
Fl93-1	-	fil*1	-	+	±	-	-	-	-	WP*2	*Fle.*3
Fl 93-2	-	fil	-	+	±	-	-	-	-	WP	*Fle.*
Fl93-3	-	fil	-	+	±	-	-	-	-	WP	*Fle.*
Fl93-4	-	fil	-	+	±	-	-	-	-	WP	*Fle.*
Fl-32	-	fil	-	+	±	-	-	-	-	WP	*Fle.*
Ab-4	-	rod	+	+	+	-	+	+	+	WBr	*Alt.*
Ab-5	-	rod	+	+	+	-	+	+	+	WBr	*Alt.*
A93-1	-	rod	+	+	+	-	-	-	+	Wbr	*Alt.*
Ps93-1	-	rod	+	+	+	-	-	-	-	WGr	*Pse.*
Ps93-2	-	rod	+	+	-	-	+	±	-	WGr	*Pse.*
F93-1	-	L-rod	-	+	-	-	-	-	-	Y	*Fla.*

*1 fil, filamentous form; L-rod, long rod.
*2 WP, whitish pink; WBr, whitish brown; WGr, whitish grey; Y, yellow.
*3 *Fle.*, *Flexibacter*; *Alt.*, *Alteromonas*; *Pse.*, *Pseudomonas*; *Fla.*, *Flavobacterium*.

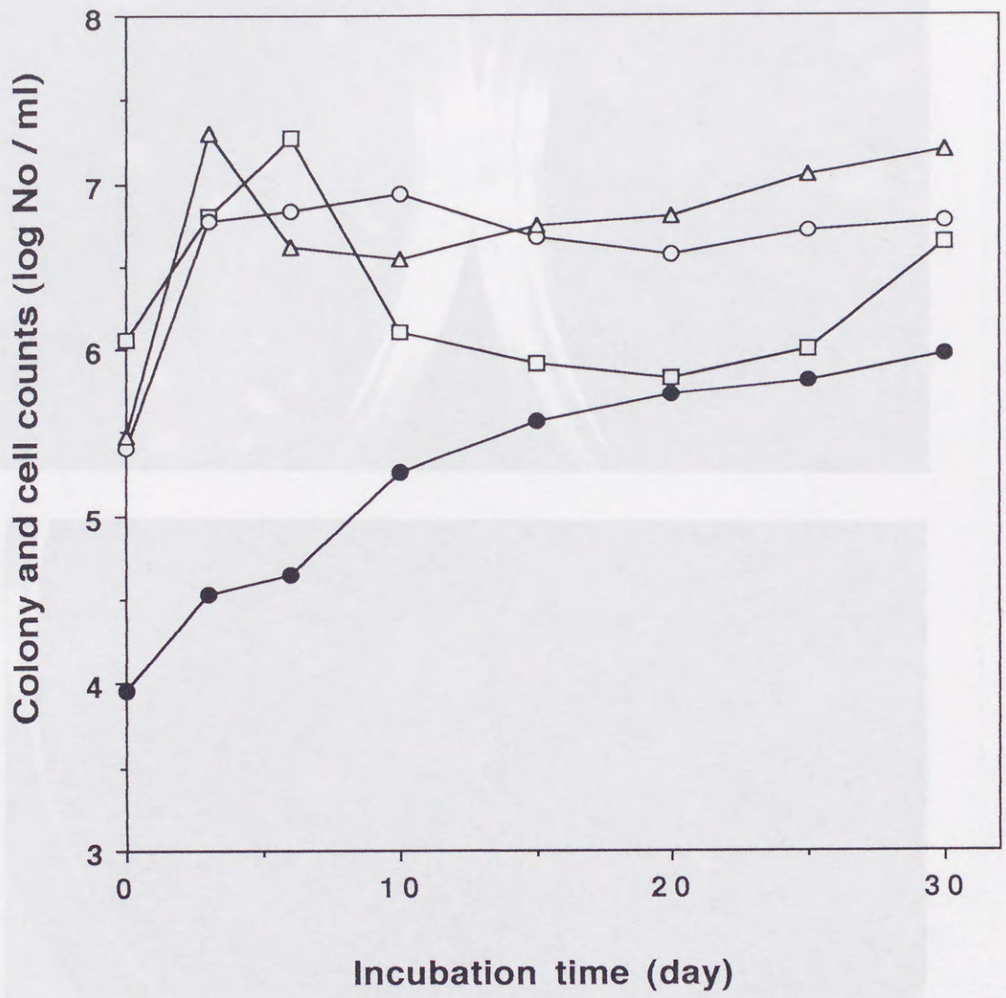

Fig. 2.1. Bacterial colony and algal cell counts in non-axenic culture of *C. marina*.

● , cell count (log No/ml) of *C. marina* ; ○ , total colony count (log No/ml) of *Flexibacter* strains; □ , total colony counts of *Alteromonas* strains; Δ , total colony count of *Pseudomonas* strains.

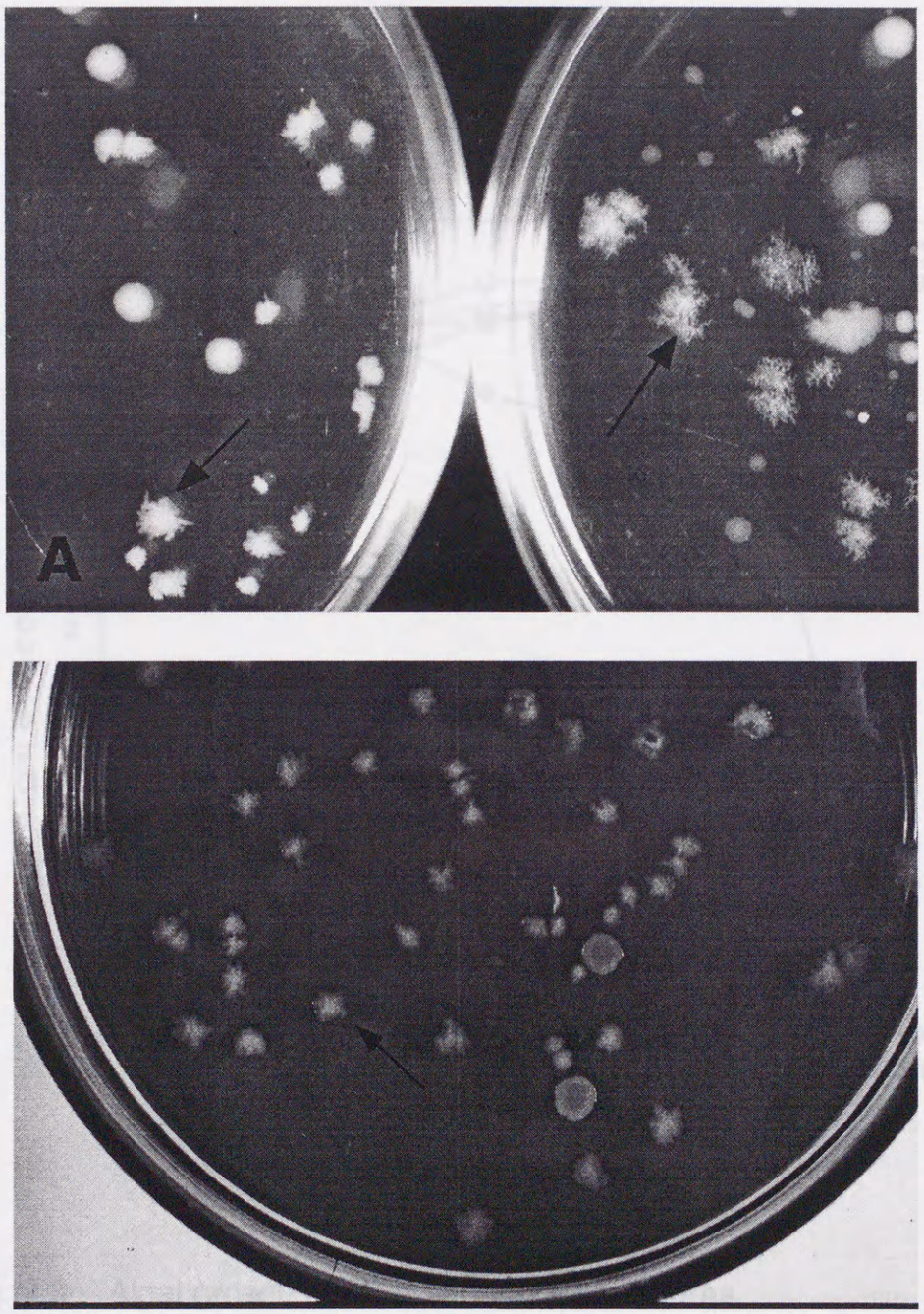

Fig. 2.2. Colony morphology of bacteria in the *C. marina* stock culture. The colonies were obtained by dilution-plating of the algal culture on ZE-CI agar plates. Arrows indicate the colonies of filamentous bacteria.

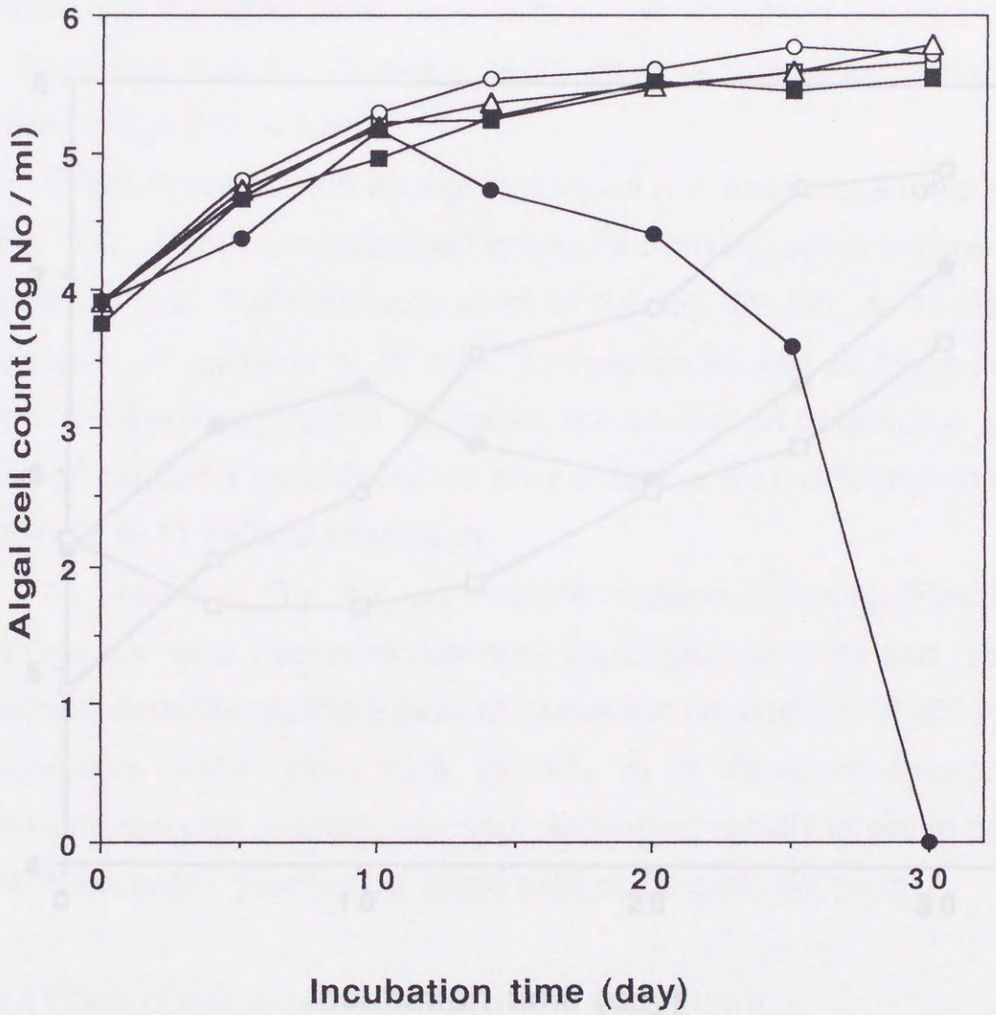

Fig. 2.3 Algal growth in mixed and axenic cultures.

□ , cell count (log No/ml) of *C. marina* in mixed culture with algal axenic strain and *Flexibacter* FI93-3; ○ , in mixed culture with *Alteromonas* Ab-5; ■ , in mixed culture with *Pseudomonas* Ps93-1; Δ , in mixed culture with *Flexibacter* FI93-3 and *Alteromonas* Ab-5; ● , cell count of *C. marina* in axenic culture.

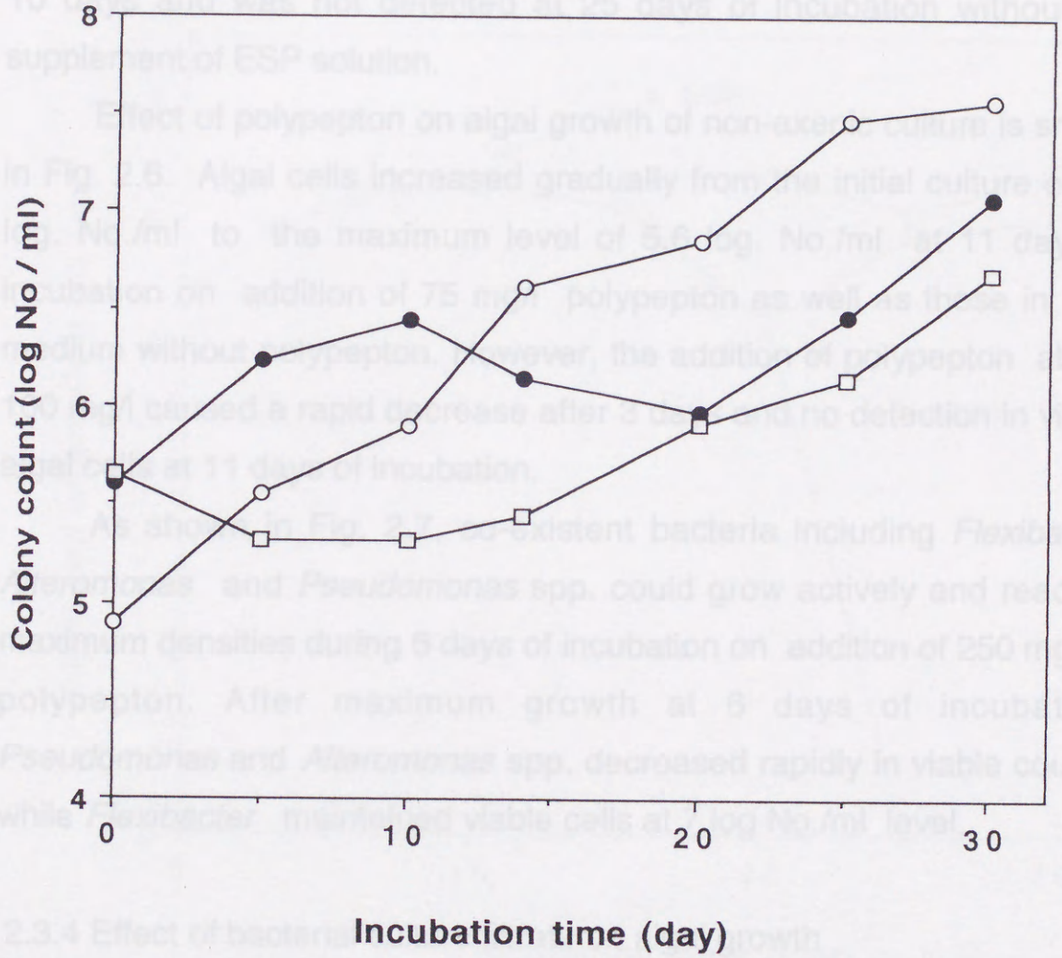

Fig. 2.4 Bacterial growth in algal mixed cultures.

●, colony count (log No/ml) of *Flexibacter* Fl93-3; □, *Alteromonas* Ab-5; ○, *Pseudomonas* Ps93-1 in mixed culture with *C. marina*. The conditions of mixed cultures with C. marina and each bacterium are the same as in Fig. 2.3.

2.3.3 Effect of enrichment solution and polypepton on algal growth

Fig. 2.5 shows the effect of the supplement of enrichment solution (ESP) on algal growth. Algal cells reached the maximum level of 5.3 log No./ml at 10 days, and the same level in viable cell number of the alga was maintained during 30 days of incubation after the supplement of ESP solution. On the other hand, algal cells in axenic culture decreased after 10 days and was not detected at 25 days of incubation without the supplement of ESP solution.

Effect of polypepton on algal growth of non-axenic culture is shown in Fig. 2.6. Algal cells increased gradually from the initial culture of 3.0 log. No./ml to the maximum level of 5.6 log. No./ml at 11 days of incubation on addition of 75 mg/l polypepton as well as those in ESS medium without polypepton. However, the addition of polypepton above 100 mg/l caused a rapid decrease after 3 days and no detection in viable algal cells at 11 days of incubation.

As shown in Fig. 2.7, co-existent bacteria including *Flexibacter*, *Alteromonas* and *Pseudomonas* spp. could grow actively and reached maximum densities during 6 days of incubation on addition of 250 mg/l of polypepton. After maximum growth at 6 days of incubation, *Pseudomonas* and *Alteromonas* spp. decreased rapidly in viable counts, while *Flexibacter* maintained viable cells at 7 log No./ml level.

2.3.4 Effect of bacterial culture filtrate on algal growth

Algal cells could not grow and were killed within 7 days of incubation in an ESS medium added with the culture supernatant of co-existent bacterial isolates grown in a nutrient rich medium (ZE-CI medium) at a concentration of 5% or more, whereas at 3% or less, the algal cell growth continued for 30 days of incubation as shown in Fig.2.8. The growth of axenic algal cells was maintained in the stationary phase

for more than 30 days by addition of 10% of a ZE-CI medium into the ESS medium. However, the algal cells in an axenic culture in the ESS medium decreased gradually after the maximum density of 5.5 log No. /ml was attained at 10 days of incubation and were not detected at 30 days of incubation.

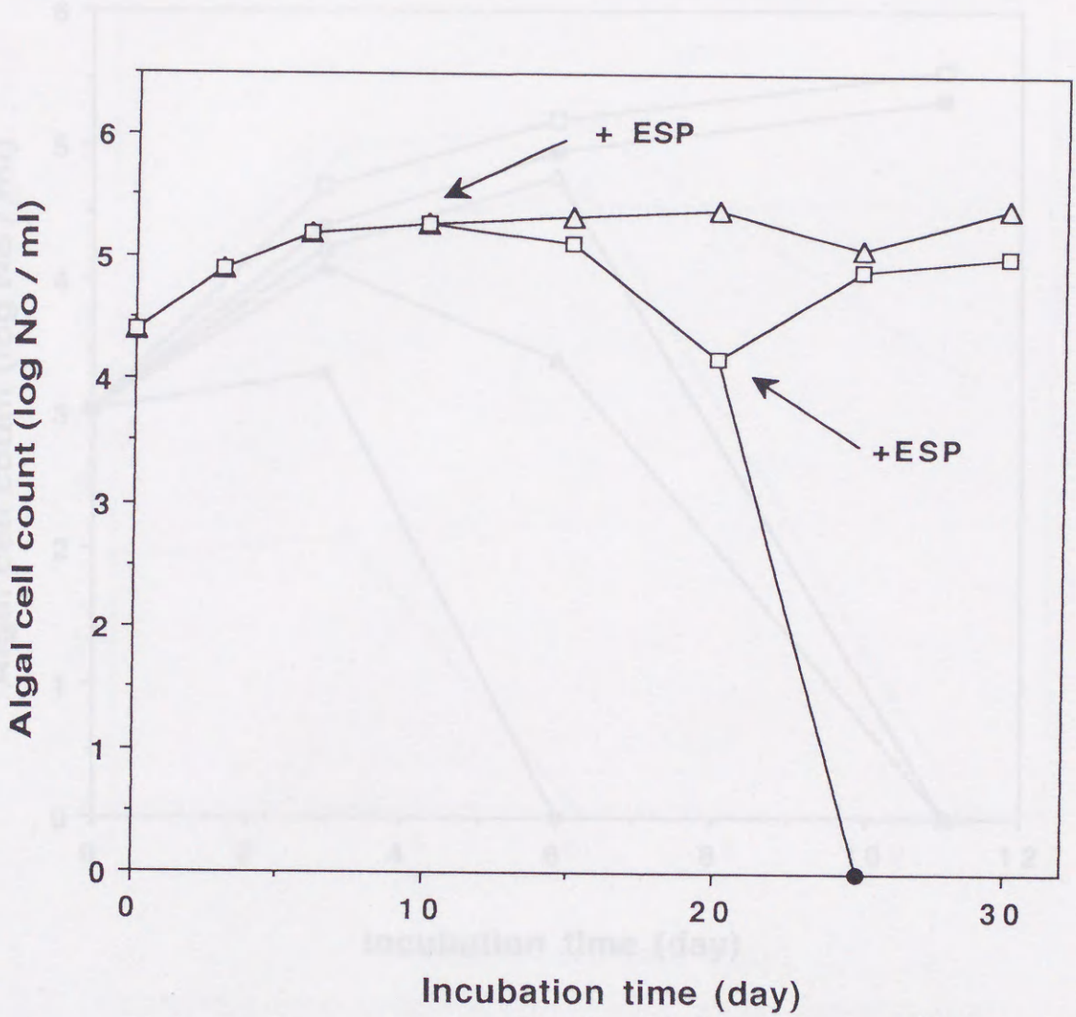

Fig. 2.5 Effect of the supplement of enrichment solution (ESP) on axenic algal growth.

Δ , algal cell count (log No/ml) in ESS medium supplied with ESP at 10 days of incubation; □ , supplied with ESP at 20 days; ● , not supplied with ESP.

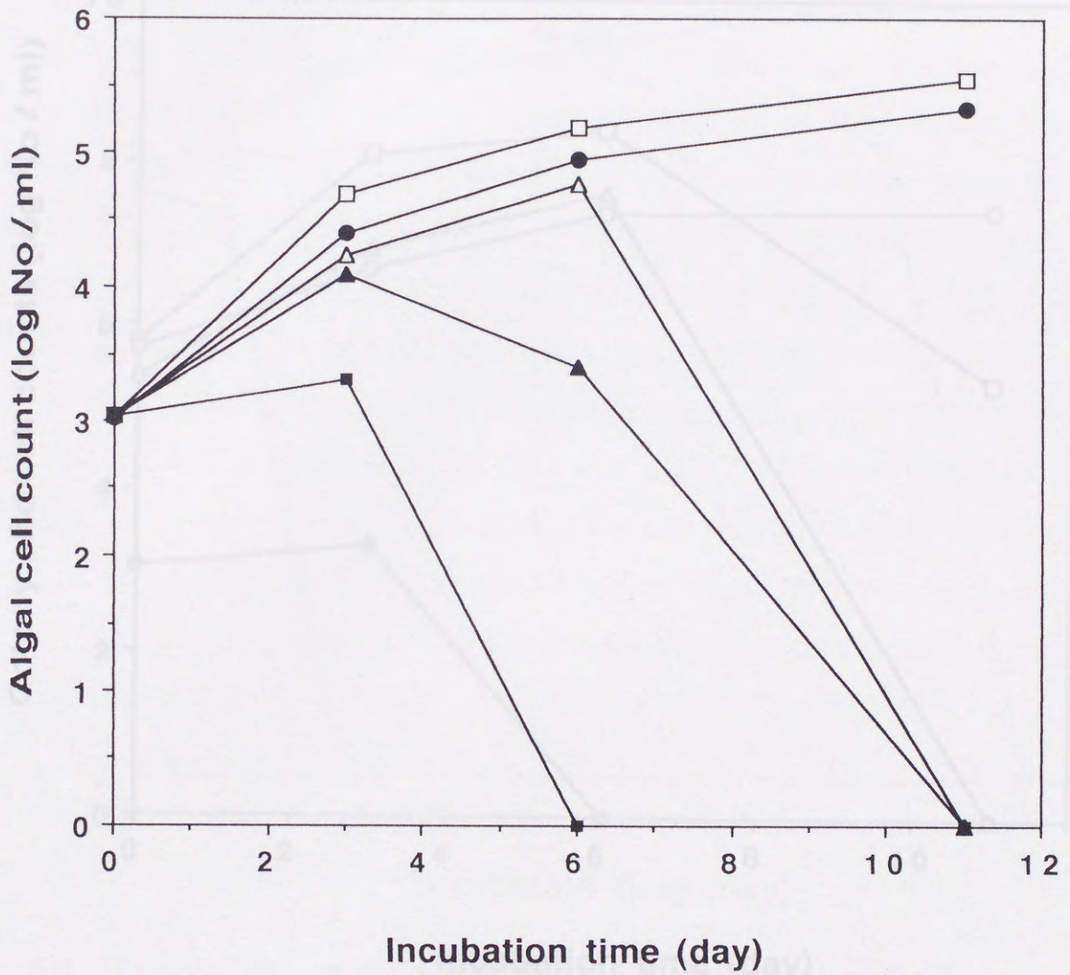

Fig. 2.6 Effect of various concentrations of polypepton on algal growth in non-axenic culture.

□ , algal cell count (log No/ml) in ESS medium added with polypepton at 75 mg/l;Δ , 100 mg/l; ▲ , 150 mg/l; ■ , 250 mg/l; ● , no addition.

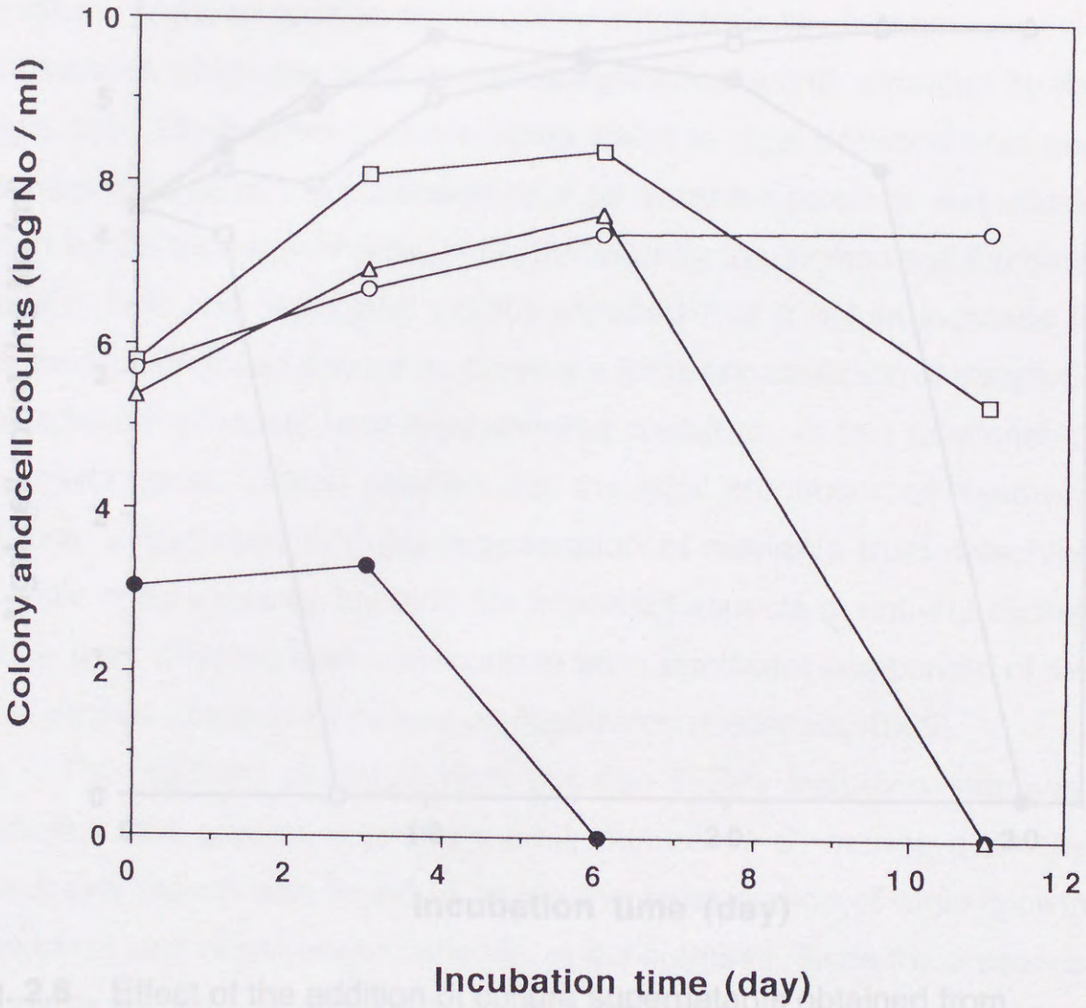

Fig. 2.7 Bacterial growth in non-axenic culture added with 250 mg/l polypepton.

○, colony count (log No/ml) of *Flexibacter* strains; □, *Alteromonas* strains; Δ, *Pseudomonas* strains; ●, algal cell count (log No/ml).

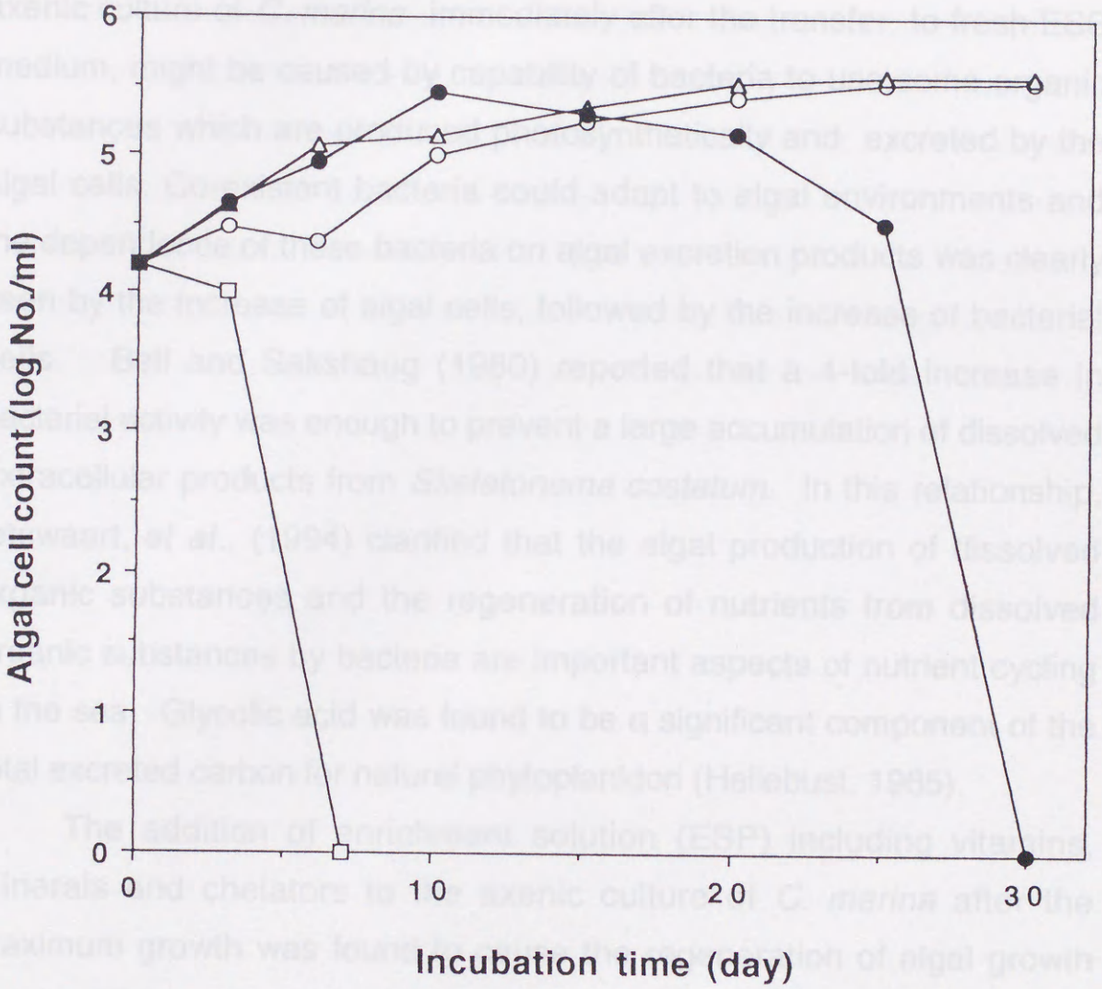

Fig. 2.8 Effect of the addition of culture supernatants obtained from bacterial culture in peptone medium (ZE-CI) on algal growth in axenic culture.

Δ, algal cell count (log No/ml) in axenic culture added with 10% of ZE-CI liquid medium; ○, algal cell count in axenic culture added with 3% culture supernatant of *Flexibacter* FI93-3; □, added with 5% culture supernatant of *Flexibacter* FI93-3; ●, algal cell count in axenic culture without the addition of bacterial culture superenatant.

2.4 Discussion

The increased density of co-existent bacteria including *Flexibacter* (filamentous bacteria), *Alteromonas* and *Pseudomonas* spp. in non-axenic culture of *C. marina* immediately after the transfer to fresh ESS medium, might be caused by capability of bacteria to use some organic substances which are produced photosynthetically and excreted by the algal cells. Co-existent bacteria could adapt to algal environments and the dependence of these bacteria on algal excretion products was clearly seen by the increase of algal cells, followed by the increase of bacterial cells. Bell and Sakshaug (1980) reported that a 4-fold increase in bacterial activity was enough to prevent a large accumulation of dissolved extracellular products from *Skeletonema costatum*. In this relationship, letswaart, *et al.*, (1994) clarified that the algal production of dissolved organic substances and the regeneration of nutrients from dissolved organic substances by bacteria are important aspects of nutrient cycling in the sea. Glycolic acid was found to be a significant component of the total excreted carbon for natural phytoplankton (Hellebust, 1965).

The addition of enrichment solution (ESP) including vitamins, minerals and chelators to the axenic culture of *C. marina* after the maximum growth was found to cause the regeneration of algal growth due to utilizing vitamins and minerals as the nutrients. Since the presence of nutrients was ascertained to stimulate the regeneration of algal growth, it may be assumed that the nutritional requirement of algal cells is supplied by co-existent bacteria as demonstrated in non-axenic algal cultures. The major source of vitamin B_{12} in open sea water has been reported to be supplied by heterotrophic bacteria and to be beneficial for algal growth (Cole, 1982). Because of the supplement of nutrients by co-

existent bacteria, the stationary phase of *C. marina* in non-axenic cultures was longer than that in axenic cultures.

However, the addition of polyppeton above 100 mg/l to non-axenic cultures resulted in the fact that the algal cells decreased rapidly as the total bacterial counts increased. From these findings, it is concluded that co-existent bacteria utilized polypepton preferably than algal excretion substances and caused the death of algal cells due to some toxic low-molecular substances produced through a polypepton utilization process. On the other hand, co-existent bacteria are thought to be capable of stimulating algal growth by production of nutrients and/or degradation of algal toxic metabolites by the bacteria grown on algal excretion substances.

Chapter 3

Effect of Filamentous Bacteria on the Growth of *Chattonella marina*

3.1. Introduction

Microalgae transform inorganic nutrients into the organic matters through photosynthesis in marine ecosystems. Some organic substances such as glycollate which are excreted into the water from algal cells, are probably utilized by bacteria shortly after excretion (Droop and Wood, 1968). On the other hand, microalgae require some essential nutrients including vitamins besides carbon dioxide and minerals (Cole, 1982). In the previous chapter the author described that the stock culture of a red-tide alga, *Chattonella marina*, harbors several bacteria and that the viability of algal cells was improved in algal cultures with these bacteria.

This algae-bacteria relationship may be based on capability of algae to produce organic compounds and oxygen, which are utilized by bacteria. In turn, bacteria supply nutrients such as vitamins, amino acids or other metabolites that are required for the growth of algae.

Some bacteria have been isolated from fresh water, sea water, suspended matters and coastal marsh muds; they provide vitamins including vitamin B_{12} in fresh and marine environments (Kashiwada, Kakimoto, and Kanazawa, 1956; Ohwada and Taga, 1972; Nishijima and Hata, 1978; Sugita *et al.*, 1994). The vitamin production and utilization capability by phytoplankton were also observed by many workers (Carlucci and Bowes, 1970; Provasoli and Carlucci, 1974; Swift and Taylor, 1974).

In this chapter, the growth of *Chattonella marina* with or without a filamentous bacterium isolated from the stock culture of *Chattonella marina* was compared in order to evaluate the effect of co-existent bacteria on algal growth.

3.2 Materials and Methods

3.2.1 Organisms

An axenic strain of *Chattonella marina* was prepared and a filamentous bacterium FI93-3 was isolated from the stock culture of *C. marina,* as previously described. *Euglena gracilis* strain *z* was obtained through the courtesy of Dr. Toshitaka Nishijima of Kochi University. Stock cultures of *Euglena* were transferred every week and incubated at 30°C under 3,000-4,000 lux of fluorescent lamps in the stock culture medium containing the basal medium as described in Table 3.1 with 15 g sucrose, 2.5 g agar, 2.0 g Tryptone and 50 ng vitamin B_{12} per liter.

3.2.2 Vitamin requirements of algal cells

Axenic cultures of *C. marina* were maintained in ESS media in the same condition as described in the previous chapter. All glassware were acid-cleaned, rinsed thoroughly, and baked at 160°C for 60 min. Prior to all experiments, axenic algal cells were starved of vitamins by culturing in a vitamin-free ESS medium for 7 days of incubation. Vitamin requirement tests were done in the vitamin-free ESS media which were added with vitamin B_{12}, biotin, thiamin at 4, 2, 200 ng/ml, respectively. The concentrations of vitamins were chosen based on a Provasoli's enrichment solution (ESP) (Provasoli, McLaughlin, and Droop, 1957).

3.2.3 Preparation of samples for vitamin B_{12} assay

Prior to all experiments, axenic algal cells were starved of vitamins by culturing in a vitamin-free ESS medium for 7 days. Then, 500 μl of the algal cell culture was added to 9.5 ml of the ESS medium without addition of bacterial cells or with addition of the 0.5 ml logarithmic phase culture of a filamentous bacterium FI93-3. Before assay of vitamin B_{12}, culture supernatants were obtained by centrifuging the axenic algal

cultures or mixed culture at 8,000 rpm for 20 min. The supernatant solutions were first filtered through a glass fiber filter (GF/C) and then by a Millipore filter (0.45 μm), after which the filtrate was freeze-stored. The supernatants obtained were diluted appropriately. Two and a half ml of the double-strength basal medium was added to 2 ml of each dilution sample and 0.5 ml of DDW was added to give a total volume of 5 ml, then measured on the vitamin B_{12} concentration by a bioassay method for *Euglena gracilis* (Hayashi and Kamikubo, 1966; Carlucci, 1974).

Table 3.1. Composition of *Euglena gracilis z* strain basal medium (double strength)

Ingredients*1	Amount (g/l)	Ingredient of metals 45*2	Amount (g/l)
KH_2PO_4	0.60	$FeSO_4(NH_4).6H_2O$	14.000
$MgSO_4 \cdot 7H_2O$	0.80	$ZnSO_4.7H_2O$	4.400
L-Glutamic Acid	6.00	$MnSO_4.H_2O$	1.550
$CaCO_3$	0.16	$CuSO_4.5H_2O$	0.310
DL-Aspartic acid	4.00	$CoSO_4.7H_2O$	0.480
DL-Malic acid	2.00	H_3BO_3	0.570
Glycine	5.00	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.640
NH_4HCO_3	1.24	$NaVO_3 \cdot 4H_2O$	0.038
Succinic acid	0.94		
Metal 45*2	0.44		
Thiamin HCl	(1.2 mg)		
Sucrose	30.00		

*1 A half amount of dry mixture was dissolved in 1liter of deionized and distilled water.
*2 Dry mixture of metals 45.

3.2.4 Bioassay for vitamin B_{12}

A half ml of the stock culture of *Euglena* gracilis strain z was added to 5 ml of a pre-incubation medium containing the basal medium with 15 g sucrose and 80 ng vitamin B_{12} per liter. After 2 to 6 days of incubation, a green pigmented culture was selected and transferred into a sterile tube for centrifugation at 3,000 rpm for 10 min. Using an aseptic technique, the supernatant was decanted and the algal cells were suspended in 10 ml

of the sterile pre-incubation medium (vitamin B_{12} free). The algal cells should be washed in this manner three times to reduce B_{12} carry-over. The washed cells were suspended at an appropriate dilution of optical density at 430 nm of wavelength to 0.10-0.15, and transferred one drop by a sterile micro-syringe to 5 ml of test sample solutions or vitamin B_{12} standard solutions, then incubated at 30°C under dark condition. After 7 days of incubation, the test tubes were agitated uniformly and optical density of the test cultures was determined at 430 nm using a spectrophotometer.

3.3 Results

3.3.1 Vitamin requirements

For determining vitamin requirement, the vitamin starved axenic algal cells of *Chattonella marina* were cultured in ESS media containing various vitamins and in a vitamin-free medium as the control. As demonstrated in Fig. 3.1, algal cells completely died within 3 days of incubation in the vitamin-free medium and within 10 days in the media with biotin or biotin and thiamin. However, the algal cells cultured in vitamin B_{12}-containing media were maintained for 25 days and completely died after 30 days of incubation. These findings indicate that vitamin B_{12} is an essential nutrient among the B group vitamins for the growth of *Chattonella marina*.

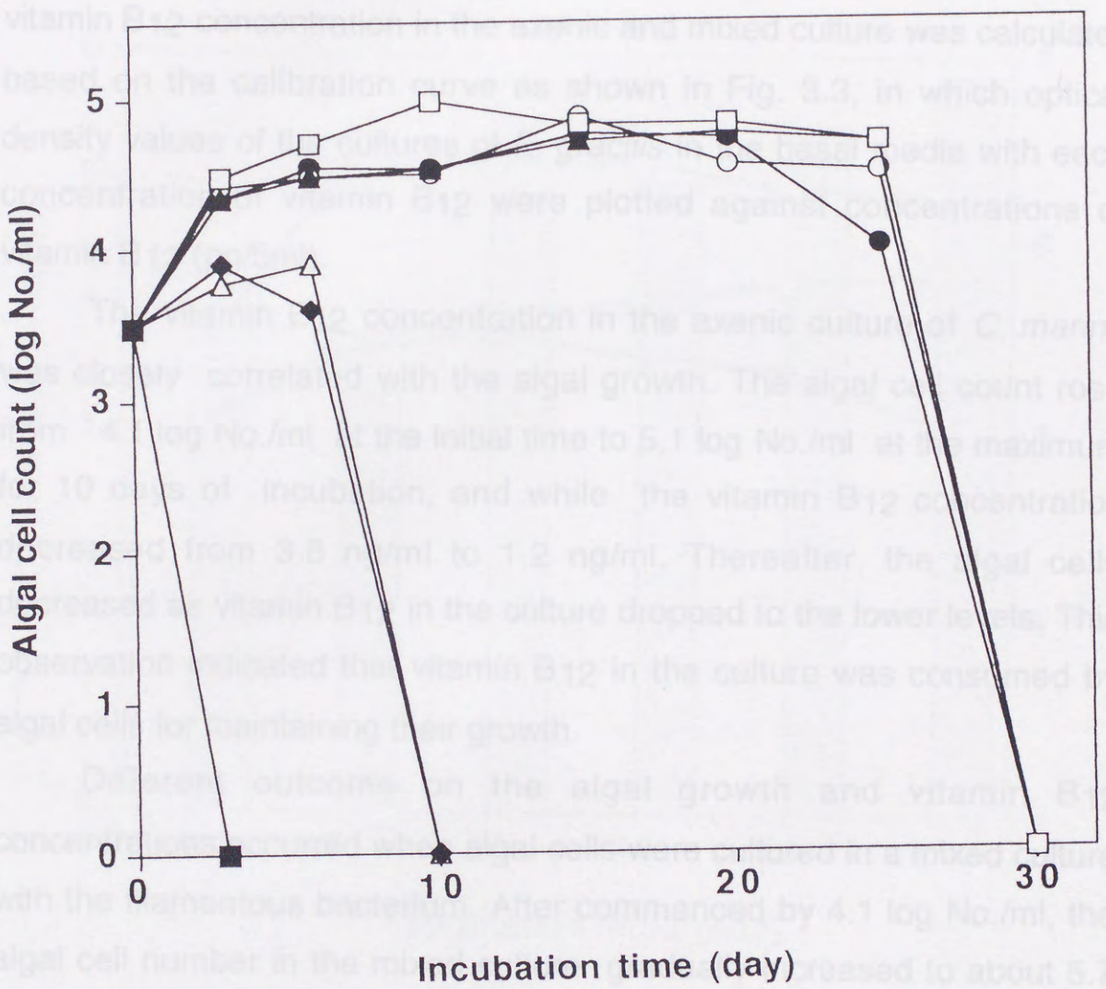

Fig. 3.1. The growth of axenic starved cells of *C. marina* in ESS media containing various vitamins.

■, ESS free-vitamins medium; ◆, ESS medium added with biotin (2 ng/ml); Δ, with biotin (2 ng/ml) and thiamin (200 ng/ml); ●, with vitamin B_{12} (4 ng/ml); ○, with thiamin (200 ng/ml) and B_{12} (4 ng/ml); ▲, with biotin (2 ng/ml) and B_{12} (4 ng/ml; □, ESP containing B_{12}(4 ng/ml), thiamin (200 ng/ml) and biotin (2 ng/ml).

3.3.2 Algal growth with vitamin B_{12} in mixed culture

Growth curves of *C. marina* in an axenic culture and in a mixed culture with a filamentous bacterium (strain FI93-3), as well as vitamin B_{12} concentration profiles in the algal cultures are shown in Fig 3.2. The vitamin B_{12} concentration in the axenic and mixed culture was calculated based on the calibration curve as shown in Fig. 3.3, in which optical density values of the cultures of *E. gracilis* in the basal media with each concentration of vitamin B_{12} were plotted against concentrations of vitamin B_{12} (pg/5ml).

The vitamin B_{12} concentration in the axenic culture of *C. marina* was closely correlated with the algal growth. The algal cell count rose from 4.1 log No./ml at the initial time to 5.1 log No./ml at the maximum for 10 days of incubation, and while the vitamin B_{12} concentration decreased from 3.8 ng/ml to 1.2 ng/ml. Thereafter, the algal cells decreased as vitamin B_{12} in the culture dropped to the lower levels. This observation indicated that vitamin B_{12} in the culture was consumed by algal cells for maintaining their growth.

Different outcome on the algal growth and vitamin B_{12} concentrations occurred when algal cells were cultured in a mixed culture with the filamentous bacterium. After commenced by 4.1 log No./ml, the algal cell number in the mixed culture gradually increased to about 5.7 log No. /ml for 25 days of incubation. In addition, the total viable count of the filamentous bacterium also increased initially from 4.8 log No./ml to 6.9 log No. /ml for 15 days of incubation and then was maintained at 6.5 log No./ml until 25 days of incubation. As the growth of bacterium was enhanced, the concentration of vitamin B_{12} in the culture was slightly dropped from 3.8 ng/ml of initial concentration to 3.3 ng/ml and then increased to 5.8 ng/ml for 25 days of incubation.

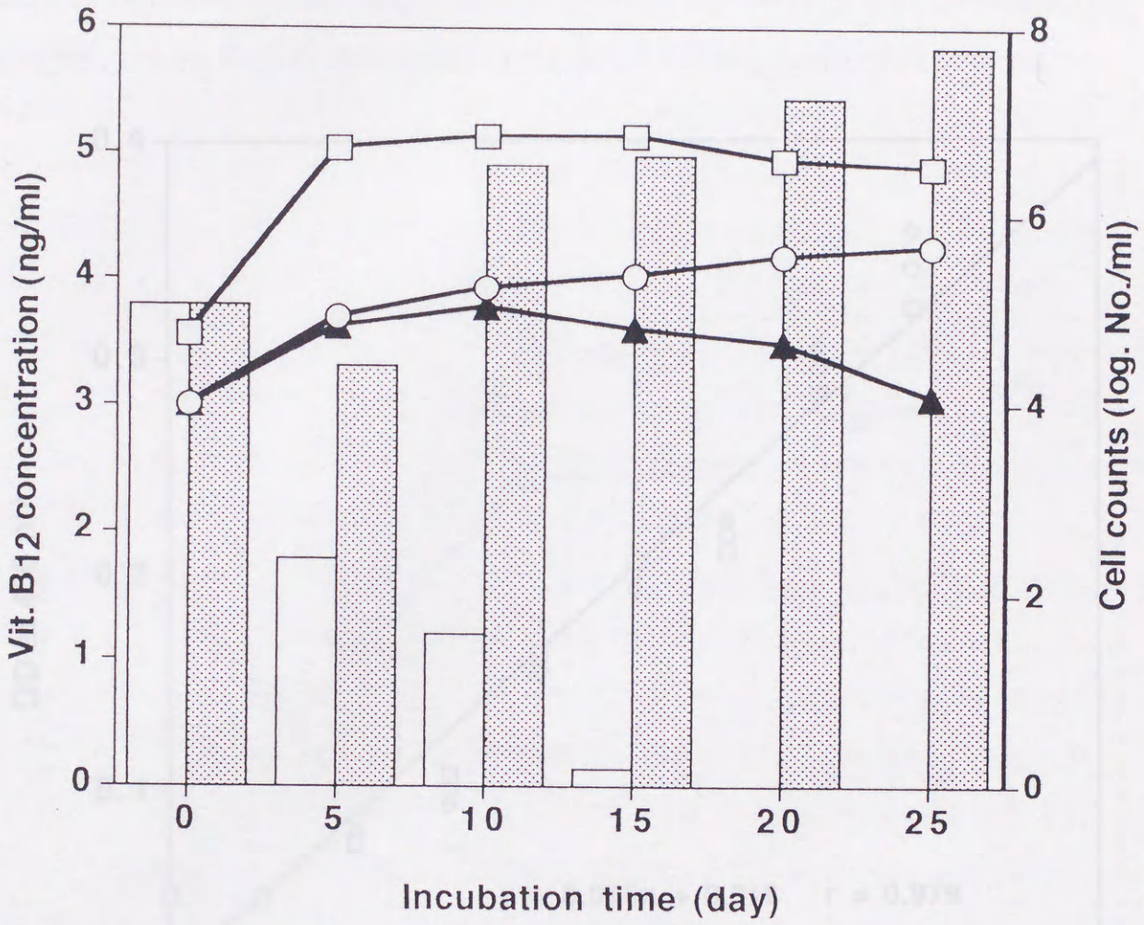

Fig. 3.2. Algal growth and vitamin B_{12} concentration in axenic and mixed cultures.

White columns and patterned columns show vitamin B_{12} concentration in the axenic culture and mixed culture of *C. marina* respectively; ○, algal cell count in the mixed culture with FI93-3; ▲, algal cell count in the axenic culture; □, colony count of FI93-3 in the mixed culture.

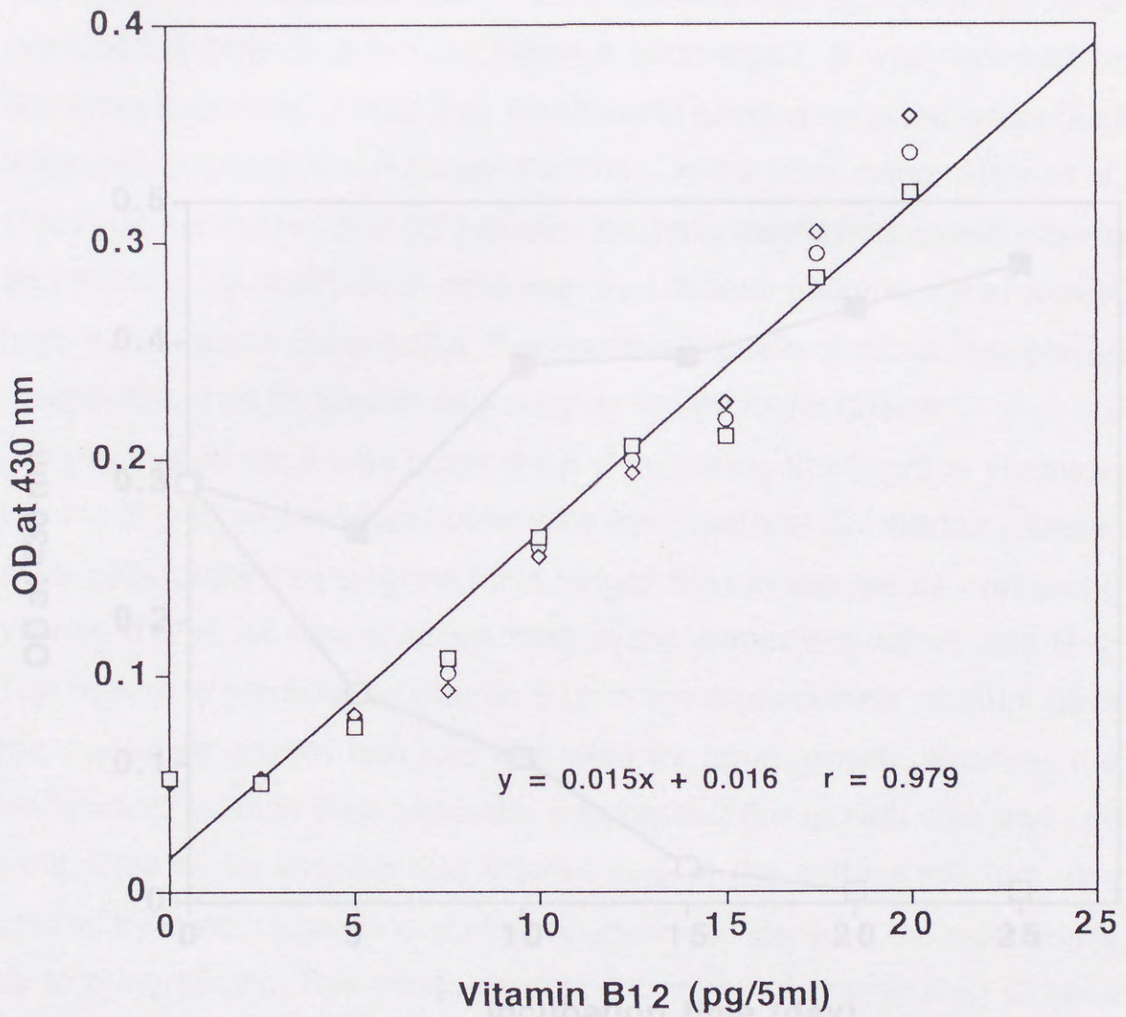

Fig. 3.3. Calibration curve of vitamin B_{12} bioassay using *Euglena gracilis* strain *z*.

Optical density of the culture of *Euglena gracilis* used for bioassay of vitamin B_{12} decreased in the culture filtrate from an axenic culture of *Chattonella marina*, while it increased in the culture filtrate of non-axenic algal culture with the filamentous bacterium as shown in Fig. 3.4. The results obtained in this study indicated that vitamin B_{12} was produced by the bacterium FI93-3, and it was effective for the growth of *C. marina*.

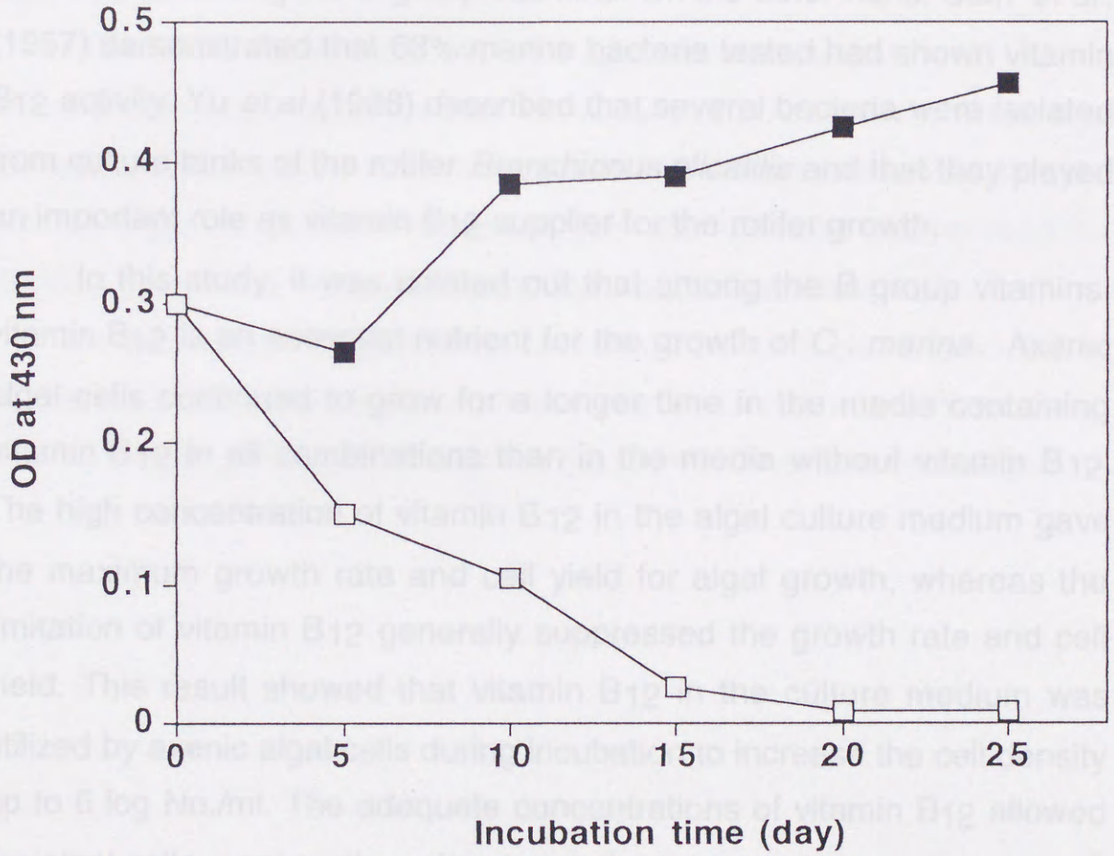

Fig. 3.4. Growth response of *Euglena gracilis* strain *z* for 7 days incubation in the culture filtrates of axenic (□) and mixed (■) cultures of *C. marina* with FI93-3 obtained at different incubation times.

3.4 Discussion

Qualitative and quantitative studies on vitamin B_{12} in the fresh and sea water environments have been carried out by a number of investigators. Vitamin B_{12}-active compounds have been shown to be essential or stimulatory for the growth of various marine microorganisms, especially microalgae. Carlucci (1974) reported that *Dunaliella teriolecta* produced vitamin B_{12} but not other 4 microalgae. It was reported by Nishijima and Hata (1986) that *Chattonella antiqua* required essentially vitamin B_{12} among the B group vitamins. On the other hand, Starr *et al.*, (1957) demonstrated that 63% marine bacteria tested had shown vitamin B_{12} activity. Yu *et al.*(1988) described that several bacteria were isolated from culture tanks of the rotifer *Branchionus plicatilis* and that they played an important role as vitamin B_{12} supplier for the rotifer growth.

In this study, it was pointed out that among the B group vitamins, vitamin B_{12} is an essential nutrient for the growth of *C . marina*. Axenic algal cells continued to grow for a longer time in the media containing vitamin B_{12} in all combinations than in the media without vitamin B_{12}. The high concentration of vitamin B_{12} in the algal culture medium gave the maximum growth rate and cell yield for algal growth, whereas the limitation of vitamin B_{12} generally suppressed the growth rate and cell yield. This result showed that vitamin B_{12} in the culture medium was utilized by axenic algal cells during incubation to increase the cell density up to 6 log No./ml. The adequate concentrations of vitamin B_{12} allowed the algal cells apparently active during the logarithmic growth phase. *C. marina* required vitamin B_{12} essentially for its cell growth and division. Therefore, this compound must be supplied by other microorganisms for *C. marina* cells to grow in the culture after it was exhausted.

Filamentous bacteria were isolated from non-axenic culture of *C. marina* and were tentatively identified as *Flexibacter* spp., as described in the previous chapter. Filamentous bacteria formed multicellular rods and small, whitish-pink and rough colonies. The addition of these bacteria to the culture of the axenic algal strain resulted in the increase of algal cell yield and extension of algal growth as compared with the axenic culture. It was expected that the algal cells utilized vitamin B_{12} added initially to the culture medium for the early incubation period until co-existent bacteria began to grow at the maximum rate to produce vitamin B_{12} vigorously.

The same filamentous bacterial species was also isolated as one of the predominant bacteria from the culture of *H. akashiwo*, which was isolated from the bloom occurred in April 1995 in Kagoshima Bay, and the culture water of *Tetraselmis* sp., which was cultured as the food for Kuruma prawn in an aquaculture facility, Kagoshima Prefecture.

These results suggest that certain co-existent bacteria including filamentous bacteria might provide some essential nutrients such as vitamin B_{12} to give an advantage for the growth of various microalgae.

Chapter 4

Characteristics of Filamentous Bacteria Co-existing with Some Marine Microalgae

4.1 Introduction

Previously the author described that stock cultures of *C. marina* (Raphidophyceae) contain several co-existents bacterial strains and that filamentous bacteria were isolated as one of the predominant bacteria. Moreover, filamentous bacteria were also isolated as predominant bacteria from the culture of *Heterosigma akashiwo* (Raphidophyceae), which caused a red tide in 1995 in Kagoshima Bay, and found to be in the cultures of *Tetraselmis* sp. (Prasinophyceae), which was cultured as food for juvenile of Kuruma prawns.

The addition of co-existent bacteria to axenic cultures of microalgae often results in a large increase in the biomass and extending growth periods of microalgae, while microalgae fail to grow for longer period in axenic culture as described in the previous chapter. Co-existent bacteria can grow depending on organic matters derived from microalgae and on the other hand they stimulate algal growth with production of growth factors for algae including vitamin B_{12}. Despite their frequent detection in association with marine microalgae, these filamentous bacteria have not been characterized to clarify the taxonomic position.

Biochemical, physiological and morphological tests have been done for a long time by microbiologists to characterize and identify the microbial isolates. The use of electron microscopy with a high magnification has been employed to observe the cell structure in detail.

In the present chapter the author attempts to characterize filamentous bacteria isolated from the cultures of marine microalgae by morphological and biochemical tests.

4.2 Materials and Methods

4.2.1 Bacterial strains

Filamentous bacteria strains FI90-32, FI93-1, FI93-3 and FI94-1 were isolated from the stock culture of *C. marina*. Filamentous strains FI95-1, FI95-5, FI95-6 and FI95-10 were isolated from the culture of *H. akashiwo*. Filamentous strains FI96-1 and FI96-2 were isolated from the culture of *Tetraselmis* sp. Bacterial strains were grown at 25°C in a ZE-CI liquid medium or in a ZE-CI agar medium as described in the previous chapter.

4.2.2 Characterization of bacterial strains

Bacteriological characterization as carried out based on morphological and physiological characters (colony, cell form, pigmentation, Gram stain and motility), biochemical reactions (catalase, oxidase, Hugh and Leifson test, indole production, MR and VP tests), and macromolecular degradation tests (casein, starch and DNA). Salinity for bacterial growth was examined using ZoBell modified liquid media (ZE-CI) without NaCl or with 3% NaCl or artificial sea water (ASW) as described earlier.

4.2.3 Pigment analysis

Bacterial cells were harvested from ZE-CI agar plates (1.5% agar) which were incubated at 25°C for 4-5 days. Pigments were extracted from cells with cold ethanol (30 ml) for one day at 4°C and filtered through a filter paper (Toyo No. 2). The filtrate was then mixed with acetone (40 ml) and *n*-hexane (20 ml) and let stand for 30 min. Pigments in *n*-hexane layer was separated and washed three times with distilled water to remove ethanol and acetone. Visible absorption spectra of extracted

pigments were recorded using a multipurpose spectrophotometer (Shimadzu, MPS-2000).

4.2.4. Photosynthetic pigment analysis

About 1.75 g of bacterial cells were harvested from ZE-CI agar plates incubated aerobically at 25°C for 4-5 days under illumination (3,000 lx in the light cycle of 14L: 10D) or in the dark condition. Pigments were extracted from cells with methanol (5 ml) for 2-3 hours at 4°C and centrifuged at 10,000 rpm for 10 min. The visible absorption spectra of extracted pigments were recorded using a multipurpose UV/VIS spectrophotometer (JASCO Ubest-30).

4.2.5 Scanning electron microscopy

Bacterial strains were cultured in a L-shape test tube containing 10 m*l* of ZE-CI medium at 25°C for 3 days with rotation. Preparation of samples for scanning electron microscopy was carried out by the modified method of Maeda *et al.*(1992). The appropriate bacterial suspension was fixed with 1% glutaraldehyde and then fixed cells were concentrated onto a membrane filter (0.2 μm, Nucleopore) under moderate vacuum. Bacterial cells on the filters were dehydrated stepwise using a series of ethanol solutions. Successively ethanol was substituted by *t*-butyl alcohol, and kept in cold storage. The filters were dried by means of a freeze-dryer (Eiko, VFD-21) and then coated by gold with an ion coater (Eiko, IB-3). Bacterial samples were observed and photographed by using a scanning electron microscope (Hitachi, Model S-4100 H).

4.2.6 Epifluorescence microscopy

The appropriate bacterial suspension was fixed with 1% glutaraldehyde, then 10 drops of 4',6-diamidino-2-phenylindole (DAPI) at the final concentration of 5 μg/ml were added. Sample was mixed well and stored in a cold room for 20 min. Bacterial cells were concentrated onto a 0.2 μm Nucleopore filter under moderate vacuum. The filter soaked with low fluorescence immersion oil were mounted on a glass slide, and examined immediately by an epifluorescence microscope (Olympus BH-2) with an ultraviolet excitation filter.

4.3 Results

4.3.1 Characterization of bacterial strains

The main physiological and biochemical characteristics of filamentous bacteria isolated from *C. marina*; *H. akashiwo* and *Tetraselmis* sp., are given in Table 4.1. Those strains were all Gram-negative, filamentous form, negative in both flagellar and gliding motility, catalase positive, no acid production from glucose in Hugh & Leifson medium, negative in indole production test as well as MR and VP tests. All strains except FI95-6 were negative in hydrolysis of casein, starch, and DNA. Most strains were also weak in oxidase activity, while strain FI95-6 was negative. Their colony morphology on ZE-CI agar plates was small round shape with irregular margin, and the colony colors were whitish pink (WP) for a majority of strains, except greenish orange (GO) for FI95-5, and orange (O) for FI95-6. All strains grew well in a peptone-yeast extract medium with sea water (ZE-CI medium) but they failed to grow in the medium containing 0-3% NaCl.

Table 4.1 Biochemical and physiological characteristics of filamentous bacteria

Characteristics	FI90-32	FI93-1	FI93-3	FI94-1	FI95-1	FI95-5	FI95-6	FI95-10	FI96-1	FI96-2
Colony color	WP*1	WP	WP	WP	WP	GO	O	WP	WP	WP
Gram	-	-	-	-	-	-	-	-	-	-
Shape	fil*2	fil	fil	fil	fil	fil	fil	fil	fil	fil
Motility	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	±	±	±	±	±	±	-	±	±	±
Hugh & Leifson	NC*3	NC	NC	NC	NC	NC	NC	NC	NC	NC
Growth in 0% NaCl	-	-	-	-	-	-	-	-	-	-
3% NaCl	-	-	-	-	-	-	-	-	-	-
3/4 ASW	+	+	+	+	+	+	+	+	+	+
Hydrolysis of casein	-	-	-	-	-	-	+	-	-	-
Hydrolysis of starch	-	-	-	-	-	-	++	-	-	-
Hydrolysis of DNA	-	-	-	-	-	-	+	-	-	-
Indole production	-	-	-	-	-	-	-	-	-	-
VP test	-	-	-	-	-	-	-	-	-	-
MR test	-	-	-	-	-	-	-	-	-	-

*1 WP, whitish pink; GO, greenish orange; O, orange. *2 fil, filamentous form. *3 NC, no change.

4.3.2 Epifluoresence and scanning electron micrographs

Epifluoresence micrograph and scanning electron micrograph of filamentous bacterium strain FI90-32 are shown in Fig. 4.1. Needle-like fine filaments with rounded ends, which were mostly 0.8-1.2 μm wide and 4-30 μm long, were observed with the microscopes, although much longer filaments were also present.

4.3.3 Cellular pigments

Cellular pigments of filamentous bacteria were extracted with ethanol and transferred into *n*-hexane. Visible absorption spectra of a crude pigment solution in *n*-hexane were recorded by using a spectrophotometer. As shown in Fig. 4.2 and Table 4.2, absorption maxima (λ max) were observed at 455, 482 and 513 nm for whitish pink (WP) strains. λ max for strains FI95-5 (GO strain) and FI95-6 (O strain) were at 412, 436 and 466 nm and at 447 and 472 nm, respectively and were apparently different from those for WP strains.

Table 4.2. Absorption maxima of bacterial pigments

Pigments (colony)	Solvent	Max (nm)
FI90-32 (WP)	n-hexane	455, 482, 513
	acetone	481, (505)
FI93-3 (WP)	n-hexane	455, 481, 512
FI95-1 (WP)	n-hexane	455, 481, 512
FI95-7 (WP)	n-hexane	455, 481, 512
FI95-10 (WP)	n-hexane	455, 481, 512
FI95-5 (GO)	n-hexane	412, 436, 512
FI95-6 (O)	n-hexane	447, 472
Flexixanthin	acetone	483, 510
Dehydro-flexixanthin	acetone	485, (505)
Spheroidenone	acetone	(455), 484, (505)

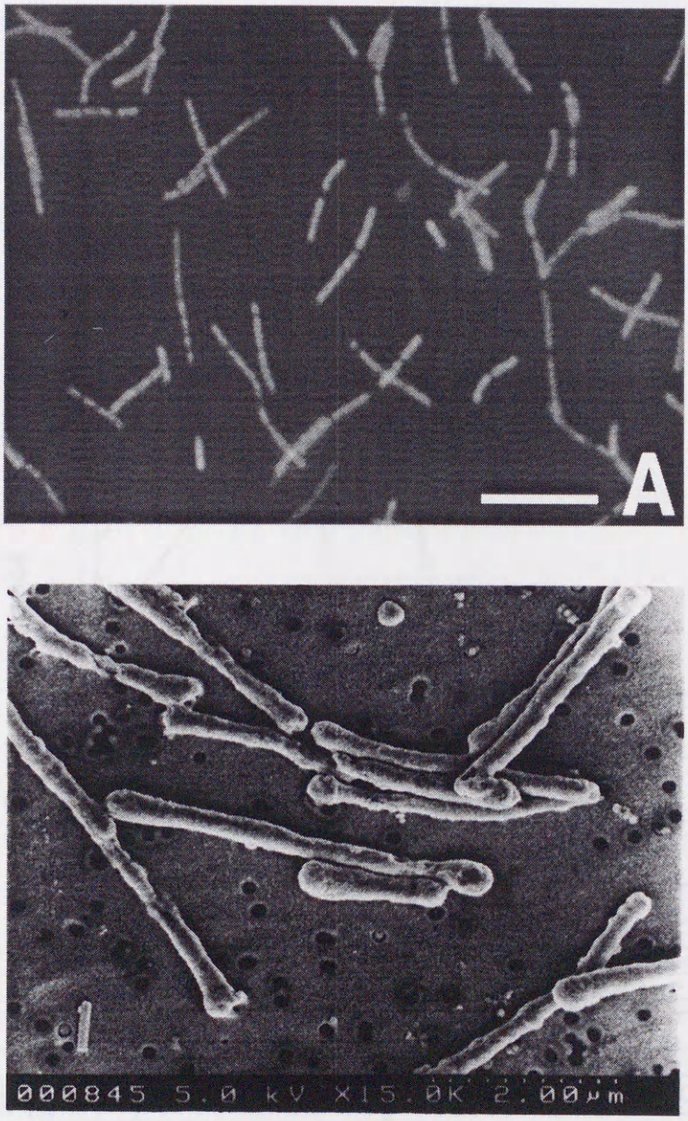

Fig. 4.1. Epifluorescence (A) and scanning electron (B) micrographs of a flamentous strain Fl90-32. Bar indicates 15 µm.

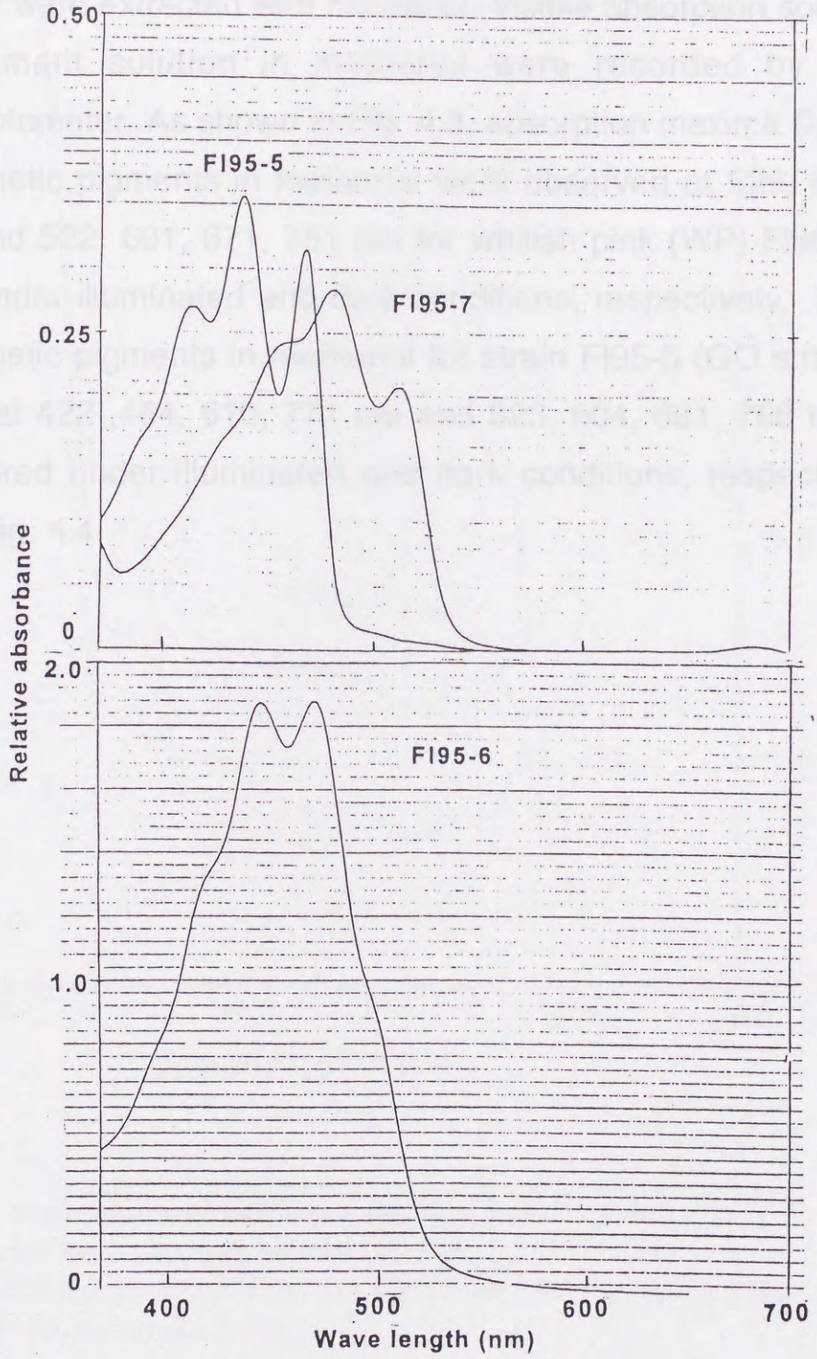

Fig. 4.2 Absorbace spectra of the *n*-hexane extracted pigment fractions derived from the filamentous bacterial strains including whitish pink (FI95-7), greenish orange (FI95-5) and orange (FI95-6) strains.

4.3.4 Photosynthetic pigment analysis

Photosynthetic pigments of filamentous bacteria cultured aerobically were extracted with methanol. Visible absorption spectra of a crude pigment solution in methanol were recorded by using a spectrophotometer. As shown in Fig. 4.3, absorption maxima (λ max) of photosynthetic pigments in methanol were observed at 529, 610, 680, 752 nm and 522, 601, 671, 751 nm for whitish pink (WP) FI96-1 strain cultured under illuminated and dark conditions, respectively. λ max of photosynthetic pigments in methanol for strain FI95-5 (GO strain) were observed at 422 ,464, 610, 771 nm and 523, 604, 681, 766 nm which were cultured under illuminated and dark conditions, respectively, as shown in Fig. 4.4.

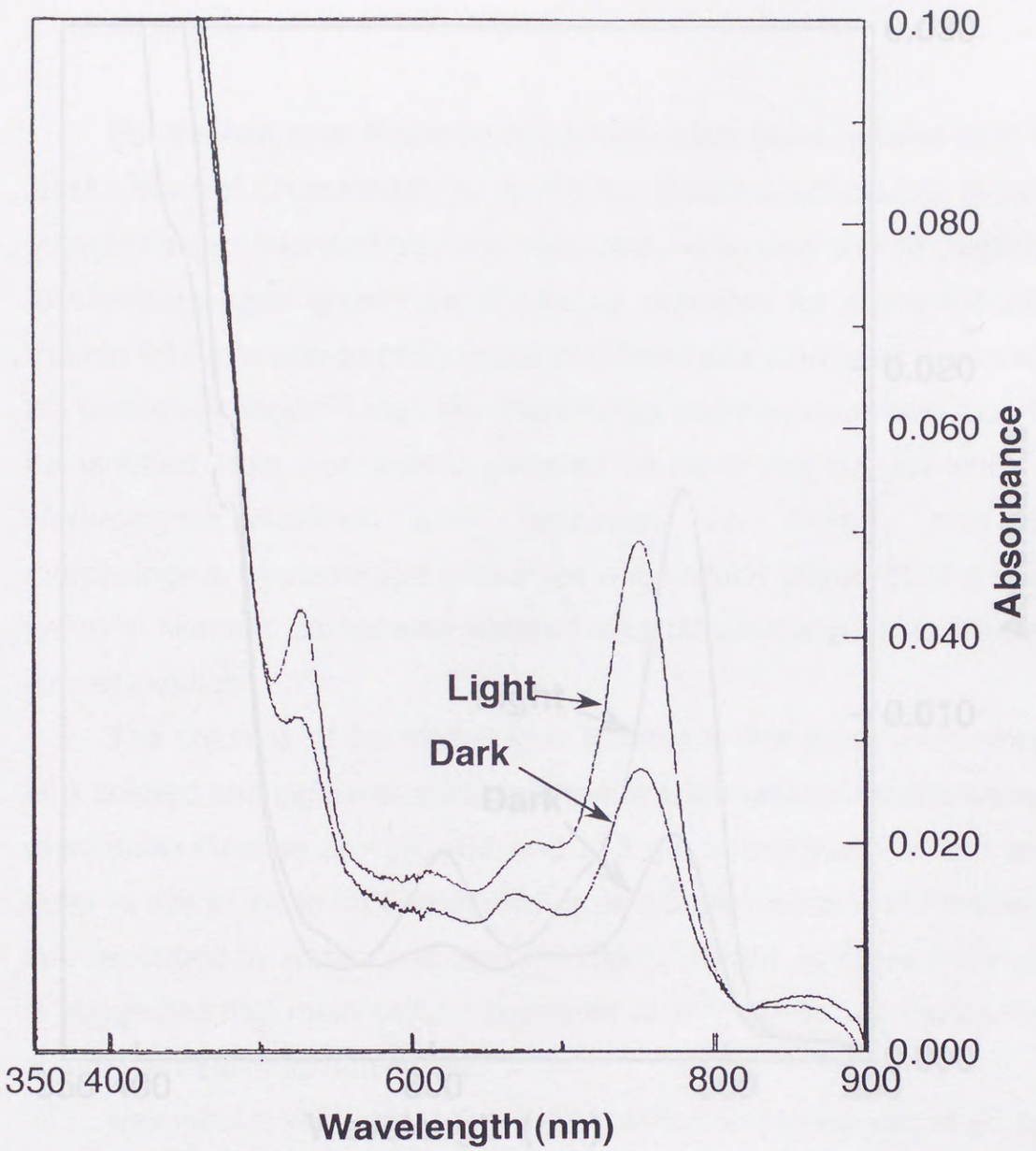

Fig. 4.3. Absorbance spectra of the methanol-extracted pigment fractions derived from the filamentous bacterial strain FI96-1.
The profiles of the pigment fractions extracted from FI96-1 cultured under light and dark conditions are shown.

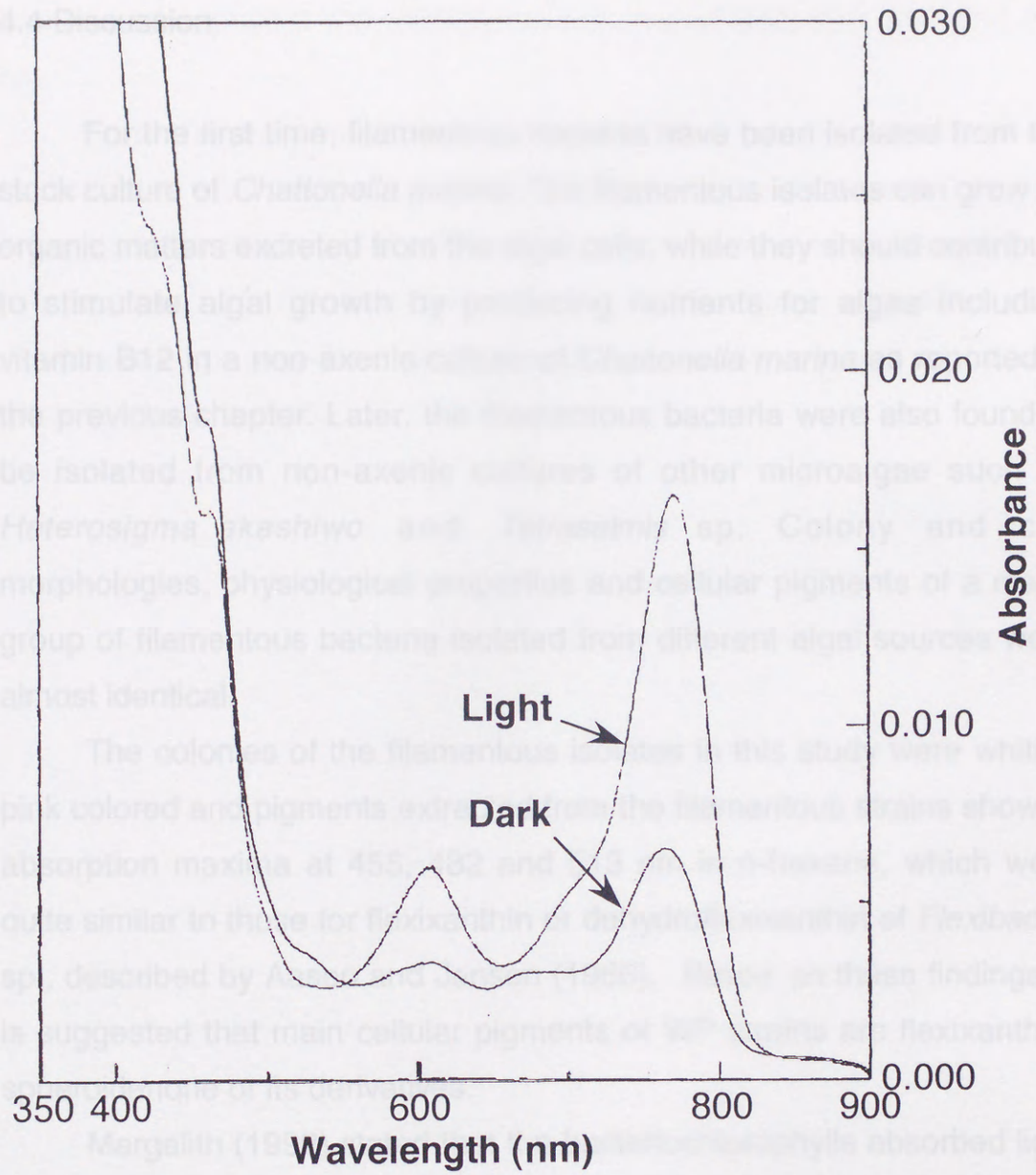

Fig. 4.4. Absorbance spectra of the methanol-extracted pigment fractions derived from the filamentous bacterial strain FI95-5.
The profiles of the pigment fractions extracted from FI95-5 cultured under light and dark conditions are shown.

4.4 Discussion

For the first time, filamentous bacteria have been isolated from the stock culture of *Chattonella marina*. The filamentous isolates can grow on organic matters excreted from the algal cells, while they should contribute to stimulate algal growth by producing nutrients for algae including vitamin B12 in a non-axenic culture of *Chattonella marina* as reported in the previous chapter. Later, the filamentous bacteria were also found to be isolated from non-axenic cultures of other microalgae such as *Heterosigma akashiwo* and *Tetraselmis* sp. Colony and cell morphologies, physiological properties and cellular pigments of a major group of filamentous bacteria isolated from different algal sources were almost identical.

The colonies of the filamentous isolates in this study were whitish pink colored and pigments extracted from the filamentous strains showed absorption maxima at 455, 482 and 513 nm in n-hexane, which were quite similar to those for flexixanthin or dehydroflexixanthin of *Flexibacter* sp., described by Aasen and Jensen (1966). Based on these findings, it is suggested that main cellular pigments of WP strains are flexixanthin, spheroidenone or its derivatives.

Margalith (1992) stated that the bacteriochlorophylls absorbed light in the near infra-red region (660-870 nm). The photosynthetic pigment analysis of bacteria as showed on FI96-1(WP) and FI95-5 (GO) strains exhibited the absorption maxima in methanol at around 752 nm and 771 nm, respectively. Shiba, *et al.*, (1979) reported that the methanol extracted pigment of the pink-pigmented bacteria had absorption maxima at 770 to 772 nm, and that was identified as bacteriochlorophyll a. While, Nagamine *et al.*, (1996) reported that the cell culture of *R.hodopseudomonas sphaeroides* was observed containing

bacteriochlorophyll a with absorption maxima at 380, 600, 800 and 850 nm.

From the presence of bacteriochlorophyll a, aerobic photosynthetic bacteria can grow heterotrophycally, and they utilize light as an auxiliary energy source (Shiba, *et al.*, 1991). The bacteria isolated commonly from the culture of *Chattonella marina, Heterosigma akashiwo,* and *Tetraselmis* sp. may belong to certain species in a group of aerobic photosynthetic bacteria.

Chapter 5

DNA Isolation, GC Content and Dot Hybridization of Filamentous Bacteria

5.1 Introduction

In recent years, the molecular approaches to microbial identification and systematics such as protein, cell wall and nucleic acid analyses, have become accepted by microbial taxonomists (Johnson, 1985; Priest and Austin, 1993; Tamaoka and Komagata, 1984; Yamamoto, Ezaki and Kato, 1991). Nucleic acid analysis has been developing as determination techniques of mol% of GC content of DNA, DNA-DNA hybridization, DNA restriction patterns and DNA base-sequence because only chromosomal DNA and RNA are unaffected by growth conditions among the various chemical component used for bacterial taxonomy.

The duplex structure of DNA and hydrogen bonding between guanine (G) and cytosine (C), and between adenine (A) and thymine (T) bases, ensures that equivalent amounts of G+C and A+T are represented in the molecule. The content of mol% G+C in the DNA molecule from bacteria varies within very broad limits from a minimum of about 25% to a maximum of approximately 75% as represented in some mycoplasma and streptomycetes, respectively (Priest and Austin, 1993). By determination of the base composition of mole %G+C, the bacterial isolates can be differentiated in genus level.

Quantitative DNA hybridization is an indispensable method for determining genetic relatedness among the type of the most phenotypically related strains. This approach is far direct and quick means to determine whether the isolated strains are identically or closely related each other. Moreover, base on the DNA hybridization, the structure of bacterial community will be understood. Unfortunately, the use of DNA-DNA hybridization method needs radioisotope for labeling DNA (Ezaki, *et al.*, 1988A), involves complex procedures and takes

several days to complete experiment((Ezaki, *et al.*, 1988[a]; Ezaki, *et al.*, 1988[b]; Ezaki, *et. al.*, 1988[c]).

Recently by using a photobiotin method for labeling DNA, the labeling procedure became quite simple, and results will be quickly analyzed. Various hybridization techniques have been used widely to identify the bacterial gene expression differences including mRNA-based isothermal subtractive hybridization (Utt, *et. al.*, 1995), dot hybridization, southern hybridization, fluorometric hybridization (Lee, *et. al.*, 1992; Ezaki, *et. al.*, 1988) and detect the presence of pathogenic bacteria, such as *E. coli* and *Legionella* species by using DNA colony hybridization and microplate hybridization methods, respectively (Hill, *et. al.*, 1983; Ezaki, *et. al.*, 1990).

In the present work, by using the purified chromosomal DNA, the determination of GC content (mol% G+C) and DNA-DNA dot hybridization were done to clarify the taxonomic and genetic relationship among bacterial isolates and other representative reference strains.

5.2 Materials and Methods

5.2.1 Bacterial strains

Filamentous bacteria strains FI90-32, FI93-1, FI93-3 and FI94-1 were isolated from the stock culture of *C. marina*. Filamentous strains FI95-1, FI95-5, FI95-6, FI95-7 and FI95-10 and Pi95-4 were isolated from the culture of *H. akashiwo*. Filamentous strains FI96-1 and FI96-2, and a rod strain Ps96-1 were isolated from the culture of *Tetraselmis* sp. Reference strains including *Cytophag lytica* (IFO 14961), *C. marinoflava* (IFO 14170), *C. uliginosa* (IFO 14962), and *Flexibacter elegans* (IFO 15055) were obtained from Institute for Fermentation, Osaka (IFO). Bacterial strains were grown at 25°C in a ZE-CI liquid medium or on a ZE-CI agar medium as described in the previous chapter.

5.2.2 DNA isolation and purification

Bacterial cells (ca. 400 mg wet weight) were harvested from ZE-CI agar plates after incubation at 25°C for 4-5 days and suspended in 8.0 ml of TE-buffer (10 mM Tris HCl and 1 mM EDTA-2Na, pH 8.0) added with 900 μl of 5% sodium dodecyl sulfate (SDS) solution and 150 μl protease K solution (6 mg/ml), and the suspension was incubated at 37°C for 60 minutes. The cell lysate was added with 1.5 ml of 5 M NaCl and 1.2 ml of CTAB-NaCl (100 mg cetyltrimethylammonium bromide and 41 mg NaCl in 1 ml of distilled water), and incubated at 65°C for 10 minutes. Chromosomal DNA was isolated and purified by using the modified Marmur method (Marmur, 1961). For enzymatic hydrolysis of RNA, 1 ml of DNA solution was mixed with 100 μl of RNAase A solution (1 mg RNAase A was dissolved in 1 ml of the mixed solution of 10 mM Tris HCl and 15 mM NaCl, pH 7.5), and incubated at 37°C for 60 minutes. After deproteinized with a phenol-chloroform-isoamylalcohol mixture, the purified DNA was dissolved in TE-buffer at 2.5 μg/ml.

5.2.3 Determination of GC contents

For hydrolyzing DNA into nucleotides, the 100 μl of nuclease P1 (Yamasa) was added to DNA solution and the mixture was incubated at 50°C for 60 minutes. After centrifugation at 8,000 rpm for 10 minutes, the mixture was then filtered through a Millipore filter (DISMIC-3, 0.45 μm) and the hydrolysate containing nucleotides was analyzed by an HPLC system (LC-10A, Shimadzu), equipped with reversed-phase Cosmosil packed column 5C18-AR (Nacalai Tesque; 4.6 x 250 mm) (Katoh *et al.*, 1983). Nucleotides were eluted with a mixture of 10 mM H_3PO_4 and 10 mM KH_2PO_4 (pH 3.0) at a flow rate 0.5 - 0.7 ml /min. Standard mixture of nucleotides (Yamasa) was used as the reference for calibrating mol% of GC content.

5.2.4 DNA -DNA dot hybridization

DNA-DNA dot hybridization was carried out based on the modified method of Ezaki (Ezaki *et al.*, 1988; Ezaki *et al.*, 1989). Fifty μl of DNA solution (1.0 μg/μl) from representative strains including FI90-32, FI93-1, FI95-5, Ps 96-1 and *F. elegans*, were dissolved with 50 μl of Photobiotin (1.0 μg/μl; Vector Laboratories) solution. The mixture in an ice bath was irradiated under a 300 - 500 W sun-lamp for 15 minutes. One hundred μl of 0.1 M Tris-HCl (pH 9.5) were added to the mixture. The mixture was then treated at least three times by adding 200 μl of *n*-butyl alcohol and centrifuged at 12,000 rpm for 3 minutes, then the upper layer of supernatant was discarded. Two hundred μl of purified biotin-labeled DNA were boiled for 5 minutes, then mixed well with 5 ml of hybridization solution (5 ml of pre-hybridization solution with 0.125 gr of Sodium dextran sulfate) and stocked in a refrigerator just prior to use. Fifty μl DNA solutions (200 μg/ml) of tested strains were boiled for 5 minutes, then added with 200 μl of 4 x SSC solution (saline sodium citrate). About 8 μl of the mixture were spotted on a membrane filter (Micron Separation Inc.) and dried at 80°C for 30 minutes. The treated filter was soaked in 5 ml of pre-hybridization solution described by Ezaki *et al.*,(1988) and shacked for 30 minutes at room temperature. The solution was discarded, and the filter was again soaked in 5 ml of biotinylated DNA solution (DNA probe) for 3 hours at 45°C. The solution was discarded, and the filter was washed several times with 2 X SSC/0.5% SDS solution at room temperature, 37°C, and 68°C. Finally, the filter was washed 3 times by soaking in 5 ml of 20 X PBS solution (16 g, NaCl : 0.4 g, KCl : 2.3 g, Na_2HPO_4 : 0.4 g, KH_2PO_4 : 100 ml , distilled water). The washed filter was placed into a petri dish and washed again three times by soaking in 5 ml of TTBS solution (0.1 M Tris, 0.15 M NaCl, and 0.1% Tween 20, pH 7.5) for 3, 3, and 4 minutes, respectively. After that the

filter was soaked in Vectstain ABC-AP solution (5 ml of TTB solution added with 1 drop of A and B reagents of Vecstain ABC-alkaline phosphatase (AP) kit. (Vector Lab.) for 30 minutes, and the mixture was discarded, then the filter was washed three times by soaking in TTBS solution. The washed filter was then placed into a new dish containing 5 ml of alkaline phosphatase (AP) solution (5 ml of 0.1 M Tris - HCl (pH 9.5). After 10 minutes, the solution was discarded and the filter was washed three times with TTBS solution. The dark blue coloration at spotted areas was observed.

5.3 Results

5.3.1 Mol % GC contents

As shown in Table 5.1, 12 isolated strains and 4 reference strains of the genera *Cytophaga* and *Flexibacter* were selected for determination of GC content of DNA.

The values of GC mol% of DNAs extracted from filamentous bacteria isolates were in a range from 58.7 to 60.0 for all WP strains and FI95-5 (GO strain), while those of FI95-6 (O strain) and Pi95-4 were 32.9 mol% and 36.7 mol%, respectively. The DNA GC mol% of reference strains were obtained as 31.9 for *C. lytica*, 37.3 for *C. marinoflava*, 42.0 for *C. uliginosa*, and 47.9 for *F. elegans*.

5.3.2 DNA-DNA dot hybridization

Taxonomic and genetic relationship among the filamentous bacteria was clarified by a DNA-DNA dot hybridization method as illustrated in Fig. 5.1 and Table 5.2. When whole cell DNAs extracted from strains FI90-32, FI93-1 and FI95-5 (WP strains) were used as DNA probes, those of all WP strains and FI95-5 (GO strain) were hybridized with the DNA probe examined but not with DNA from FI95-6 (O strain). When DNAs extracted

from a reference strain of *F. elegans* and an isolate Ps96-1 were applied as DNA probes, neither of whole cell DNAs from all WP filamentous strains reacted with them. Furthermore, DNA probes prepared from WP strains such as FI90-32, FI93-1 and FI95-5 were hybridized with none of DNA molecules from reference strains (*C. lytica*, *C. marinoflava*, *C. uliginosa*, and *F. elegans*).

Table 5.1 GC% of filamentous bacteria and other type strains

No.	Strains	Sources	GC%
1	FI90-32 (WP)	*C. marina*	59.6
2	FI93-1 (WP)	*C. marina*	58.7
3	FI93-3 (WP)	*C. marina*	58.9
4	FI94-1 (WP)	*C. marina*	60.0
5	FI95-1 (WP)	*H. akashiwo*	59.4
6	FI95-5 (GO)	*H. akashiwo*	59.9
7	FI95-6 (O)	*H. akashiwo*	32.9
8	FI95-10 (WP)	*H. akashiwo*	59.4
9	Pi95-4 (P)	*H. akashiwo*	36.7
10	FI96-1 (WP)	*Tetraselmis* sp	59.8
11	FI96-2 (WP)	*Tetraselmis* sp	60.0
12	Ps96-1 (O)	*Tetraselmis* sp	33.8
13	*Cytophaga lytica*	IFO 14961	31.9
14	*Cytophaga marinoflava*	IFO 14170	37.3
15	*Cytophaga uliginosa**	IFO 14962	42.0
16	*Flexibacter elegans*	IFO 15055	47.9

**Flavobacterium uliginosum*

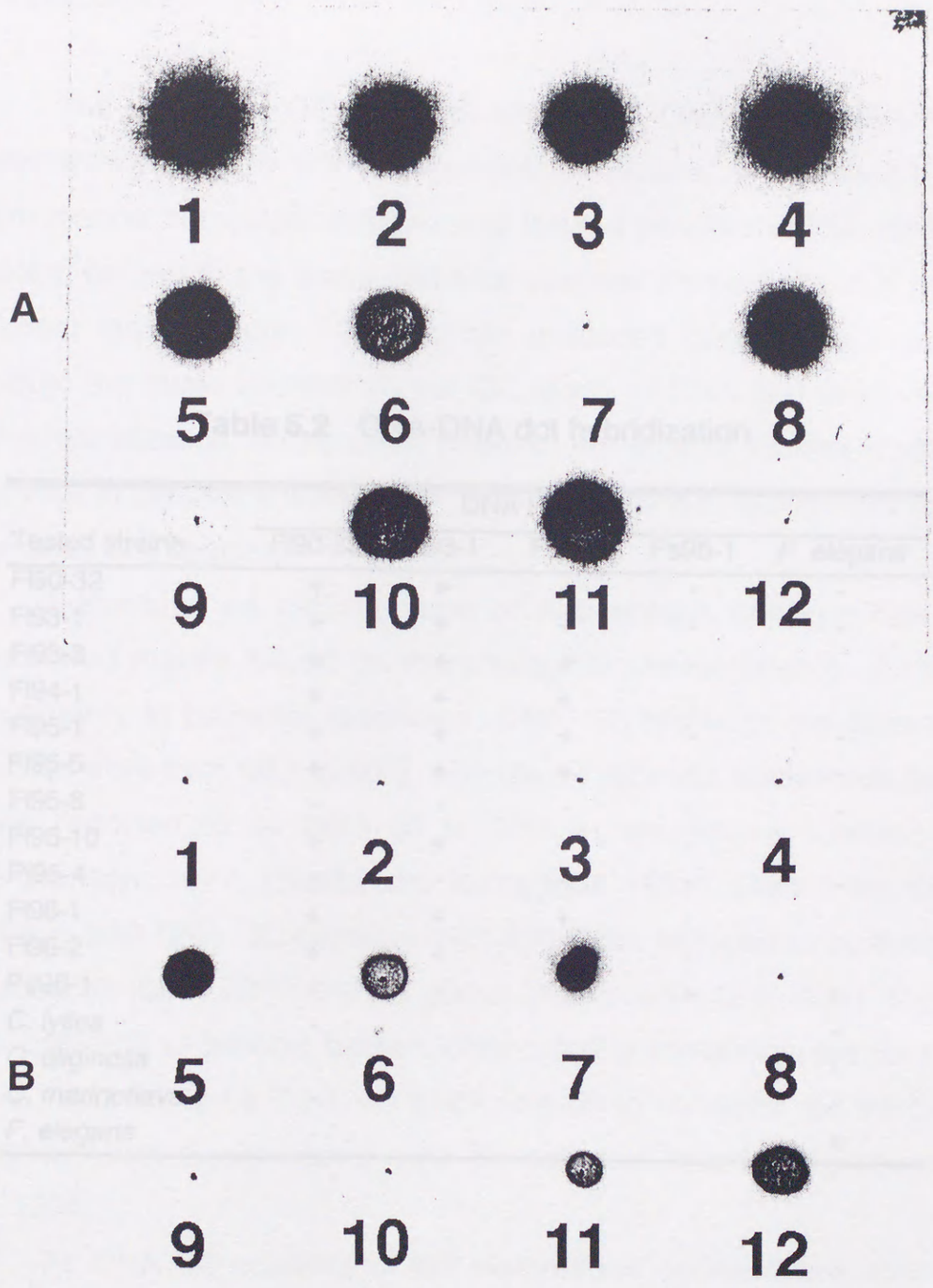

Fig. 5.1. DNA-DNA dot hybridization of whole cell DNAs of filamentous bacteria with biotin-labeled DNA probe from a strain Fl90-32.

A: 1, Fl90-32; 2, Fl-93-1; 3, Fl-93-3; 4, Fl94-1; 5, Fl95-1; 6, Fl95-5; 7, Fl95-6; 8, Fl95-10; 9, Pi95-4; 10, Fl96-1; 11, Fl96-2; 12, Ps96-1.

B: 1, *C. lytica*; 2, *C. uliginosa*; 3, *C. marinoflava*; 4, *F. elegans*; 5, Fl90-32; 6, Fl95-5; 7, Fl93-1; 8, Ps96-1; Pi95-4; 10, Fl95-6; 11, Fl96-1; 12, Fl95-1.

Table 5.2 DNA-DNA dot hybridization

Tested strains	DNA Probes				
	FI90-32	FI93-1	FI95-5	Ps96-1	*F. elegans*
FI90-32	+	+	+	-	-
FI93-1	+	+	+	-	-
FI93-3	+	+	+	-	
FI94-1	+	+	+	-	
FI95-1	+	+	+	-	-
FI95-5	+	+	+	-	-
FI95-6	-	-	-	-	-
FI95-10	+	+	+	-	
Pi95-4	-	-	-	-	
FI96-1	+	+	+	-	-
FI96-2	+	+	+	-	
Ps96-1	-	-	-	+	-
C. lytica	-				-
C. uliginosa	-				-
C. marinoflava	-				-
F. elegans	-				+

5.4 Discussion.

The results of GC mol% of whole cell DNA and DNA-DNA dot hybridization indicate that the filamentous bacteria, which were isolated from marine microalgal cultures and formed peculiar whitish pink (WP) colony, belong to the same bacterial species. Particularly, it is of great interest that although FI95-5 strain produced greenish orange (GO) colony, the strain showed similar GC mol% of DNA and positive DNA-DNA hybridization reaction with DNAs of WP strains. Further studies are required to determine whether WP and GO strains of filamentous bacteria compose one species.

Generally, as classification of filamentous bacteria has been performed mainly based on morphological characteristics, it remains uncertainty in bacterial taxonomy. DNA GC mol% of the filamentous isolates were from 58.7 to 60.0, while those for most filamentous bacteria were reported to be from 30 to 50% in references (Larkin, 1989; Christensen, 1977; Oyaizu and Komagata, 1981). Marine filamentous bacteria with DNA GC contents over 55% were reported to be the genus *Lysobacter* (65 to 70%) and the genus *Vitreoscilla* (42 to 63%). The DNA GC contents of aerobic bacteriochlorophyll a containing bacteria have been reported to be 70.0, 60 to 67 and 56 to 60 mol% for the genera *Roseococcus*, *Erythrobacter* and *Roseobacter*, respectively (Yurkov, *et al.*, 1994).

As DNA GC contents of WP filamentous isolates were apparently different from those of *Flexibacter* spp. reported, it is difficult to identity these WP strains to be the genus *Flexibacter* and *Cytophaga* spp. The determination of base sequences of specific DNA such as 16S rDNA, which has been reported in various bacterial strains, should contribute to

clarify the taxonomic position of filamentous bacteria isolated in this study (Van Den Eynde, *et al*., 1990; Gherna and Woese, 1992).

Chapter 6

Restriction Fragment Length Polymorphism (RFLP) Analysis of 16S rDNA Genes from Filamentous Bacteria Co-existing with Marine Microalgae

6.1 Introduction

The studies about filamentous bacteria have been the subject of the author's interest because of their biological effect on marine microalgae and ecological distribution as potential bacteria enhancing the growth of marine microalgae. The great interest was shown on filamentous isolates as FI95-5 strain which produced greenish orange (GO) colony and gave similar GC mol% of DNA and positive DNA-DNA hybridization reaction with DNAs of major whitish pink (WP) strains. Therefore, the difference among them needs to be elucidated.

The conventional identification and the GC% and DNA-DNA hybridization analysis for the chromosomal DNA have been done in order to clarify the taxonomic position. By using a dot hybridization technique, the DNA-DNA hybridization was carried out for differentiation among the test strains and *Cytophaga-Flexibacter* as reference strains. This approach explained successfully the relationship between isolated strains and reference strains, but the appropriate taxonomic position of the filamentous strains remained uncertain.

Generally, DNA-DNA dot hybridization using random DNA probes derived from test strains has been employed to investigate the overall genetic interrelationship among isolated strains. Dot hybridization assay is particularly useful for preliminary categorizations of test strains on the basis of genetic interrelationship. Test strains having intensive sequence homology can be separated from unrelated or less related strains. More detailed genotypic information can be obtained by restriction fragment length polymorphism (RFLP) which has recently provided useful informations for distinguishing strains in a variety of bacterial species and for closer investigation of the genetic interrelationships (Masters *et. al.*, 1991; Mavingui, *et al.*, 1992; Moyer, *et. al.*, 1994).

Although the greenish orange (GO) strain FI95-5 has been considered to be identical with whitish pink (WP) strains in morphological, physiological, and biochemical tests as well as GC% and dot hybridization analysis, but it showed apparently different in some traits such as pigmentation. In the present chapter, RFLP was done to determine the diversity, especially among greenish orange strain (FI95-5) and other representative whitish pink strains.

6.2 Materials and Methods

6.2.1 Bacterial strains and DNA isolation

Five selected filamentous strains including FI90-32, FI93-3; FI95-1, FI95-5 and FI96-1 isolated from *C. marina*, *H. akashiwo* and *Tetraselmis* sp., respectively were chosen as representative strains based on the GC analysis and dot hybridization results. DNA isolation and purification of filamentous strains and *Saprospira*-like bacterium SS91-40 were conducted as described in the previous chapter.

6.2.2 16S ribosomal DNA amplification by polymerase chain reaction (PCR)

The 16S ribosomal DNA of the isolated filamentous bacteria by PCR was amplified by PCR using the GeneAmp PCR reagent kit (Perkin-Elmer). The PCR primer's were designed according to the consensus sequences at both ends of 16S rDNA strands from *Escherichia coli*, *Thermus thermophilus*, *Bacillus subtilis*, and *Clostridium perfringens*. The sequences of these primers were as follows: the forward primer 5'-TTGTTGGAGAGTTTGATCCTG-3' and the reverse primer 5'-AAAGGAGGTGATCCAGCCGCACCTTC-3' obtained from Lifetech Oriental, Japan.

PCR amplification was performed in a 100 μl reaction mixture containing 100 ng of template DNA, 10 μl of 10x Chelating buffer (100 mM Tris-HCl, pH 8.3, 1M KCl, 7.5 mM EGTA, 0.5% Tween 20, 50%[V/V] glycerol), 5U of r*Tth* DNA polymerase, 0.15 mM forward primer, 0.15 mM reverse primer, 40 mM dATP, 40 mM dGTP, 40 mM dCTP and 40 mM dTTP, and 2 mM $MgCl_2$ (Perkin-Elmer). The mixture was incubated in the thermal cycler, for 35 cycles of 1 minutes at 95°C, for denaturation 1 minutes at 60°C for primer annealing and 2 minutes at 72°C for extension followed by an additional extension at 72°C for 18 minutes.

6.2.3 Purification of DNA fragments using glass fine powder

The PCR products were applied on 0.8% (wt/vol) agarose gel to be electrophoresed in 0.5 x TBE buffer. A gel piece containing the DNA fragments of interest was excised, then placed in a microcentrifuge tube and weighed. For each 100 mg of gel, 200 μl NaI were added and the gel was dissolved by incubation at 55°C. Five μl of glass powder suspension were added to the dissolved gel. For binding the DNA fragments to glass fine powder, they were resuspended uniformly and incubated for 5 minutes on ice. Pellets of glass powder were collected by centrifugation at 10,000 rpm for 5 seconds, then the glass pellets were washed by adding 250 μl of EASYTRAP™ Ver 2 (TAKARA SHUZO CO., LTD. Biomedical Group) buffer and centrifuged at 10,000 rpm for 5 seconds. For DNA elution from glass powder, 5 μl of TE buffer were added to it and incubated at 55°C for 5 minutes. The supernatant containing DNA was collected after centrifuging the pellets at 10,000 rpm for 5 seconds.

6.2.4 Preparation of 16S rDNA genes for RFLP and sequence analysis

Restriction fragment length polymorphism analysis (RFLP) was done directly by using 16S rDNA products amplified by a PCR technique

or those cloned into plasmid. The 16S rDNAs derived from the plasmid were isolated by the following steps:

A. Cloning

PCR amplified 16S rDNA fragments were ligated to linearized plasmid DNA (vector). The recombinant molecules were then transformed into host cells of *E. coli*. Detailed procedures are given below.

A.1 Ligation and transformation

Fifty μg of PCR amplified 16S rDNA fragments were ligated to 100 μg of pT7 Blue vector (Novagen) using the DNA ligation Kit Ver.2 (Takara Shuzo, Japan) and incubated overnight at 16°C. After that, the transformation was done by mixing the ligation products and 100 μl of competent cells (*E. coli*, JM109 strain), and incubated on ice for 40 minutes. The cells were heat-treated by transferring the mixture to a 37°C water bath for exactly 2 minutes, then placed onto an ice-bath for 2 minutes, followed by the addition of 900 μl of SOC medium (2.0 g Bacto-trypton, 0.5 g Bacto-yeast extract, 200 μl 5M NaCl, and 250 μl 1M KCl, glucose 2 μg in 100 ml of distilled water), and incubated at 37°C for 1 hour with gentle shaking. The mixture was then centrifuged at 4,500 rpm for 5 minutes and cells were precipitated, and the supernatant was discarded. The precipitated cells were mixed with 100 μl LB-broth medium (1.0 g Bacto-tryipton, 0.5 g Bacto-yeast, 0.5 g NaCl in 100 ml of distilled water). During this period, competent cells were recovered to the ordinary states and the antibiotic resistance gene needed to replicate on selective plates was expressed. Using a sterile glass spreader, several amounts of cells from the mixture were spread evenly over the surface of LB agar plates supplemented with appropriate antibiotics (2 ml of ampicillin (25 mg/ml)

was added into 1/ of LB-broth with 15 gr agar), and incubated at 37°C for 12 to 20 hours. Obtained colonies were subjected to plasmid DNA purification as described below.

B. Plasmid purification by a large scale alkaline lysis method

E. coli containing plasmid DNA as described above was grown in a 200 ml LB-ampicillin broth medium at 37°C for 12 to 20 hours. Cells from 200 ml culture were collected by centrifugation at 16,000 rpm for 10 minutes at 4°C. After discarding supernatant, the cell pellet was resuspended in 3.2 ml of TES solution (50 mM TRIS (PH 8.0), 50 mM sucrose, !0 mM EDTA.2Na), and added with a fresh lysozyme solution (20 mg/ml), then incubated for 5 minutes at room temperature. The mixture was then added with 8 ml of a fresh alkaline-SDS solution (0.2 M NaOH, 1% SDS), and mixed gently, then incubated on ice for 5 minutes. Moreover, the mixture was added with 6 ml of a potassium acetate-glacial acetic acid solution (29.4 mg potassium acetate, 11.5 ml glacial acetic acid, and add with distilled water to 100 ml), and incubated on ice for 15 minutes. The white precipitate was spun down by centrifugation at 15,000 rpm for 5 minutes at 4°C. The supernatant was transferred in a new tube and the precipitate was discarded.

The supernatant was extracted with a TE buffer-saturated phenol solution and separated by centrifugation at 15,000 rpm for 10 minutes at 4°C, then the upper aqueous phase was transferred to a new tube. This extraction was repeated one more time. The upper aqueous phase was transferred to a new tube, followed by extraction of the aqueous phase with an equal volume of chloroform. After that, nucleic acid fraction was precipitated by adding 2.5 volumes of ethanol, and centrifuged at 15.000 rpm for 10 min. at 4°C. The precipitate was then added with 1 ml TE buffer and 1.3 μl of RNase (20 μg/ml), and incubated for 30 minutes at

37°C, then added with 0.6 ml of PEG solution (20 g PEG 6000, 100 mM NaCl) and incubated overnight at 5°C.

The precipitated plasmid DNA was collected by centrifuging at 15,000 rpm for 10 minutes at 4°C. The supernatant was removed and the pellet was saved and washed with 70% ethanol followed by centrifugation at 15,000 rpm for 5 minutes at 4°C. Then, the plamid DNA was dissolved in 100 µl TE buffer and stored at - 20°C until needed.

6.2.5 Purification of 16S rDNA from plasmid DNA by restriction enzymes

The 16S rDNA gene fragment inserted into the plasmid vector was digested by restriction enzymes. *Bam*HI and *Sal*I derived from *Bacillus amyloliquefaciens* H and *Streptococcus albus* G, respectively were chosen as digestion enzymes based on their digestible sequences. Fifty µl of plasmid DNA solution were added with 2.5 µl *Bam*HI (12 unit/µl), 3.0 µl *Sal*I (10 unit/µl), 10 µl 10xH buffer and 34.5 µl distilled water, then incubated overnight at 37°C. The mixture was applied on 0.8% (wt/vol) agarose gel containing ethidium bromide (0.5 µg/ml) and electrophoresed in 0.5xTBE buffer, then visualized by UV excitation. A gel piece containing the DNA fragment of interest was excised, and purified by using a glass fine powder method as described above.

6.2.6 Restriction fragment length polymorphism (RFLP) analysis of 16S rDNA

The 16S rDNAs prepared from DNA plasmid DNA were treated for 3 hours at 37°C with 13 restriction enzymes from MBI Fermentas including *Alu*I, *Bgl*II, *Bsu*RI, *Dra*I, *Mbo*I, *Mlu*I; *Msp*I, *Nco*I, *Nhe*I, *Pst*I, *Xba*I, *Kpn*2I and *Sma*I. On the other hand, for restriction endonucleases *Kpn*2I and *Sma*I, the 16S rDNA reaction mixture was treated for 3 hours at 55°C and 30°C, respectively. The digested DNA was separated by

electrophoresis together with 100 bp ladder maker (Pharmacia Biotech) on 2.0 % (wt/vol) NuSieve GTG agarose gels (FMC Bio Products). Gels were stained with ethidium bromide (0.5 μg/ml) and visualized by UV excitation.

6.3 Results

6.3.1 16S rDNA amplification by PCR

Five filamentous strains were chosen to amplify 16S rDNA by a PCR technique. Previously, the author used r*Tth* and Ampli*Taq* DNA polymerases obtained from *Thermus thermophilus* and *Thermus aquaticus*, respectively for extending and duplicating the segment of DNA between the primers. Results indicated that *rTth* DNA polymerase amplified template DNA more effectively than Ampli*Taq* DNA polymerase.

6.3.2 Restriction fragment length polymorphism (RFLP) analysis of 16S rDNA

Electrophoretic profiles of the 16S rDNA derived from DNA plasmid are shown in Fig. 6.1. The band patterns compared with λ /*Hind*III maker are exhibited at around 1,700 bp. As shown in Fig. 6.2, the 16S rDNAs were not digested by 7 restriction endonucleases including *Bgl*II, *Dra*I, *Kpn*2I, *Mbo*I, *Nhe*I, *Pst*I, and *Xba*I. On the other hand, 16S rDNAs were digested by 6 restriction endonucleases including *Alu*I, *Bsu*RI, *Mlu*I; *Msp*I, *Nco*I, and *Sma*I. The photograph and illustrated figures of electrophoresis for digested 16S rDNA fragments with *Alu*I, *Bsu* RI, *Msp*I and *Sma*I are demonstrated in Fig. 6.3 A, B, C and D and Fig. 6.4. In this experiment the 16S rDNA derived from filamentous strains and a representative strain (SS91-40) of *Saprospira* sp., were used. DNA fragment lengths of the restriction endonuclease-digested bands are summarized in Table 6.1.

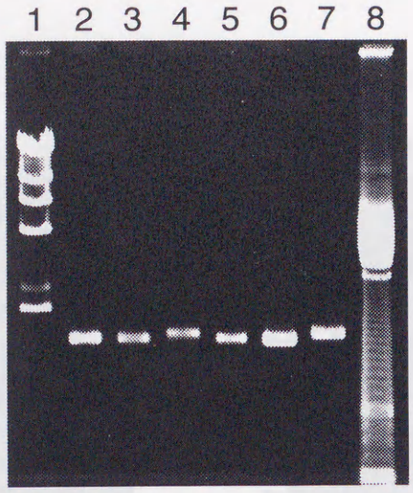

Fig. 6.1. PCR-amplified 16S ribosomal DNA fragments derived from the filamentous bacteria.
Lane 1, λ/*Hin*dIII marker; lane 2, FI90-32; lane 3, FI93-3; lane 4, FI95-1; lane 5, FI95-5; lane 6, FI96-1; lane 7, SS91-40 (as a representative); lane 8, 100 bp ladder marker.

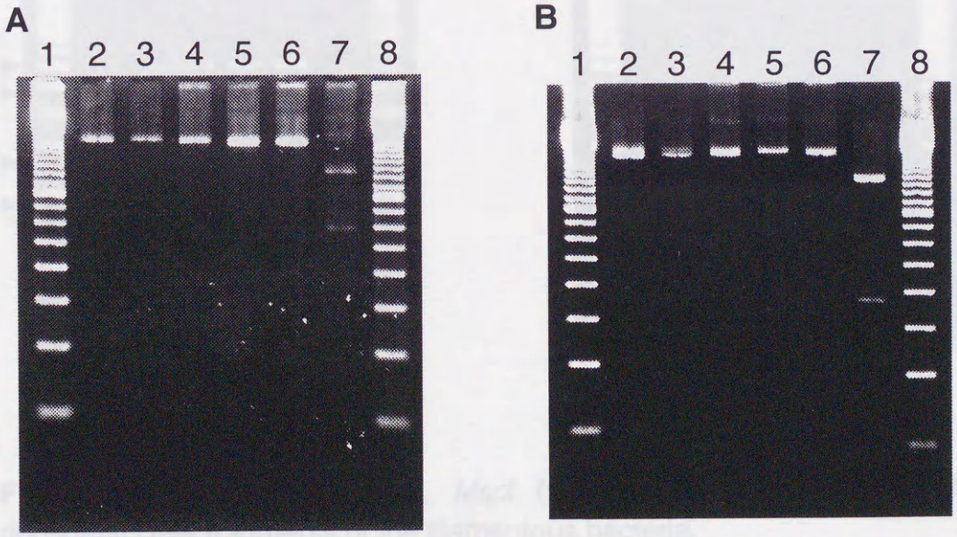

Fig. 6.2. *Bsp*MII (**A**) and *Mbo*I (**B**)-digested 16S ribosomal DNA fragments of the filamentous bacteria.
Lane 1 and 8, 100 bp ladder markers; lane 2, FI90-32; lane 3, FI93-3; lane 4, FI95-1; lane 5, FI95-5; lane 6, FI96-1; lane 7, SS91-40 (as a representative).

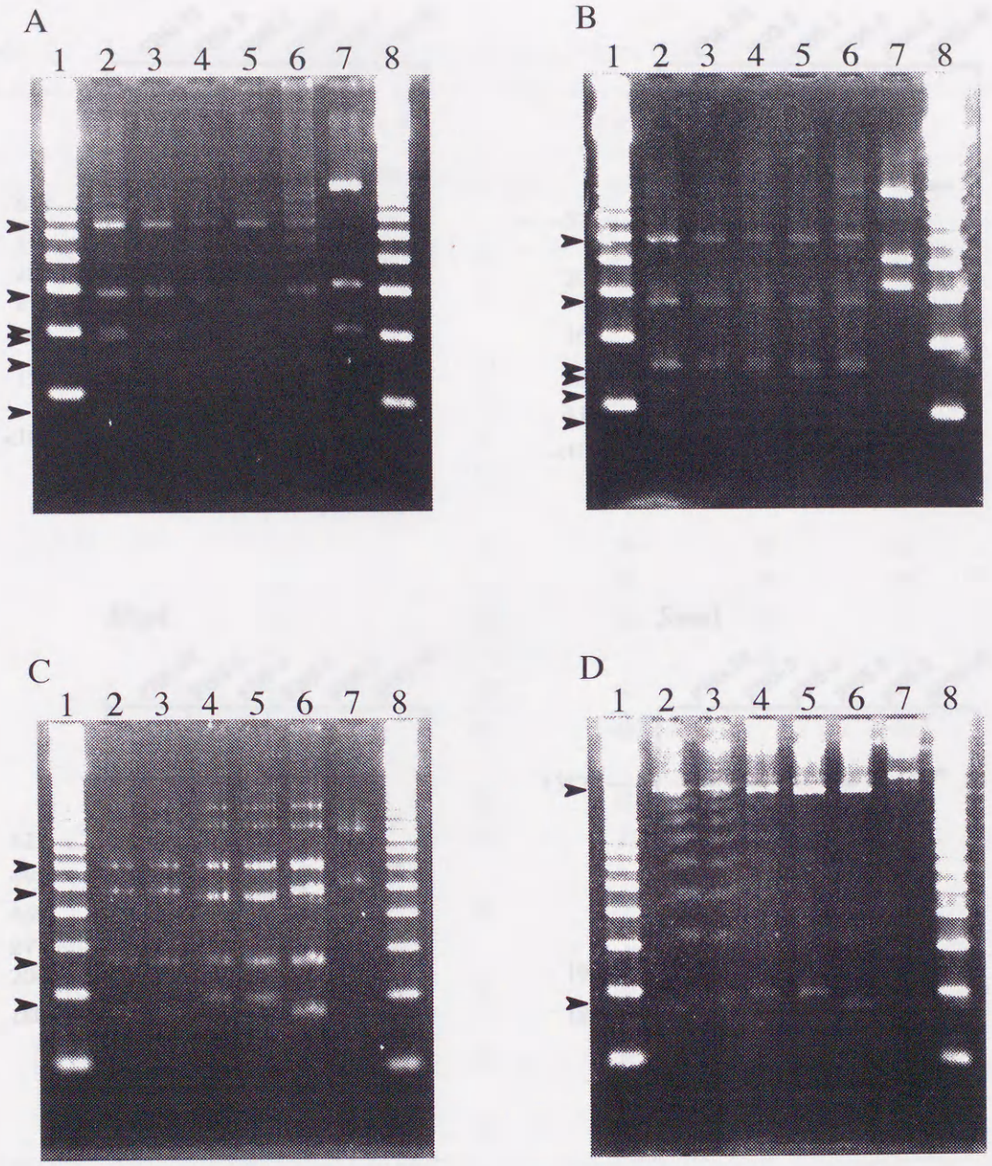

Fig. 6.3. *Alu*I (**A**), *Bsu*RI (**B**), *Msp*I (**C**), and *Sma*I (**D**)-digested 16S ribosomal DNA fragments of the filamentous bacteria.
Lane 1 and 8, 100 bp ladder markers; lane 2, FI90-32; lane 3, FI93-3; lane 4, FI95-1; lane 5, FI95-5; lane 6, FI96-1; lane 7, SS91-40 (as a representative). Positions of the restriction endonuclease-digested DNA fragments were indicated as *arrowheads* at left.

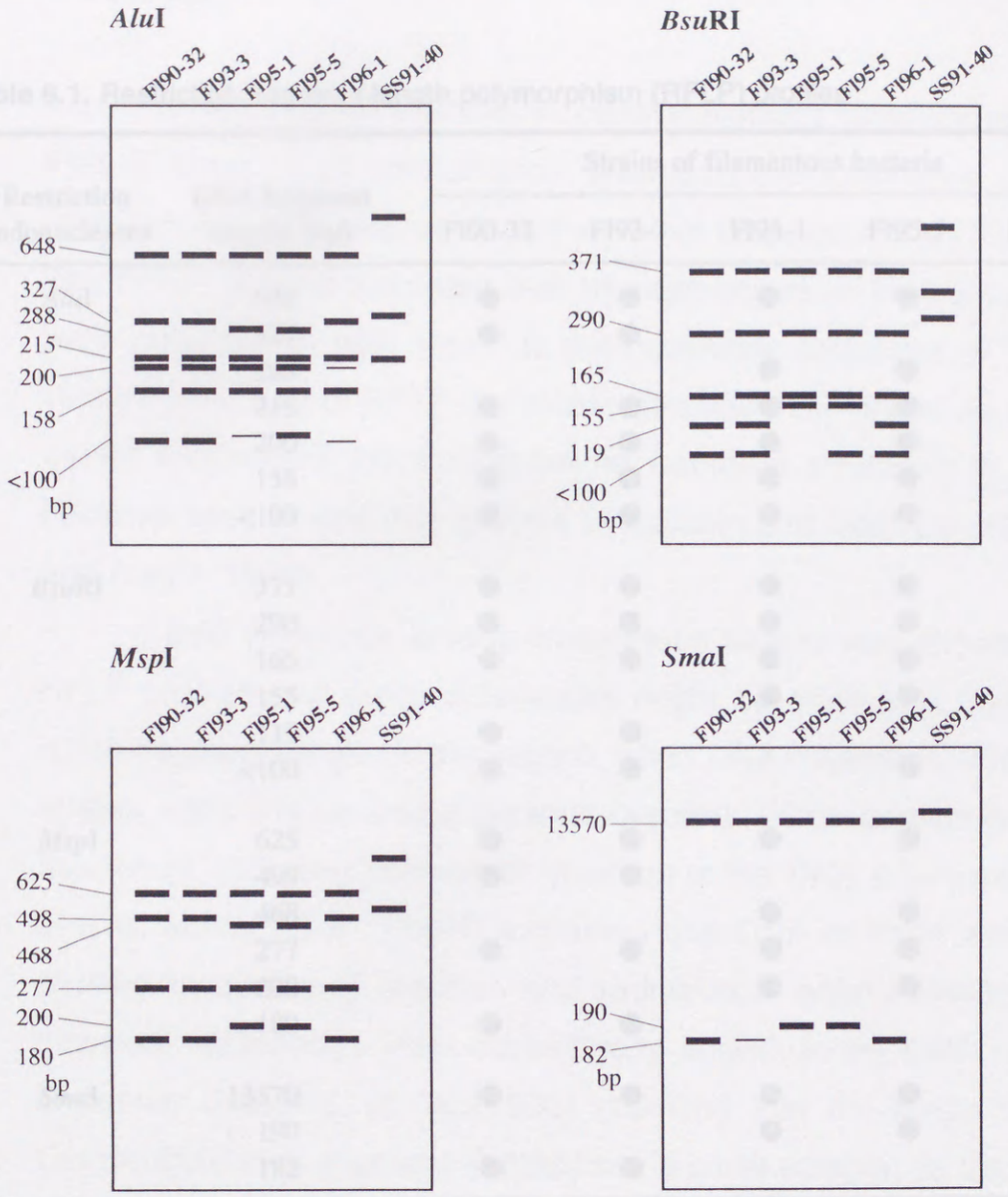

Fig. 6.4. Restriction fragment length polymorphism (RFLP) profiles of the filamentous bacteria.
Restiction endonucleases and strains used for total DNA digestion strains are indicated at the top, and the length of each band is at the left. Faint bands are shown as the thin lines.

Table 6.1. Restriction fragment length polymorphism (RFLP) profiles

Restriction endonucleases	DNA fragment lengths (bp)	Strains of filamentous bacteria				
		Fl90-32	Fl93-3	Fl95-1	Fl95-5	Fl96-1
*Alu*I	648	●	●	●	●	●
	327	●	●			●
	288			●	●	
	215	●	●	●	●	●
	200	●	●	●	●	
	158	●	●	●	●	●
	<100	●	●	●	●	●
*Bsu*RI	371	●	●	●	●	●
	290	●	●	●	●	●
	165	●	●	●	●	●
	155			●	●	
	119	●	●			●
	<100	●	●		●	●
*Msp*I	625	●	●	●	●	●
	498	●	●			●
	468			●	●	
	277	●	●	●	●	●
	200			●	●	
	180	●	●			●
*Sma*I	13570	●	●	●	●	●
	190			●	●	
	182	●	●			●

DNA fragment lengths at which the restriction endonuclease-digested bands were obtaind are indicated as ●.

6.4 Discussion

Although the differences in cellular pigment were shown between FI95-5 strain and whitish-pink strains, polymorphisms among all representative filamentous strains were not detected in the RFLP profiles. The RFLP profiles indicated that all representative filamentous strains were quite similar with regard to the nucleotide sequence of 16S rDNA. Recently the use of RFLP technique has been convinced as one of the useful techniques for distinguishing bacterial strains in a variety of bacterial species and sub species (Kaijalanin and Lidstrom, 1989; Priest and Austin, 1993) .

A little difference among filamentous strains was shown in some RFLP profiles, but these differences might be originated from the 16S rDNA fragment length. In this regard, when DNA fragments of filamentous strains were cloned into a plasmid (vector), some accidents might be happened, including deletion or insertion in the DNA fragments of some strains. Maloy, *et al.*, (1994) explained that DNA damage was possibly caused by chemical reaction and radiation in environments and that incorrect nucleotides were occasionally added during DNA replication. Moreover, Masters, *et. al.*,(1991) reported that the large number of *Deinococcus radiopugnans* strains from a small sample, as distinguished by RFLPs, was unlikely to be based on the result of point mutations added and subtracted in *Eco*RI sites of the chromosome.

Based on the above results, the small difference among 16S rDNA fragments shown by RFLP profiles was not informative regarding nucleotide sequences to distinguish filamentous strains. Therefore, based on the 16S rDNA analysis by RFLP technique, the author suggested that the 5 filametous strains were very similar phylogenetically. Since this experiment was conducted by using one clone for each strain, it could be

interesting to examine the other clones derived from 5 representative strains. It is interesting to note if some differences among clones or especially the differences between greenish orange (FI 95-5) and whitish pink strains can be detected by comparing with the other clones.

Chapter 7

Sequence Analysis of 16S rDNA and Phylogenetic Study of Filamentous Bacteria

7.1 Introduction

Information about the diversity of various bacterial strains has been obtained by using a RFLP technique. However, as stated in the previous chapter apparent differentiations among the filamentous strains reflected by fragment profiles in RFLP were not recognized. By using nucleotide sequences of 16S rDNA, the comparison among the filamentous strains may be undertaken. The comparison of rDNA sequences is a powerful tool for deducing phylogenetic and evolutionary relationships among eubacteria, archaebacteria, and eucaryotic organisms (Weisburg, *et. al.*, 1991). Various methods have been developed by some authors including PCR specific for 16S rDNA fragments followed by direct or cloning-assisted sequence analysis.

The phylogenetic analysis has been done based on the sequence comparison of 5S, 16S and 23S rDNA molecules in modern taxonomy of bacteria. For bacteriologists, 16S rDNA and 23S rDNA were commonly used as an ideal molecule for estimating the relationship of genetic distance, diversity and community structure among microorganisms (Andresson and Fridjonsson, 1994; Moyer, *et al*, 1994) because the 16S and 23S rRNA genes contained the patchworks of conserved and non-conserved regions and provided the suitable molecules for new approaches such as PCR amplification, oligonucleotide probe hybridization, and DNA sequencing (Kabir, *et al.*, 1995)

There is a much larger data base (DDBJ. DNA Data Bank of Japan) available for sequences of 16S rDNA than for those of 23S rDNA. Therefore, 16S rDNA is more popular, although the analysis of genetic variations in this region has not necessarily been appropriate to differentiate strains within species (Laguerre *et al*, 1996). The success for discriminating among the strains of bacteria was reported by Laguere, *et.*

al., (1966) when they used PCR-RFLP analysis of intergenic spacer between gene coding for 16S and 23S rRNAs (16S and 23S rDNAs).

In this chapter, the author amplified 16S rDNA from three representative strains of filamentous bacteria including FI90-32, FI95-5 and FI96-1 by using the PCR technique in order to compare nucleotide sequences of 16S rDNA among them and to construct their phylogenetic relationship with other related bacteria of which sequences are registered in a data base.

7.2 Material and Methods

7.2.1 Bacterial strains

FI90-32, FI95-5 and FI96-1 isolated from cultures of *C. marina*, *H. akashiwo*, and *Tetraselmis* sp., respectively were chosen as representative strains based on the 16S rDNA restriction patterns as shown in the previous chapter. Plasmid DNAs inserted with 16S rDNA fragment of FI90-32, FI95-5 or FI 96-1 were used for sequence analysis.

7.2.2 Thermal cycle sequence reactions

The 16S rDNA molecules were amplified and cloned into the plasmid vector as described previously. For thermal cycle sequencing, the forward primer 3'-TGACCGGCAGCAAAATG-5', the reverse primer 5'-GGAAACAGCTATGACCATG-3' (Lifetech Oriental) and the M13 primer (Takara Biotech) were used. In order to perform full sequence analysis, the forward primer 5'-CATTGTCACTACCATTGTAGC-3' and the reverse primer 5'- GGAAACTGAGAGTAATACCGT-3' were also used as the inner primers designed from the consensus sequence of three clones of filamentous strains and purchased from Lifetech Orientalis.

Thermal cycle sequencing was performed in 20 μl of reaction mixtures containing 8 μl of plamid DNA (37.5 ng/μl), 4 μl of primer and 8

μl of premix solution (Perkin Elmer). All reactions were conducted according to the protocol of ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). The mixture was incubated in GeneAmp PCR system 9600, a programmable thermal controller (Perkin-Elmer, Norwalk, Conn.) for 25 cycles according to the manufacture's protocol. Temperature cycles for amplification were composed of denaturation at 96°C for 10 seconds, primer annealing at 50°C for 5 seconds, chain extension/amplification at 60°C for 4 minutes, and held at 4°C, for a total of 25 cycles. Then, the extension products were ready to be purified.

7.2.3 Purification of extension products

The extension products were ready to be purified by using the ethanol precipitation method according to the manufacture's protocol. The extension products were transferred in a 1.5 ml microcentrifuge tube by adding 2.0 μl of 3M sodium acetate (CH3COONa), pH 4.6 and 50 μl of 95% ethanol, then mixed, and placed on ice for 10 min. The mixture was centrifuged at 15,000 rpm in 4°C for 30 minutes. The supernatant was aspirated carefully with a micropipetter and removed as completely as possible. Then, the pellet was rinsed by adding 250 μl of 70% ethanol, followed by centrifugation at 15,000 rpm in 4°C for 10 minutes. The supernatant was discarded carefully with a micropipetter and then the pellet was dried in a vacuum dryer.

7.2.4 DNA sequence analysis

The dried pellet was added with 12 μl of loading buffer (Template Suppression Reagent, Perkin Elmer), then mixed and spun. The samples were heated at 90°C for two minute to denature and placed on ice until ready to load. The fluorescence-labeled fragments were sequenced with a

DNA sequencer ABI PRISM 310 Genetic Analyzer (Perkin Elmer). The obtained sequences were analyzed by the Gene Works software (Teijin System Technology, Inc.)

7.2.5 Phylogenetic analysis

Nucleotide sequences of the 16S rDNAs were aligned by the multiple sequence alignment program Clustal W version 1.5 (Thompson, *et al.*, 1994). Evolutionary genetic distance matrices and similarity values were calculated by using the DNADIST program and Gene Works software (Teijin System Technology, Inc.) Phylogenetic trees were calculated with the neighbor-joining algorithm (Saitou and Nei, 1987) by using the NEIGHBOR and DRAWTREE programs of the PHYLIP package version 3.5c software (Felsenstein, 1993).

7.3 Results

Sequences of 16S rDNA cloned into plasmid DNAs were analyzed for PCR products with ca. 1,418 bp of bacterial ribosomal DNA sequence. At first, the partial 16S rDNA sequence was obtained by use of the forward primer 3'-TGACCGGCAGCAAAATG-5' and the reverse primer 5'-GGAAACAGCTATGACCATG-3' for sequencing primers, but the full sequences were not read in this experiment. In order to perform the 16S rDNA full sequence analysis, the inner primers (the forward primer 5'-CATTGTCACTACCATTGTAGC-3' and the reverse primer 5'-GGAAACTGAGAGTAATACCGT-3') designed from the consensus sequence of three clones of filamentous strains were used.

When about 1.3 Kbp sequences of 16S rDNA were compared, three filamentous strains showed a high degree of similarity. Total nucleotide sequence with 1,418 bp of small sub-unit ribosomal DNA (16S

rDNA) from a filamentous strain (FI90-32) is presented in Fig. 7.1. A very high similarity among the 16S rDNA sequences was found when sequences of the 16S ribosomal DNA from three filamentous strains were compared with those of other related bacteria including *Roseobacter algicola* and *Rhodobacter capsulatus* as presented in Fig. 7.2. Moreover, the similarity and distance matrices inferred from multiple-aligned sequences of the 16S ribosomal DNA are illustrated in Table 7.1. In these cases, the similarity among the 16S rDNA sequences of filamentous isolates showed a high degree of similarity (99.2 - 99. 4%). The similarity between α , β, γ-Proteobacteria groups and filamentous isolates revealed that *Roseobacter algicola, R. denitrificans* and *R. litoralis* from α-Proteobacteria group showed the highest similarity value (92.5 - 94.1%) and the shortest distance at about 0.058 to 0.066 against filamentous strains.

Neighbor-joining algorithm was used to perform the phylogenetic analysis utilizing 16S rDNA sequences from three filamentous isolates and various bacteria including α , β, γ-Proteobacteria and Gram positive bacteria groups (Fig. 7.3). The results in phylogenetic tree demonstrated that the filamentous strains belonged to α subdivision of the Proteobacteria and that they were clustered with the genus *Roseobacter* group.

```
GCTCAGAACG AACGCTGGCG GCAGGCCTAA CACATGCAAG TCGAGCGAGG ACTTCGGTTC     60

TAGCGGCGGA CGGGTTAGTA ACGCGTGGGA ACGTACCCTT TTCTACGGAA TAGCCTCGGG    120

AAACTGAGTA GTAATACCGT ATACGCCCTT CGGGGGAAAG ATTTATCGGA GAAGGATCGG    180

CCCGCGTTAG ATTAGATAGT TGGTGGGGTA ATGGCCTACC AAGTCTACGA TCTATAGCTG    240

GTTTGAGAGG ATGATCAGCA ACACTGGGAC TGAGACACGG CCCAGACTCC TACGGGAGGC    300

AGCAGTGGGG AATCTTAGAC AATGGGCGCA AGCCTGATCT AGCCATGCCG CGTGAGTGAT    360

GAAGGCCTTA GGGTCGTAAA GCTCTTTCGC CTGTGAAGAT AATGACTGTA GCAGGTAAAG    420

AAACCCCGGC TAACTCCGTG CCAGCAGCCG CGGTAATACG GAGGGGGTTA GCGTTGTTCG    480

GAATTACTGG GCGTAAAGCG CGCGTAGGCG GACATTTAAG TCAGAGGTGA AATCCCAGGG    540

CTCAACCCTG GAACTGCCTT TGATACTGGG TGTCTTGAGT TCGAGAGAGG TGAGTGGAAT    600

TCCGAGTGTA GAGGTGAAAT TCGTAGATAT TCGGAGGAAC ACCAGTGGCG AAGCGGCTCA    660

CTGGCTCGAT ACTGACGCTG AGGTGCGAAA GTGTGGGGAG CAAACAGGAT TAGATACCCT    720

GGTAGTCCAC ACCGTAAACG ATGAATGCCA GTCGTCGGGC AGTATACTGT TCGGTGACAC    780

ACCTAACGGA TTAAGCATTC CGCCTGGGGA GTACGGTCGC AAGATTAAAA CTCAAAGGAA    840

TTGACGGGGG CCCGCACAAG CGGTGGAGCA TGTGGTTTAA TTCGAAGCAA CGCGCAGAAC    900

CTTACCAACC CTTGACATCC TCAGCTAAAT CCAGAGATGG ATGGTTCCCT TCGGGGACTG    960

AGTGACAGGT GCTGCATGGC TGTCGTCAGC TCGTGTCGTG AGATGTTCGG TTAAGTCCGG   1020

CAACGAGCGC AACCCACATC TTCAGTTGCC ATCAGGTTAT GCTGGGCACT CTGGAGAAAC   1080

TGCCCGTGAT AAGCGGGAGG AAGGTGTGGA TGACGTCAAG TCCTCAGGCC CTTACGGGTT   1140

GGGCTACACA CGTGCTACAA TGGTAGTGAC AATGGGTTAA TCCCAAAAAG CTATCTCAGT   1200

TCGGATTGGG GTCTGCAACT CGACCCCATG AAGTCGGAAT CGCTAGTAAT CGCGTAACAG   1260

CATGACGCGG TGAATACGTT CCCGGGCCTT GTACACACCG CCCGTCACAA CCATGGGAGT   1320

TGGGTTCACC CGAAGGCCGT GCGCCAACCT GCAAAGGAGG CAGCGGACCA CGGTGAGCTC   1380

AGCGACTGGG GTGAAGTCGT AACAAGGTAG CCGTAGGG.. ..........  ..........   1418
```

Fig. 7.1. Small subunit ribosomal DNA (16S rDNA) nucleotide sequences of filamentous bacteria FI90-32.

```
F190-32          GCTCAGAACGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGAGGACTTCGGTTC
F195-5           GCTCAGAACGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGAGGACTTCGGTTC
F196-1           GCTCAGAACGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGAGGACTTCGGTTC
Rose.algic             ACGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGAGCTCTTCGGAGC
Rb.capsul            AACGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGAGACCTTCGGGTC
                     ************************************************  ******  *

F190-32          TAGCGGCGGACGGGTTAGTAACGCGTGGGAACGTACCCTTTTCTACGGAATAGCCTCGGG
F195-5           TAGCGGCGGACGGGTTAGTAACGCGTGGGAACGTACCCTTTTCTACGGAATAGCCTCGGG
F196-1           TAGCGGCGGACGGGTTAGTAACGCGTGGGAACGTACCCTTTTCTACGGAATAGCCTCGGG
Rose.algic       TAGCGGCGGACGGGTTAGTAACGCGTGGGAACGTGCCCTTCACTGCGGAATAGCCACTGG
Rb.capsul        TAGCGGCGGACGGGTGAGTAACGCGTGGGAACGTGCCCTTTGCTACGGAATAGCCCCGGG
                 *************** ****************** *****  ** ********** * **

F190-32          AAACTGAGTAGTAATACCGTATACGCCCTTCGGGGGAAAGATTTATCGGAGAAGGATCGG
F195-5           AAACTGAG-AGTAATACCGTATACGCCCTTAGGGGGAAAGATTTATCGGAGAAGGATCGG
F196-1           AAACTGAG-AGTAATACCGTATACGCCCTTCGGGGGAAAGATTTATCGGAGAAGGATCGG
Rose.algic       AAACGGTG-AGTAATACCGCATACGCC-TTCGGGGGAAAGATTTATCGGTGAAGGATCGG
Rb.capsul        AAACTGGG-AGTAATACCGTATGTGCCCTTNGGGGGAAAGATTTATCGGCAAAGGATCGG
                 **** * * ********** **  *** ** ******************  *********

F190-32          CCCGCGTTAGATTAGATAGTTGGTGGGGTAATGGCCTACCAAGTCTACGATCTATAGCTG
F195-5           CCCGCGTTAGATTAGATAGTTGGTGGGGTAATGGCCCACCAAGTCTACGATCTATAGCTG
F196-1           CCCGCGTTAGATTAGATAGTTGGTGGGGTAATGGCCTACCAAGTCTACGATCTATAGCTG
Rose.algic       CCCGCGTTAGATTAGATAGTTGGTGGGGTAACGGCCTACCAAGTCTACGATCTATAGCTG
Rb.capsul        CCCGCGTTGGATTAGGTAGTTGGTGGGGTAATGGCCTACCAAGCCGACGATCCATAGCTG
                 ******** ****** *************** **** ****** * ****** *******

F190-32          GTTTGAGAGGATGATCAGCAACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGC
F195-5           GTTTGAGAGGATGATCAGCAACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGC
F196-1           GTTTGAGAGGATGATCAGCAACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGC
Rose.algic       GTTTTAGAGGATGATCAGCAACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGC
Rb.capsul        GTTTNAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGC
                 **** ************** ****************************************

F190-32          AGCAGTGGGGAATCTTAGACAATGGGCGCAAGCCTGATCTAGCCATGCCGCGTGAGTGAT
F195-5           AGCAGTGGGGAATCTTAGACAATGGGCGCAAGCCTGATCTAGCCATGCCGCGTGAGTGAT
F196-1           AGCAGTGGGGAATCTTAGACAATGGGCGCAAGCCTGATCTAGCCATGCCGCGTGAGTGAT
Rose.algic       AGCAGTGGGGAATCTTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGAT
Rb.capsul        AGCAGTGGGGAATCTTAGACAATGGGGGAAACCCTGATCTAGCCATGCCGCGTGAGCGAT
                 **************** ********* * ** ******* ***************** ***

F190-32          GAAGGCCTTAGGGTCGTAAAGCTCTTTCGCCTGTGAAGATAATGACTGTAGCAGGTAAAG
F195-5           GAAGGCCTTAGGGTCGTAAAGCTCTTTCGCCTGTGAAGATAATGACTGTAGCAGGTAAAG
F196-1           GAAGGCCTTAGGGTCGTAAAGCTCTTTCGCCTGTGAAGATAATGACTGTAGCAGGTAAAG
Rose.algic       GAAGGCCTTAGGGTCGTAAAGCTCTTTCGCCAGAGATGATAATGACAGTATCTGGTAAAG
Rb.capsul        GAAGGCCTTAGGGTTGTAAAGCTCTTTCAGGTGGGAAGATAATGACGGTACCACCAGAAG
                 ************** *************    * ** ********* *** *     ***

F190-32          AAACCCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGGGTTAGCGTTGTTCG
F195-5           AAACCCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGGGTTAGCGTTGTTCG
F196-1           AAACCCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGGGTTAGCGTTGTTCG
Rose.algic       AAACCCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGGGTTAGCGTTGTTCG
Rb.capsul        AAGCCCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGGGCTAGCGTTGTTCG
                 ** ********************************************* ************

F190-32          GAATTACTGGGCGTAAAGCGCGCGTAGGCGGACATTTAAGTCAGAGGTGAAATCCCAGGG
F195-5           GAATTACTGGGCGTAAAGCGCGCGTAGGCGGACATTTAAGTCAGAGGTGAAATCCCAGGG
F196-1           GAATTACTGGGCGTAAAGCGCGCGTAGGCGGACATTTAAGTCAGAGGTGAAATCCCAGGG
Rose.algic       GAATTACTGGGCGTAAAGCGCACGTAGGCGGATCAGAAAGTTGGGGGTGAAATCCCGGGG
Rb.capsul        GAATTACTGGGCGTAAAGCGCACGTAGGCGGATCAGAAAGTCAGAGGTGAAATCCCAGGG
                 ********************** **********     ****  * *********** ***

F190-32          CTCAACCCTGGAACTGCCTTTGATACTGGGTGTCTTGAGTTCGAGAGAGGTGA-GTGGAA
F195-5           CTCAACCCTGGAACTGCCTTTGATACTGGGTGTCTTGAGTTCGAGAGAGGTGAAGTGGAA
F196-1           CTCAACCCTGGAACTGCCTTTGATACTGGGTGTCTTGAGTTCGAGAGAGGTGA-GTGGAA
Rose.algic       CTCAACCCCGGAACTGCCTCCAAAACTCCTGGTCTTGAGTTCGAGAGAGGTGA-GTGGAA
Rb.capsul        CTCAACCTTGGAACTGCCTTTGAAACTCCTGGTCTTGAGGTCGAGAGAGGTGA-GTGGAA
                 *******  **********   * ***    ******** ************* ******

F190-32          TTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGC-GAAG-CGGC
F195-5           TTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGC-GAAGGCGGC
F196-1           TTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGCCGAAGGCGGC
Rose.algic       TTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGC-GAAGGCGGC
Rb.capsul        TTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGC-GAAGGCGGC
                 ************************************************* **** ****

F190-32          TCACTGGCTCGATACTGACGCTGAGGTGCGAAAGTGTGGGGAGCAAACAGGATTAGATAC
F195-5           TCACTGGCTCGATACTGACGCTGAGGTGCGAAAGTGTGGGGAGCAAACAGGATTAGATAC
F196-1           TCACTGGCTCGATACTGACGCTGAGGTGCGAAAGTGTGGGGAGCAAACAGGATTAGATAC
Rose.algic       TCACTGGCTCGATACTGACGCTGAGGTGCGAAAGTGTGGGGAGCAAACAGGATTAGATAC
Rb.capsul        TCACTGGCTCGATACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATAC
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F190-32         CCTGGTAGTCCACACCGTAAACGATGAATGCCAGTCGTCGGGCAGTATACTGTTCGGTGA
F195-5          CCTGGTAGTCCACACCGTAAACGATGAATGCCAGTCGTCGGGCAGTATACTGTTCGGTGA
F196-1          CCTGGTAGTCCACACCGTAAACGATGAATGCCAGTCGTCGGGCAGTATACTGTTCGGTGA
Rose.algic      CCTGGTAGTCCACACCGTAAACGATGAATGCCAGTCGTCGGGTAGCATGCTATTCGGTGA
Rb.capsul       CCTGGTAGTCCACGCCGTAAACGATGAATGCCAGTCGTCGGCAGGCATGCCTGTCGGTGA
                ************* ***************************   * ** *   *******

F190-32         CACACCTAACGGATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAG
F195-5          CACACCTAACGGATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAG
F196-1          CACACCTAACGGATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAG
Rose.algic      CACACCTAACGGATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAG
Rb.capsul       CACACCTAACGGATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAG
                ************************************************************

F190-32         GAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAG
F195-5          GAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAG
F196-1          GAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAG
Rose.algic      GAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAG
Rb.capsul       GAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAG
                ************************************************************

F190-32         AACCTTACCAACCCTTGACATCCTCAGCTAAAT--CCAGAGATGGATGGTTCCCTTCGGG
F195-5          AACCTTACCAACCCTTGACATCCTCAGCTACAT--CCAGAGATGGATGGTTCCCTTCGGG
F196-1          AACCTTACCAACCCTTGACATCCTCAGCTAAAT--CCAGAGATGGATGGTTCCCTTCGGG
Rose.algic      AACCTTACCAACCCTTGACATCCTCAGCTACAT--CGAGAGATCGATGGTCCTC----GG
Rb.capsul       AACCTTACCAACCCTTGACATCGAGATCGCGGTTACCAGAGATGGTTTCCTTCAGTTCGG
                ********************   * *    *  * ****** * *           **

F190-32         --GACTGAGTGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTCGGTTA
F195-5          --GACTGAGTGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTCGGTTA
F196-1          --GACTGAGTGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTCGGTTA
Rose.algic      --GACTGAGTGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTCGGTTA
Rb.capsul       CTGGATCTCAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTCGGTTA
                  *  *    **************************************************

F190-32         AGTCCGGCAACGAGCGCAACCCACATCTTCAGTTGCCATCAGGTTATGCTGGGCACTCTG
F195-5          AGTCCGGCAACGAGCGCAACCCACATCTTCAGTTGCCATCAGGTTATGCTGGGCACTCTG
F196-1          AGTCCGGCAACGAGCGCAACCCACATCTTCAGTTGCCATCAGGTTATGCTGGGCACTCTG
Rose.algic      AGTCCGGCAACGAGCGCAACCCACATCCCTAGTTGCCAGCAG-TTCGGCTGGGCACTCTA
Rb.capsul       AGTCCGGCAACGAGCGCAACCCACACTTTCAGTTGCCATCAN--TCAGTTGGGCACTCTG
                *************************     ******** **   *  * **********

F190-32         GAGAAACTGCCCGTGATAAGCGGGAGGAAGGTGTGGATGACGTCAAGTCCTCAGGCCC-T
F195-5          GAGAAACTGCCCGTGATAAGCGGGAGGAAGGTGTGGATGACGTCAAGTCCTCATGGCCCT
F196-1          GAGAAACTGCCCGTGATAAGCGGGAGGAAGGTGTGGATGACGTCAAGTCCTCATGGCCCT
Rose.algic      TGGAAACTGCCCGTGATAAGCGGGAGGAAGGTGTGGATGACGTCAAGTCCTCATGGCC-T
Rb.capsul       GAAGAACTGCCGATGATAAGTCGGAGGAAGGTGTGGATGACGTCAAGTCCTCATGGCCCT
                    *******  *******  ********************************* * ** *

F190-32         TACGGGTTGGGCTACACACGTGCTACAATGGTAGTGACAATGGGTTAATCCCAAAAAGCT
F195-5          TACGGGTTGGGNTACACACGTGNTACAATGGTAGTGACAATGGGTTAATCCCAAAAAGCT
F196-1          TACGGGTTGGGCTACACACGTGCTACAATGGTAGTGACAATGGGTTAATCCCAAAAAGCT
Rose.algic      TACGGGTTGGGCTACACACGTGCTACAATGGCAGTGACAATGGGTTAATCCCAAAAAACT
Rb.capsul       TACGGGTTGGGCTACACAACGTGCTACAATGGTGGTGACAATGGGCCAATCCCAAAAAGCC
                *********** ********** ********  ***********  *********** *

F190-32         ATCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAATCGCTAGTAATCG
F195-5          ATCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAATCGCTAGTAATCG
F196-1          ATCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAATCGCTAGTAATCG
Rose.algic      GTCTCAGTTCGGATTGTCGTCTGCAACTCGACGGCATGAAGTCGGAATCGCTAGTAATCG
Rb.capsul       ATCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAATCGCTAGTAATCG
                 ***************  **************  ***************************

F190-32         CGTAACAGCATGACGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACAACC
F195-5          CGTAACAGCATGACGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACA-CC
F196-1          CGTAACAGCATGACGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACA-CC
Rose.algic      CGTAACAGCATGACGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACA-CC
Rb.capsul       CGTAACAGCATGACGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACA-CC
                ************************************************************ **

F190-32         ATGGGAGTTGGGTTCAC-CCGAAGGCCGTGCGCCAACCTGCAAAGGAGGCAGCGGACCAC
F195-5          ATGGGAGTTGGGTTCAC-CCGAAGGCCGTGCGCCAACCTGCAAAGGGGGCAGCGGACCAC
F196-1          ATGGGAGTTGGGTTCNNGCCGAAGGCCGTGCGCCAACCTGCAAAGGAGGCAGCGGACCAC
Rose.algic      ATGGGAGTTGGGTTCAC-CCGAAGGCCGTGCGCCAACCTTTCGAGGAGGCAGCGGACCAC
Rb.capsul       ATGGGAATTGGGTCTAC-CCTAAGATGGTGCGCCAACCTGCAAAGGAGGCAGCCAGCCAC
                ****** ******      ** ***   ************    *** ******   ****

F190-32         GGTGAGCTCAGCGACTGGGGTGAAGTCGTAACAAGGTAGCCGTAGGG
F195-5          GGTGAGCTCAGCGACTGGGGTGAAGTCGTAACAAGGTAGCCGTAGGG
F196-1          GGTGAGCTCAGCGACTGGGGTGAAGNCGTAACAAGGTAGCCGTAGGG
Rose.algic      GGTG
Rb.capsul       GGTAGGCTCAGTGACTGGGGTG
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Fig. 7.2. DNA sequence alignment of 16S ribosomal DNA obtained from the filamentous bacteria. *Roseobacter algicola* and *Rhodobacter capsulatus* are abbreviated as Rose.algic and Rb.capsul, respectively.

Table 7.1. Similarity and distance matrices inferred from multiple-aligned sequences of the 16S ribosomal DNA

	90-32	95-5	96-1	*R.a*	*R.d*	*R.l*	*O.a*	*R.c*	*R.s*	*P.a*	*P.d*	*E.c*	*N.g*
Fl90-32		99.2	99.4	93.8	92.7	94.0	93.4	91.5	91.7	92.2	90.8	65.3	66.2
Fl95-5	0.005		99.2	93.8	92.5	93.8	93.3	91.5	91.7	92.0	90.7	66.4	66.6
Fl96-1	0.004	0.003		93.8	92.8	94.1	93.5	91.6	91.9	92.3	90.8	64.9	66.3
Roseobacter algicola	0.058	0.059	0.058		93.2	94.4	92.1	89.6	91.1	91.7	90.5	65.9	65.5
Roseobacter denitrificans	0.066	0.060	0.062	0.053		96.8	91.3	89.4	89.8	91.5	90.5	63.8	65.1
Roseobacter litoralis	0.066	0.066	0.064	0.053	0.019		93.0	90.2	91.2	92.6	91.2	64.2	67.4
Octadecobacter arcticensis	0.069	0.067	0.067	0.079	0.075	0.068		89.5	90.4	91.8	90.0	65.1	66.6
Rhodobacter capsulatus	0.086	0.085	0.085	0.106	0.105	0.107	0.114		95.5	92.9	93.1	65.8	65.1
Rhodobacter sphaeroides (*rrnA*)	0.085	0.084	0.084	0.092	0.101	0.097	0.105	0.051		93.1	93.2	69.0	68.0
Paracoccus aminophilus	0.082	0.081	0.080	0.088	0.081	0.081	0.092	0.076	0.076		96.2	65.0	66.9
Paracoccus denitrificans	0.096	0.096	0.095	0.101	0.092	0.095	0.110	0.074	0.074	0.040		64.0	67.0
Escherichia coli	0.264	0.262	0.264	0.261	0.267	0.274	0.262	0.261	0.250	0.255	0.254		82.2
Neisseria gonorrhoeae	0.272	0.268	0.273	0.275	0.288	0.285	0.287	0.288	0.273	0.261	0.264	0.217	

The upper and lower half triangles show the similarity values (%) and the genetical distances (nucleotide substitution/position), respectively.

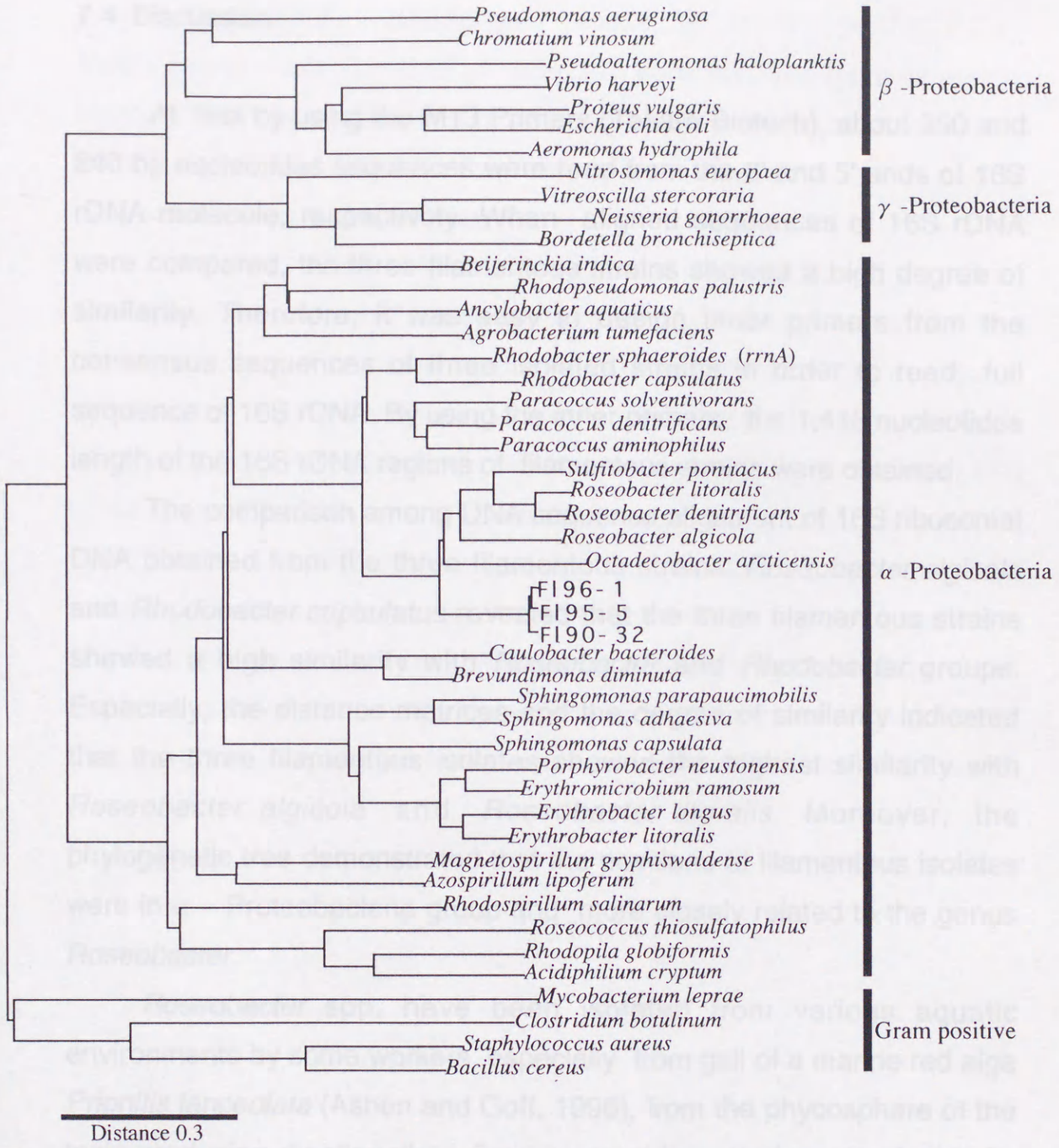

Fig. 7.3. Phylogenetic relationships inferred from 16S ribosomal DNA nucleotide sequence similarities.

7.4 Discussion

At first by using the M13 Primers (Takara Biotech), about 350 and 240 bp nucleotides sequences were read from the 3' and 5' ends of 16S rDNA molecule, respectively. When aligned sequences of 16S rDNA were compared, the three filamentous strains showed a high degree of similarity. Therefore, it was easy to design inner primers from the consensus sequences of three isolated strains in order to read full sequence of 16S rDNA. By using the inner primers, the 1,418 nucleotides length of the 16S rDNA regions of filamentous strains were obtained.

The comparison among DNA sequence alignment of 16S ribosomal DNA obtained from the three filamentous strains, *Roseobacter algicola* and *Rhodobacter capsulatus* revealed that the three filamentous strains showed a high similarity with *Roseobacter* and *Rhodobacter* groups. Especially, the distance matrices and the degree of similarity indicated that the three filamentous isolates showed the highest similarity with *Roseobacter algicola* and *Roseobacter litoralis*. Moreover, the phylogenetic tree demonstrated that the positions of filamentous isolates were in α – Proteobacteria group and more closely related to the genus *Roseobacter*.

Roseobacter spp. have been isolated from various aquatic environments by some workers, especially from gall of a marine red alga *Prionitis lanceolata* (Ashen and Goff, 1996), from the phycosphere of the toxin-producing dinoflagellate *Prorocentrum lima* (Lafay, *et al.*, 1996), from sea water at Otsuchi Bay, Iwate, Japan (Shiba *et al.*, 1996), and from fresh water cyanobacterial mats in a thermal alkaline sulfide spring (Yurkov, *et al.*, 1994).

The utilization of 16S rDNA sequence has been convinced in the modern taxonomy of bacteria as a standard method for the investigation

of their phylogenetic relationships (Woese, 1987; Burgess, 1993; Fox, 1992). Nucleotide sequences of 16S rDNA contained two types of region, highly conserved regions that have been used to define the relationship among the distant taxa, and variable regions that have been used to differentiate genera and species.

Since the results obtained in this experiment indicated that three filamentous strains could not be differentiated furthermore, it is interesting to use other genetic regions such as 23S rDNA and 5S rDNA in order to differentiate among the filamentous strains. By using sequence analysis of variable regions in 23S rDNA, or intergenic spacer region between 16S rDNA and 23S rDNA, more informations may be obtained to perform identification and differentiation among the greenish orange strain and whitish pink strains. Moreover, since the filamentous isolates have been found as bacteriochlorophyll a-containing bacteria, genetic analysis of genes encoding photosynthetic system may give more information to differentiate among the greenish orange and whitish pink strains.

Chapter 8

Conclusions

1. The laboratory stock culture of *Chattonella marina* harbors several bacterial strains and they have been surviving in hundred times of transfer process. Among them, filamentous bacteria as well as *Alteromonas*, and *Pseudomonas* spp. were isolated as the predominant bacteria.

2. In the state of mixed culture of axenic alga and co-existent bacterial strains, the living algal cells were maintained over 30 days after transferred into a fresh ESS liquid medium, whereas they began to decrease at about 10 days and were not detected at about 30 days in an axenic culture.

3. When more than 100 mg/l of polypepton was added to a non-axenic culture, the algal cell counts decreased as the total bacterial counts increased. While, filamentous bacteria were detected in higher levels after prolonged incubation of the non-exenic culture added with polypeptone than other co-existent bacteria.

4. The addition of enrichment solution (ESP) into axenic cultures, algal cells were stimulated to grow even after reached to the maximum cell number.

5. Algal cells completely died within 3 days of incubation in the vitamin-free medium, within 10 days in the media with biothin or biotin and thiamin, and within 25 days in the vitamin B_{12}-containing media.

6. Vitamin B_{12} was consumed completely within 15 days by algal cells in an axenic culture. While, the algal growth was maintained longer

by the presence of vitamin B_{12} produced by co-existent bacteria in non-axenic cultures.

7. Multicellular filamentous bacteria were isolated not only from the culture of *C. marina* but also from those of *H. akashiwo* and *Tetraselmis* sp.

8. Filamentous bacteria strains formed whitish pink colonies and produced flexixanthin-like or spheroidenone-like pigment. They were also found to contain bacteriochlorophyll a.

9. GC% of filamentous bacterial DNA was between 58.7-60.0, except for FI95-6 with 32.9 mol%.

10. DNA-DNA dot hybridization among the filamentous bacterial strains was observed but neither with the FI95-6 nor with the reference strains (*Cytophaga-Flexibacter* group).

11. Based on DNA-DNA dot hybridization with the reference strains, all filamentous bacteria were identified as neither *Cythophaga* nor *Flexibacter* spp.

12. Although the differences in cellular pigments were shown between FI95-5 strain (greenish orange) and whitish-pink strains, polymorphisms among all representative filamentous strains were not detected in the RFLP profiles. The RFLP profiles indicated that all representative filamentous strains were quite similar with regard to nucleotide sequences of 16S rDNA. A few differences among the

filamentous strains were shown in some RFLP profiles, but these differences may be originated from the 16S rDNA fragment lengths.

13. The aligned sequences of the 1,418 nucleotides length of 16S rDNA regions from three filamentous strains showed a high degree of similarity. The comparison among DNA sequence alignments of 16S ribosomal DNAs obtained from the three filamentous strains, *Roseobacter algicola* and *Rhodobacter capsulatus* revealed that the three filamentous strains showed a high similarity with *Roseobacter* and *Rhodobacter* groups.

14. The distance matrices and the degree of similarity among the 16S rDNAs indicated that the three filamentous isolates showed the highest similarity with *Roseobacter algicola* and *Roseobacter litoralis*.

15. The phylogenetic tree demonstrated that the positions of filamentous isolates were in α – Proteobacteria group and that they were more closely related to the genus *Roseobacter*.

Summary

The laboratory stock culture of *Chattonella marina* harbored several bacterial strains and it has been surviving in hundred times of transfer process. Filamentous bacteria as well as *Alteromonas* and *Pseudomonas* spp., were isolated as the predominant bacteria from a non-axenic culture of *C. marina*. Moreover, they were also detected as the predominant bacteria in a red tide alga, *H. akashiwo*, which was isolated from the bloom occurred at April 1995 in Kagoshima Bay, and in the culture water of *Tetraselmis* sp., which was generally cultured as a food for Kuruma prawn in Kagoshima.

In the state of mixed cultures of an axenic algal strain and co-existent bacterial isolates, the living algal cells were maintained over 30 days in an enrichment sea water (ESS) medium. On the other hand, algal cells began to decrease at 10 days and were not detected at 30 days in axenic cultures.

On the addition of enrichment solution (ESP) into axenic cultures, algal cells were stimulated to grow even after reached to the maximum cell number. When more than 100 mg/l of polypepton was added to non-axenic cultures, the algal cell counts decreased as the total bacterial counts increased.

The mutual interactions between bacteria and microalgae through the biological and biochemical processes were clarified by conducting bioassay on vitamin B_{12} by using *Euglena gracilis,* strain *z*. The results indicated that vitamin B_{12} was consumed completely within 15 days by algal cells in an axenic culture. On the contrary, algal growth was maintained longer by the presence of vitamin B_{12} produced by co-existent bacteria in non-axenic cultures compared with an axenic culture in ESS medium with vitamin B_{12}.

The effect of sea water on the growth of filamentous multicellular bacteria which have been isolated from the cultures of three marine

microalgae including *C. marina*, *H. akashiwo* and *Tetraselmis* sp, indicated that the all strains showed good growth in the artificial sea water medium and failed to grow in the medium with 3% NaCl or without NaCl. Major pigments extracted from the cells of filamentous isolates were considered to be flexixanthin-like or spheroidenone-like carotenoid and bacteriochlorophyll a according to their absorption spectra.

Most of the filamentous strains had 58.7 to 60.0 mol% of GC content. DNA probes prepared from the representative strains were hybridized with DNAs of a major group of filamentous isolates but not with those of *Cytophaga-Fexibacter* reference strains. These findings indicated that a major group of filamentous bacteria isolated from the cultures of different microalgae belonged to the same bacterial species.

Although the differences in cellular pigments were shown between FI95-5 strain (greenish orange) and whitish-pink strains, polymorphism among all representative filamentous strains was not observed in the RFLP profiles. The RFLP profiles indicated that all representative filamentous strains were quite similar with regard to nucleotide sequences of 16S rDNA.

The comparison among DNA sequence alignments of 16S ribosomal DNAs obtained from the three filamentous strains, *Roseobacter algicola* and *Rhodobacter capsulatus* revealed that the three filamentous strains showed a high similarity among them and with *Roseobacter* and with *Rhodobacter* groups. Especially, the distance matrices and the degree of similarity among the 16S rDNAs showed the highest similarity with *Roseobacter algicola* and *Roseobacter litoralis*. Moreover, the phylogenetic tree demonstrated that the positions of filamentous isolates were in α - Proteobacteria group and that they were clustered with *Roseobacter* spp.

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In the name of my Lord, most gracious, most merciful. Praise be Lord, the cherisher and sustainer of the world. Amen.

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