Perspectives of the Development of 16S rDNA Probe Specific for Algicidal and/or Algal-lytic Gliding Bacteria

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Sequence analyses of small subunit ribosomal RNA gene (16S rDNA) were performed on 16 strains of gliding marine bacteria isolated at the time of bloom of *Skeletonema costatum* in Ariake Sea. The strains were comprised of 8 strains which have capabilities to kill and lyse *Skeletonema costatum*, 5 strains with algicidal or algal-lytic activity and 3 strains without activity. The strains were divided into 4 clusters including 1, 2, 3, and 10 strains. Cluster A, C and D were close to *Cytophaga latercula*, *Flexibacter maritimus* and *Cytophaga marinoflava*, respectively. Cluster B was close to *Flavobacterium aquatile*. Neither algicidal nor algal-lytic activity was restricted to any of the clusters. The strains of cluster B contained flexirubin and did not require addition of Na⁺ in growth peptone media.

Key words: algicidal bacteria, algal-lytic bacteria, 16S rDNA

Red tides have inflicted serious damages to the aquaculture in Japanese coastal seawaters. For example, at the time of the bloom of *Skeletonema costatum*, inorganic nutrients are consumed vigorously and become quickly extinct, and then the extinction results in a color fading of *Porphyra* in aquaculture.¹⁾ This color fading is called “iro-ochi” in Japanese.

As a possible tool for repressing or predicting the bloom of *S. costatum* and also the other algae, algicidal and/or algal-lytic bacteria have attracted much attention. The population of a certain species of algae is quickly diminished in coincidence with the increase of such algicidal and/or algal-lytic bacteria.²⁾ Such bacteria are inhibitory to a given range of algal species, and isolated at the bloom of the algae. The species of algal-lytic and/or algicidal bacteria isolated so far is distributed among a wide variety of taxonomic divisions.^{3–10)}

Mitsutani has recently isolated some gliding bacteria which kill and/or lyse *S. costatum* from the Ariake Sea, with a double-layered agar plate medium containing the cells of *S. costatum*.¹⁾ The algicidal bacteria digest cytoplasmic materials of the alga, while the algal protoplasts were produced by the algal-lytic bacteria. A limited number of phenotypic characteristics, examined hitherto, suggested that these bacteria were monophyletic. Hence, the bacteria seemed to be an available tool for predicting or repressing the bloom of *S. costatum* if a specific DNA probe is developed for the bacteria. Development of such a DNA probe is a prerequisite for quickly monitoring the bacteria in environments.

In this experiment, we determined the sequence of small subunit ribosomal RNA gene (16S rDNA) of these algicidal and/or algal-lytic bacteria, together with the gliding bacteria without algicidal or algal-lytic activity, for estimating the perspectives for developing a DNA probe in 16S rDNA for algicidal and/or algal-lytic bacteria. Phylogenetic diversity of the bacteria is a crucial point for developing a specific DNA probe.

Materials and Methods

Bacterial Strains

Bacterial strains were isolated from the Ariake Sea, with a double-layered agar plate medium which contained *Skeletonema costatum* NIES-324 strain.¹⁾ The algal strain was axenically cultured in a modified SWM-III medium^{11,12)} at 15°C before addition to the agar medium. An intermittent illumination, 12L:12D, was provided at 35 $\mu\text{Em}^{-2}\text{S}^{-1}$ with an incandescent lamp. Bacterial strains forming a plaque around colony were isolated, and purified with the modified SWM-III medium supplemented with 0.1% of casitone (Difco) and 0.05% of yeast extract (Difco) or PPES-II medium.¹³⁾

Examination of Algal-lytic and Algicidal Activity

Definition of algal-lytic and algicidal activity was referred to Mitsutani and Mitsutani *et al.*^{1,3)} Algicidal bacteria digest cytoplasmic materials of the alga, while algal-lytic bacteria produce algal protoplast. The activities of the isolated strains were ascertained in a liquid culture of *S. costatum*. Briefly, the bacterial strains grown in the above peptone media was collected at an exponential growth phase, washed and suspended in SWM-III. The suspensions were inoculated into the culture of *S. costatum*. The numbers of algal cells were determined under a phase-contrast microscopy. Decrease of the algal cells was defined as an indication of the activity.

DNA Extraction

The bacterial strains were grown in PPES-II liquid medium. The cells were centrifuged and washed in a sterilized artificial seawater.¹⁴⁾ DNA was extracted based on the method of Keller and Manak,¹⁵⁾ or by boiling the cells in sterilized distilled water.

PCR Amplification of 16S rDNA

PCR amplification of 16S rDNA was accomplished us-

ing an automatic thermal cycler (model 480, Perkin-Elmer Co.), with 30 thermal cycles of 94°C for 60 s, 60°C for 45 s and 72°C for 90 s. Hot start at 94°C for 180 s, and an extension at 72°C for 300 s after the cycles was also performed. A 100 μ l of the reaction mixture contained 0.1 to 1.0 μ g of template DNA, 20 nmol each of dNTP, 100 pmol each of PCR primer, 1.35 unit of recombinant *Taq* DNA polymerase (Takara Co. Ltd., Shiga, Japan), 10 μ l of 10-fold concentrated PCR buffer prepared by the manufacturer. The primers used in this experiment were 27F (5'-AGAGTTTGTATCMGTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTACGACTT-3') which were correspondent to the position 8 to 27 and 1513 to 1492 in the *Escherichia coli* 16S rDNA number,¹⁶⁾ respectively. Molecular size of PCR amplicon was determined with an agarose electrophoresis including a molecular marker at a constant voltage of 100 V.

PCR Direct Sequencing and Phylogenetic Analysis of 16S rDNA

The excess dNTP and the remaining primers in PCR products were removed by filtration through a cartridge filter Suprec-02 (Takara Co. Ltd.). The purified products were directly sequenced by using a Dye terminator cycle sequencing kit (Perkin-Elmer Co.), with universal primer 338R (5'-CTGCTGCCTCCCGTAGGAGT-3') corresponding to the position of 357 to 338 in the *Escherichia coli* 16S rDNA number.¹⁶⁾ PCR amplification with a combination of 27F and RVM517R (5'-CAGGAAACAGCTATGACCGTATTACCGCGGATGCTGGC-3') primers was also repeated before sequencing by using Dye primer cycle sequencing kit after the purification with Suprec-02.

The search for 16S rDNA sequences similar to those of the gliding bacteria was performed using Similar Rank in RDP (Ribosomal Database Project) database.¹⁷⁾ The 16S rDNA sequences were aligned using a software Clustal W (v. 1.6).¹⁸⁾ Kimura's two-parameter model¹⁹⁾ was applied to the calculations of evolutionary distance. A phylogenetic tree was constructed by the neighbor-joining method.²⁰⁾ Bootstrap analyses of 1000 replicates were carried out using Clustal W.

Accession Numbers of 16S rDNA Sequences

The 16S rDNA sequences used for the phylogenetic analysis include the sequences of *Capnocytophaga canimorsus* (GenBank accession no. L14637), *Cap. gingivalis* (L14639), *Cap. ochracea* (U41350), *Cytophaga hutchinsonii* (M58768), *Cy. latercula* (M58769), *Cy. marinoflava* (M58770), *Cy. uliginosa* (M62799), *Escherichia coli* (X80725), *Flavobacterium columnare* (M58781), *Fla. flevense* (M58767), *Fla. hydatis* (M58764), *Fla. johnsoniae* (M59051), *Fla. gondwanense* (M92278), *Fla. salegens* (M92279), *Flectobacillus glomeratus* (M58775), *Flexibacter maritimus* (M64629), *Gelidibacter algens* (U62914), *Myroides odoratus* (M58777) and *Psychroserpens burtonensis* (U62913) in GenBank nucleotide sequence database, and *Cy. lytica* (RDP short ID, *Cy. lytica*), *Fla. aquatile* (F. aquatile), *Fla. saccharophilum* (F. saccharo) and *Fla. succinicans* (F. succinic) in RDP database.

The nucleotide sequences determined in this study have been submitted to the DDBJ nucleotide sequence database under accession numbers from AB008031 to AB008046.

Flexirubin Test

Existence of flexirubin was examined with the cells of slope and liquid cultures according to the method described by Reichenbach *et al.*²¹⁾ Briefly, a 20% KOH was poured onto the slope culture, and then observed. Color shift to red or orange indicated the presence of flexirubin. In case of liquid cultures, the bacterial cells were centrifuged and serially extracted with methanol and chloroform. A drop of 20% KOH was added to the extracts. Spectra were examined before and after the addition of KOH. Peak shift toward a longer wavelength was recognized as an indication of the presence of flexirubin.

Other Test

Gram staining was performed with a Hucker's modification.²²⁾ Motility was examined with a hanging drop preparation under a phase microscope. Gliding motility was examined with a plate medium containing 0.8% agar in SWM-III medium. Capability of acid production from carbohydrate was examined using MOF medium.²³⁾ Kovacs's oxidase test²⁴⁾ was performed with a filter strip prepared by Nissui Co. Color change was checked after 30 s. Catalase test was performed with 3% H₂O₂ solution according to the recommendation of Taylor and Achanzar.²⁵⁾ Briefly, bacterial cultures were streaked onto the strip of filter paper. The filter paper was immersed into 3% H₂O₂ solution. Bubbles from the surface of the cells on filter paper indicated the presence of catalase in bacterial cells. Cation requirement for growth was examined with the method of Colwell and Wiebe.²⁶⁾ Quinone was extracted from a freeze-dried cell with chloroform/methanol (3:1), and purified on a thin layer chromatography based on the method of Collins *et al.*²⁷⁾ After extraction from the gel and filtration through a filter (Sunprep 4-FH, Millipore), chemical identification was performed with a high performance liquid chromatography using a reverse-phase column (Ultrasphere ODS, Beckman). Methanol/isopropylalcohol was flowed at 1 ml/min at room temperature. Quinones were monitored at 275 nm.

Results and Discussion

Figure 1 shows a phylogenetic relationship between the species of *Flavobacterium-Cytophaga* group and the gliding bacteria isolated from the Ariake Sea. The alignment was inferred from the sequences of 410 to 420 bp-length fragments of 16S rDNA which were correspondent to the position of 28 to 433 in the *E. coli* number.¹⁶⁾ The phylogenetic alignment of the species of *Flavobacterium-Cytophaga* group was slightly different from that obtained from full sequence of 16S rDNA.^{28,29)} The marine bacteria, *Cytophaga latercula* and *Cy. marinoflava*, were closely related to each other in the alignment based on the full sequence,^{28,29)} while these were distant in this alignment. In our experiment, the distance between *Cy. latercula* and *Cy. marinoflava* was comparable to the distance between *Cy. latercula* and *Flavobacterium aquatile* which has been described as a terrestrial bacterium. The discrepancy could be caused by the inclusion of only the most variable region and the exclusion of conservative one in this experiment. Although it is not clear the alignment of which experiment describes a real evolutionary relationship, we have not con-

Fig. 1. Phylogenetic position of algalicidal and/or algal-lytic bacteria within *Flavobacterium-Cytophaga* group, based on the partial 16S rDNA sequences (the position from 28 to 443 in the *E. coli* 16S rDNA).

ducted any further experiments, because the construction of a phylogenetic tree was not our purpose.

Total of 16 strains were divided into 4 clusters. Cluster A was comprised of strains A5, 11, 14, 15, 20, and strains A12, 32, 35, 41, and A38. The sequences of strains A5, 11, 14, 15 and 20 were identical to each other and very similar to *Cy. latercula*. The strains A12, 32, 35 and 41 were also identical to each other. Strains A16, 17 and 43 formed cluster B, which was relatively close to *Fla. aquatile*. Strains A37 and 45 formed a third cluster, cluster C, which was a little distant from *Flexibacter maritimus*. Strain A23 (namely cluster D) may be grouped with *Cy. marinoflava*. The clusters A, B and C included algalicidal and/or algal-lytic bacteria, while cluster D did not.

Intercluster distances (K_{nuc} values) between clusters A, B and C ranged from 0.132 to 0.197 (Table 1). The distances among *Cy. latercula*, *Flex. maritimus* and *Fla. aquatile* were estimated as from 0.132 to 0.175 in this experiment, while 0.065 to 0.092 in the full sequence analyses of 16S rDNA.²⁸⁾

Although the strains were distributed among different

Table 1. Similarity and K_{nuc} value calculated based on the partial sequence of 16S rDNA

Strain	Similarity (%) and K_{nuc} value*															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1: <i>Cytophaga latercula</i>	—															
2: A12	0.000	—														
3: A32	0.000	0.000	—													
4: A35	0.000	0.000	0.000	—												
5: A41	0.000	0.000	0.000	0.000	—											
6: A38	0.030	0.029	0.029	0.029	0.029	—										
7: A5	0.037	0.037	0.037	0.037	0.037	0.022	—									
8: A11	0.037	0.037	0.037	0.037	0.037	0.022	0.022	—								
9: A14	0.037	0.037	0.037	0.037	0.037	0.022	0.022	0.000	—							
10: A15	0.037	0.037	0.037	0.037	0.037	0.022	0.022	0.000	0.000	—						
11: A20	0.037	0.037	0.037	0.037	0.037	0.022	0.022	0.000	0.000	0.000	—					
12: <i>Flexibacter maritimus</i>	0.132	0.132	0.132	0.132	0.132	0.120	0.120	0.106	0.106	0.106	0.106	—				
13: A37	0.165	0.164	0.164	0.164	0.164	0.160	0.150	0.150	0.150	0.150	0.150	0.132	—			
14: A45	0.153	0.152	0.152	0.152	0.152	0.143	0.132	0.132	0.132	0.132	0.132	0.087	0.032	—		
15: <i>Cytophaga marinoflava</i>	0.165	0.164	0.164	0.164	0.164	0.164	0.159	0.159	0.159	0.159	0.159	0.172	0.160	0.171	—	
16: A23	0.165	0.164	0.164	0.164	0.164	0.164	0.162	0.162	0.162	0.162	0.162	0.201	0.204	0.206	0.206	—
17: <i>Flavobacterium aquatile</i>	0.175	0.174	0.174	0.174	0.174	0.174	0.160	0.160	0.160	0.160	0.160	0.165	0.199	0.183	0.151	0.165
18: A16	0.153	0.152	0.152	0.152	0.152	0.149	0.144	0.144	0.144	0.144	0.144	0.154	0.197	0.179	0.150	0.164
19: A17	0.153	0.152	0.152	0.152	0.152	0.149	0.144	0.144	0.144	0.144	0.144	0.154	0.197	0.179	0.150	0.164
20: A43	0.153	0.152	0.152	0.152	0.152	0.149	0.144	0.144	0.144	0.144	0.144	0.154	0.197	0.179	0.150	0.164

* The values on the upper right are percentages of similarities, and the values on the lower left are K_{nuc} values.

clusters, neither algicidal nor algal-lytic activity was restricted in any of the clusters, as in Table 2. Also A41 and A45, which were neither algicidal nor algal-lytic, were included in cluster A and C, respectively. Cluster A and C included algicidal and/or algal-lytic bacteria. There is no data whether the species of *Cytophaga*, *Capnocytophaga*, *Flectobacillus*, *Flexibacter* and *Flavobacterium*, which were located between 4 clusters, were algicidal or algal-lytic. Hence development of any specific probe for algicidal or algal-lytic activity is still difficult, even though evolutionary distances among the bacterial species were converged in the range of 0.065 to 0.092, as suggested from the full sequences. Even these values are quite large and suggest difficulty.

Table 3 summarizes phenotypic characteristics of the gliding bacteria. All of the strains were gram-negative rod and did not show flagella-motility. Gliding motility was observed. Catalase and oxidase were present. Acids were

produced aerobically from some carbohydrates. All characteristics mentioned above are found commonly in the bacteria in marine-type *Cytophaga* group.^{21,28,30} The major quinone in the gliding bacteria examined was menaquinone 6 which has been detected in the strains of *Cy. latercula*, *Cy. marinoflava* and *Fla. aquatile*, but not in *Cy. hutchinsonii*.²⁸ *Cy. hutchinsonii* contains menaquinone 7.

Depending on the phenotypic characteristics of bacteria, the four clusters seemed to be divided into two major groups. The strains of cluster B were different from the others in non-requirement of Na⁺ for growth, despite that they were isolated from marine environment with a seawater medium. Also flexirubin was found in the strains of cluster B as a major pigment, but not in the other clusters. Flexirubin has not yet been reported to be present in *Fla. aquatile*.³⁰ Phenotypic and phylogenetic data in the experiment suggested that some of the gliding bacteria were not included in any of the known species. Description of these bacterial strains as a new species with further experiment should be valuable in bacterial taxonomy.

In this experiment, data indicated a wide distribution of algicidal and algal-lytic activities among *Flavobacterium*-*Cytophaga* group. Mitsutani also has isolated algicidal bacteria included in the genus *Alteromonas*,¹ from the seawater sample collected at the station where the gliding bacteria in this experiment were isolated. Hence it should be difficult to investigate the ecological phenomena of algicidal and algal-lytic activities with a single DNA probe. Distinction between algal-killing and non-killing bacteria by using 16S rDNA as a target gene is also impossible. Search for other genetic materials is necessary for developing a probe for algicidal and/or algal-lytic activities. Or, a search for a specific indicative strain, which has algicidal and/or algal-lytic activity and coordinates always with algal bloom would be valuable for developing a probe for algal and/or algal-lytic activities.

Table 2. Algicidal and algal-lytic activities of gliding bacteria

Strain	Cluster	Algicidal activity	Algal-lytic activity
A5	A	—	+
A11	A	+	+
A12	A	+	+
A14	A	+	+
A15	A	+	—
A20	A	+	+
A32	A	+	+
A35	A	+	+
A38	A	+	+
A41	A	—	—
A16	B	+	—
A17	B	+	+
A43	B	+	—
A37	C	+	—
A45	C	—	—
A23	D	—	—

Table 3. General characteristics of gliding bacteria isolated at the time of bloom of *Skeletonerna costatum*

Strain	A12	A32	A35	A41	A5	A11	A14	A15	A20	A38	A23	A37	A45	A16	A17	A43
Cluster	A	A	A	A	A	A	A	A	A	A	D	C	C	B	B	B
Gram	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	ND	ND	+	ND	+	+	+
OF test																
glucose	O	O	O	ND	ND	—	—	—	—	O	ND	O	ND	O	O	O
galactose	O	ND	ND	O	—	—	—	—	—	O	—	—	O	—	—	—
maltose	O	ND	ND	O	O	O	O	O	O	O	O	O	O	O	O	O
cellobiose	O	ND	ND	O	—	—	—	—	—	O	—	—	O	—	—	O
xylose	O	ND	ND	O	—	—	—	—	—	O	—	—	O	—	—	—
Motility	glid	glid	glid	glid	glid	glid	glid	glid	glid	glid	glid	glid	glid	glid	glid	glid
Cell form	long	long	long	long	long	long	long	long	long	long	long	long	long	long	long	long
Requirement of																
K ⁺	+	+	+	+	+	—	—	—	—	+	—	+	—	—	—	—
Na ⁺	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—
Mg ²⁺	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—
Ca ²⁺	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Growth at																
pH5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
pH6	—	—	—	—	—	—	—	+	+	—	—	—	+	+	+	+
pH8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Quinone	MK6	MK6	MK6	MK6	MK6	MK6	MK6	MK6	MK6	MK6	MK6	MK6	MK6	MK6	MK6	MK6
Flexirubin	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+

ND, not done; O, oxidative; glid, gliding motility; MK6, menaquinone 6.

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