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Phylogenetic Analysis of Algicidal Bacteria (Family *Flavobacteriaceae*) and Selective Detection by PCR Using a Specific Primer Set

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Sequencing the 16S rDNA and phylogenetic analysis were performed on algicidal bacteria belonging to the family *Flavobacteriaceae*, *Chattonella*-killing *Cytophaga* sp. strain J18/M01, *Heterocapsa*-killing *Cytophaga* sp. strains AA8-2 and AA8-3, and *Gymnodinium* growth-inhibiting *Flavobacterium* sp. strain 5N-3. These algicidal strains were clustered phylogenetically within the marine Cytophaga/Flavobacter/Bacteroides (CFB) group. *Flavobacterium* sp. 5N-3 was phylogenetically clustered with *Psychroserpens burtonensis* but not with the algicidal gliding bacteria. The sequence similarity between strain 5N-3 and *P. burtonensis* was only 92%. The strains J18/M01 and AA8-2 (AA8-3) exhibited the high level of sequence similarity of 98.5%. Despite the differences of host algae and of locality and year of isolation, these algicidal bacteria were phylogenetically located on the same terminal branch.

The PCR amplification of the 16S rDNA of the CFB strains using a single set of J18/M01-specific primers resulted in specific detection of the J18/M01, indicating that the J18/M01-specific sequence in the 16S rDNA is useful for selective detection of the strain J18/M01.

Key words: Algicidal bacteria, *Cytophaga*, *Flavobacterium*, 16S rDNA, PCR, *Chattonella antiqua*, *Heterocapsa circularisquama*

In recent years, algicidal marine bacteria for red tide phytoplanktons have been isolated from the coastal sea of Japan.[1,2] A bacterium strain J18/M01 with algicidal activity against *Chattonella antiqua* was isolated from Harima-Nada, eastern Seto Inland Sea in 1990 and was tentatively identified as *Cytophaga* sp.[3] *Cytophaga* sp. J18/M01 directly attacks *C. antiqua* cells and preys upon various species of marine algae.[4,5] Other gliding bacteria, *Cytophaga* sp. AA8-2 and AA8-3, were isolated from Ago Bay, Mie Prefecture, in August of 1995 as *Heterocapsa circularisquama*-killing bacteria by Imai *et al.*[6] Another non-gliding Cytophagales, *Flavobacterium* sp. 5N-3, which inhibits the growth of *Gymnodinium mikimotoi*, was isolated from a water sample of Uranouchi Inlet, Kochi Prefecture collected in 1989.[7] It is possible that algicidal bacteria could be useful tools in reducing the impact of harmful phytoplankton blooms on aquaculture. In order to use the bacteria for controlling red tide occurrences in natural environments, the species or strains have to be specifically identified and enumerated in these environments. In the present study, we determined the almost-complete sequences of the small-subunit ribosomal RNA gene (16S rDNA) of *Cytophaga* sp. J18/M01, AA8-2 and AA8-3, and *Flavobacterium* sp. 5N-3, in order to characterize the genetic relationships among the members of the Cytophaga/Flavobacter/Bacteroides (CFB) group. Moreover, a specificity of primers designed to detect specific sequence in 16S rDNA was also investigated for detecting and differentiating the algicidal strain J18/M01 from phylogenetically related bacterial strains by means of PCR.

Materials and Methods

Bacterial Strains and Growth Conditions

Algicidal bacteria *Cytophaga* sp. (J18/M01, AA8-2 and AA8-3) and *Flavobacterium* sp. 5N-3 were cultured in 1 L of peptone broth[8] and FeTY medium,[9] respectively, at 20°C in the dark. The following strains were obtained from American Type Culture Collection, Rockville, Md., USA (ATCC), the Institute of Fermentation, Osaka, Japan (IFO), and Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan (IAM): *Cytophaga aquatilis* (*F. hydatis*) IAM12365^T (T=type strain), *F. aquatile* IAM12316^T, *C. marinoflava* IFO14170^T, *C. johnsonae* (*F. johnsoniae*) IFO14942^T, *C. succinicans* (*F. succinicans*) IFO14905^T, *C. lytica* IFO14961^T, *C. uliginosa* IFO14962^T, *F. branchiophilum* IFO15030^T, *F. odoratum* (*Myroides odoratus*) IFO14945^T, *C. latercula* ATCC23177^T, *F. salegens* ATCC51522^T, *F. gondowanense* ATCC51278^T,

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Flexibacter maritimus ATCC43398^T. These CFB strains were cultured in a broth media according to the instructions of the culture collection from which they were obtained.

DNA Extraction

Cell pellets harvested by centrifugation at 14,400 × *g* for 25 min were suspended in 5 m*l* of NET buffer (50 mM Tris-HCl, 150 mM NaCl, 100 mM Na_2-EDTA, pH 8.0) containing 0.5% sodium dodecyl sulfate and 1 mg of proteinase K, and incubated at 56 for 30 min. After the addition of 0.9 m*l* of 5 M NaCl and 0.75 m*l* of 10% cetyltrimethyl ammonium bromide in 0.7 M NaCl, incubation was carried out at 65°C. Solutions were extracted with phenol/chloroform/isoamylalcohol (25/24/1) three or four times. The genomic DNA was precipitated by the addition of one-sixth volume of 2-propanol. After rinsing with 80% ethanol, the DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM Na_2-EDTA, pH 8.0) containing RNase A (100 μg/m*l*) and incubated at 37°C for 1 h. Final purification was performed with chloroform/isoamylalcohol (24/1) and ethanol precipitation.

PCR Amplification and Sequencing

The almost-complete 16S rDNA was amplified using the purified DNA as a template with a single set of 8F (5′-AGAGTTTGATCATGGCTCAG-3′) and 1512R (5′-ACGGTTACCTTGTTACGACTT-3′) primers corresponding to the conserved regions at the 5′ and 3′ ends of the prokaryotic gene[10] with the following thermal cycling conditions: 25 cycles of denaturation (1 min at 94°C), annealing (2 min at 55°C), extension (3 min at 72°C), with a final elongation step of 7 min at 72°C. The PCR amplification of the 16S rDNA of *Cytophaga* sp. J18/M01, AA8-2 and AA8-3, and *Flavobacterium* sp. 5N-3 resulted in a single band of about 1.5 kbp when analyzed by 1% agarose gel electrophoresis and staining with ethidium bromide solution. A part of the unpurified PCR amplicon was cloned using the TA Cloning® Kit (Invitrogen, USA) for sequencing the 5′ and 3′ ends of the gene. Sequencing was performed for 4 clones and the purified PCR products from each strain using a *Taq* DyeDeoxy™ Terminator Cycle Sequencing Kit and DNA sequencer 373As (Perkin-Elmer Co., USA) by using a set of primers complementary to conserved regions located within the bacterial 16S rDNA.[10]

The 16S rDNA sequence data determined in this study will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB017146, AB017147, AB017048, and AB017597.

Phylogenetic Analysis

The almost-complete sequences of 16S rDNA determined were aligned to a collection of CFB group 16S rDNA sequences obtained from the EMBL/GenBank/DDBJ databases. Evolutionary distance values were calculated according to the 2-parameter model of Kimura.[11] The unrooted phylogenetic tree was reconstructed using the neighbor-joining method[12] as implemented in the program CLUSTAL W.[13] Confidence limits on the tree topology were estimated by bootstrap analysis[14] with 1000 repetitions.

Specificity of J18/M01-specific Primers

The specificity of the primers designed from the specific sequence of 16S rDNA was tested for detecting the algicidal strain J18/M01 by means of PCR amplification under the conditions described above and agarose gel electrophoresis was performed with 2% gel.

Results and Discussion

Figure 1 shows a phylogenetic tree indicating the position of the algicidal bacteria. This tree was almost the same as obtained by Manz *et al.*[15] According to the phylogenetic analysis, the algicidal bacteria belonged to the family *Flavobacteriaceae* which was emended by Bernardet *et al.*[16] The 16S rDNA sequences of *Cytophaga* sp. AA8-2 and AA8-3 were identical. The algicidal bacteria were clustered phylogenetically with *C. marinoflava*, *C. lytica*, *C. latercula*, *C. uliginosa*, *Flavobacterium gondowanense*, *F. salegens*, *Flexibacter maritimus*, *Gelidibacter algens*, and *Psychroserpens burtonensis*. All bacteria in this cluster were marine isolates and could be discriminated from the phylogenetically neighbor clusters comprised of freshwater or soil isolates of *Flavobacterium* species (Fig. 1). The strain J18/M01 was the closest relative to *Cytophaga* sp. AA8-2 (AA8-3), with the very high percentage homology of 98.5%. Although the strains J18/M01 and AA8-2 were isolated with different host algae (*C. antiqua* and *H. circularisquama*) from distant areas (Harima-Nada and Ago Bay) in different years (1990 and 1995), they were phylogenetically located on the same terminal branch of algicidal gliding bacteria. This branch was constructed with only the algicidal gliding bacteria, suggesting that this group of bacteria possessed algicidal activity. The 16S rDNA sequence similarities between these algicidal bacteria and *C. latercula*, which is proximal to this branch, were less than 91%. These results indicate that the algicidal gliding bacteria are a phylogenetically distinct group in the marine CFB. On the other hand, *Flavobacterium* sp. 5N-3 was clustered phylogenetically with *Psychroserpens burtonensis* rather than with the algicidal gliding bacteria. The sequence similarity between strain 5N-3 and *P. burtonensis* was only 92%. This result implies that strain 5N-3 is also phylogenetically distinct from other marine CFB.

We found at least four positions of specific to the 16S rDNA sequence of the strain J18/M01, corresponding to *Escherichia coli* position numbers 128-149 (J1), 185-208 (J2), 461-478 (J3), and 834-865 (J4). Using the BLAST program,[17] homology searching for these positions identified no sequence in bacterial 16S rDNA. We therefore chose two of these positions, J2 and J4, as the sequences of primer sets for PCR amplification to selectively detect the strain J18/M01.

The PCR amplification of the 16S rDNA and analysis by agarose gel electrophoresis of the CFB strains using the set of bacterial universal 8F and 1512R primers resulted in production of a single band of about 1.5 kbp from all strains tested, but resulted in production of a specific product only from the J18/M01 strain with the set of J2 (5′-CTGTGATCTCGCATGGGATTATAG-3′) and J4 (5′-CCACTCAGTCCGAAAACCAAA-3′) primers (Fig. 2). Between strains J18/M01 and AA8-2 there were 8

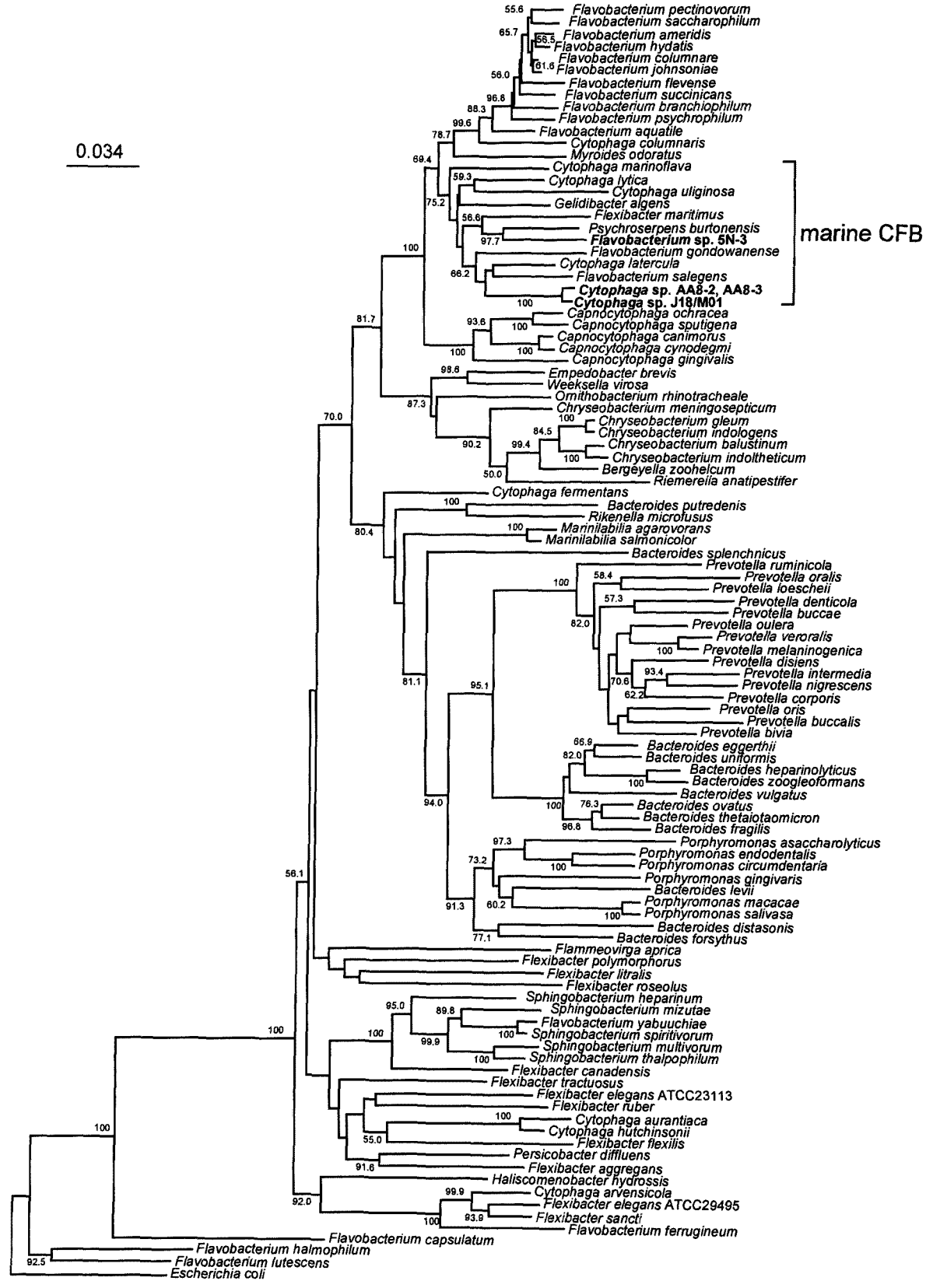

Fig. 1. Phylogenetic tree showing the position of algicidal gliding bacteria, *Cytophaga* sp. J18/M01, AA8-2, AA8-3, and *Flavobacterium* sp. 5N-3, which are indicated by bold-faced type, among the CFB group, based on 16S rDNA sequences using the neighbour-joining method.

Numbers on the branches represent percentage of 1000 bootstrap repetitions. Confidence limits of less than 50% are not shown. The distance scale indicates the expected number of changes per sequence position.

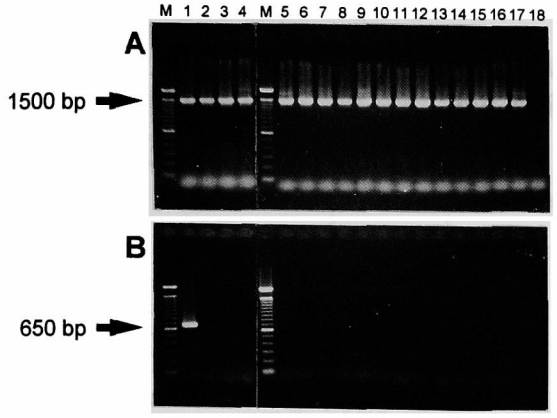

Fig. 2. PCR amplifications of the 16S rDNA from algicidal isolates and their related CFB bacteria using the set of 8F and 1512R primers (A) and the set of J2 and J4 primers specific to the strain J18/M01 (B) analyzed by 2% agarose gel electrophoresis and staining with ethidium bromide.

Lanes: M, 100-bp ladder size marker; 1, *Cytophaga* sp. J18/M01; 2, *Cytophaga* sp. AA8-2; 3, *Cytophaga* sp. AA8-3; 4, *Flavobacterium* sp. 5N-3; 5, *Cytophaga aquatilis* (*F. hydatis*) IAM12365^T; 6, *F. aquatile* IAM12316^T; 7, *C. marinoflava* IFO14170^T; 8, *C. johnsonae* (*F. johnsoniae*) IFO14942^T; 9, *C. succinicans* (*F. succinicans*) IFO14905^T; 10, *C. lytica* IFO14961^T; 11, *C. uliginosa* IFO14962^T; 12, *F. branchiophilum* IFO15030^T; 13, *F. odoratum* (*Myroides odoratus*) IFO14945^T; 14, *C. latercula* ATCC23177^T; 15, *F. salegens* ATCC51522^T; 16, *F. gondowanense* ATCC51278^T; 17, *Flexibacter maritimus* ATCC43398^T; 18, no template control.

and 2 mismatches in the J2 and J4 primer regions, respectively. Strains AA8-2 and AA8-3 were never detected by the primer set in despite of only 22 mismatches in 1485 nucleotides between the strains AA8-2 and J18/M01. Maeda *et al.*[18] stated that it should be difficult to investigate the ecology of aligicidal and algal-lytic *Cytophaga* in the lump with a single 16S rDNA probe because of a wide distribution of aligicidal and algal-lytic activities among *Flavobacterium* and *Cytophaga* group. The result of our PCR procedure indicates that the set of J2 and J4 primers could be a useful tool for the species or strain specific detection and enumeration of *Cytophaga* sp. J18/M01 in natural environments by *in situ* hybridization or *in situ* PCR techniques. However, there are still some problems with the use of this PCR procedure to natural environments such as the specificity of the probes to unknown marine bacteria existing in the environments, relationship between PCR amplification and cell abundance, and extraction efficiency of DNA from bacterial cells, which have to be settled. Further studies on the ecology of the marine CFB group having algicidal activity should be made using antibodies against the cell surface or specific probes based on rRNA sequence.

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