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Phylogenetic analysis of intracellular bacteria of a harmful marine microalga, *Heterocapsa circularisquama* (Dinophyceae)

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ABSTRACT: *Heterocapsa circularisquama*, a noxious marine dinoflagellate, has frequently caused red tides and killed cultured bivalves in western Japanese embayments. Observations by electron and epifluorescence microscopy revealed that many bacterial particles were detected inside the *H. circularisquama* cells. To elucidate the identity and origin of the intracellular bacteria associated with *H. circularisquama*, bacterial 16S ribosomal RNA genes (16S rDNA) were directly amplified by polymerase chain reaction from 5 clonal cultures of the algal strains that had been isolated from different localities. After cloning, randomly selected clones including the bacterial 16S rDNA fragments were sequenced. The results showed that intracellular bacterial populations consisted of only 2 ribotypes of bacteria, even though the algal strains were established from different localities. One ribotype (bac-G), which was dominant in the intracellular bacterial population, belonged to the gamma-proteobacteria group, and the other (bac-F) clustered with the *Flexibacter-Cytophaga-Bacteroides* group. Both of these are novel species of endosymbiotic bacteria because of their unique 16S rDNA sequences. Furthermore, the populations of extracellularly associated bacteria were also composed of bac-G and bac-F, indicating that they originated from the intracellular bacteria. Fluorescence *in situ* hybridization targeting 16S rRNA indicated that bac-G appeared to localize on the algal nuclear surface, while bac-F was distributed in the cytoplasmic space of algal cells. These results strongly suggest that only a few species of specific bacteria reside and share their habitat in the *H. circularisquama* cells as endosymbionts.

KEY WORDS: *Heterocapsa circularisquama* · Dinoflagellate · Endosymbionts · Intracellular bacteria · Gamma-proteobacteria subdivision · *Flexibacter-Cytophaga-Bacteroides* group · 16S rDNA · FISH

INTRODUCTION

The harmful dinoflagellate *Heterocapsa circularisquama* Horiguchi has frequently formed large-scale and long-term (more than 2 mo) red tides at several embayments in central and western Japan (Matsuyama et al. 1996). These red tides have severely damaged both natural and cultured bivalves, e.g. pearl oysters, short-necked clams and oysters (Matsuyama et al. 1996).

Many species of microalgae generally get some benefits from co-existing bacteria through remineralized nutrients (Azam et al. 1983, Grossart 1999), vitamins (Haines & Guillard 1974), and/or other unknown growth factors. Some microalgae also graze bacteria directly (mixotrophy) (Cole 1982, Caron et al. 1993, Keller et al. 1994). In many cases, co-existing bacteria benefit from microalgae through photosynthetic products (Bell et al. 1974, Jensen 1983). Electron microscopic observation revealed that most *Heterocapsa circularisquama* cells of culture strains possessed some bacteria-like particles (BLPs) intracellularly (Horiguchi 1995, Imai et al. 1999, Maki & Imai 2001a). We previously

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reported that the intracellular BLPs grow or survive in *H. circularisquama* (Maki & Imai 2001a). This implies the possibility that the intracellular BLPs are endosymbionts of *H. circularisquama*. Although there may be a special relationship between the intracellular bacteria and *H. circularisquama*, and the bacteria influence algal metabolism and reproduction, unfortunately it is not feasible to cultivate and identify the intracellular bacteria through conventional cultivation methods (Maki & Imai 2001b).

Recently, microbial species compositions in a variety of natural samples have been analyzed through 16S ribosomal RNA gene (16S rDNA) sequence analyses without cultivation (Amann et al. 1995). The culture-independent analyses with molecular biological techniques have suggested that some novel bacteria, which have yet to be cultivated, exist in the ocean (Giovannoni et al. 1990, Fuhrman et al. 1993), hot springs (Ward et al. 1990), freshwater lakes (Shin et al. 1997), soil (Torsvik et al. 1990), and various other environments. In the case of symbiotic relationships, the bacterial communities associated with fish (Haygood & Distel 1993, Aznar et al. 1994), insects (Ohkuma & Kudo 1996, Fukatsu & Nikoh 2000) and protozoa (Amann et al. 1991, Embley et al. 1992) have been analyzed through 16S rDNA information, even though most of these bacteria were unculturable. Intracellular bacterial populations of the dinoflagellate *Noctiluca scintillans* were also investigated by the phylogenetic analysis (Seibold et al. 2001). Moreover, fluorescence *in situ* hybridization (FISH) analyses targeting ribosomes of certain microorganisms revealed that some marine organisms, such as marine bivalves (Distel & Cavanaugh 1994, Sipe et al. 2000), tubeworms (Di Meo et al. 2000), shipworms (Distel et al. 1991) and an oligochaete worm (Dubilier et al. 2001), have various symbiotic bacteria in their tissues. With regard to unicellular organisms, endosymbiotic bacteria inside ciliate cells (Amann et al. 1991, Springer et al. 1992) or ectosymbiotic bacteria of anaerobic ciliates (Fenchel & Ramsing 1992) were also detected using the FISH technique.

In this study, the 16S rDNA of bacteria associated with 5 different strains of *Heterocapsa circularisquama*, isolated from different localities in Japan, were analyzed to identify the bacteria without cultivation. Although the clonal cultures of *H. circularisquama* were established by thorough micropipette washing, the co-existing bacteria (intracellular and extracellular bacteria) were still observed under epifluorescence microscopy (Imai et al. 1999, Maki & Imai 2001a,b). The 16S rDNA information newly obtained in this study suggested that 2 specific new species of bacteria occupied *H. circularisquama* cells. Moreover, to investigate the distribution of the bacteria inside the algal cells, we applied the FISH technique using fluorescently labeled nucleotide probes, specifically designed for the 16S rRNA of intracellular bacteria.

MATERIALS AND METHODS

Cultures of *Heterocapsa circularisquama* strains. The 5 clonal cultures of *Heterocapsa circularisquama* strains (HY9423, HU9433, HU9436, HA92-1 and HI9428) used in this study were kindly provided by Drs. T. Uchida and Y. Matsuyama at the National Research Institute of Fisheries and Environments of the Inland Sea. The cultures originated from seawater at 4 different localities in 1992 and 1994 (Fig. 1). Each algal culture was established by picking a single algal cell from a natural seawater sample and placing it into modified SWM-3 culture medium (Chen et al. 1969, Imai et al. 1996) using micropipettes; the cell was kept in the

Fig. 1. Localities of sampling sites (●) from which the *Heterocapsa circularisquama* strains were obtained. Algal strains were isolated from: Yastushiro Sea (HY9423), Imari Bay (HI9428), Uranouchi Inlet (HU9433 and HU9436) in 1994 by Dr. T. Uchida, and from Ago Bay (HA92-1) in 1992 by Dr. Y. Matsuyama

medium for 30 s and then transferred to a new medium, according to the pipette-washing protocol (Droop 1954, Hoshaw & Rosowski 1973). The algal cell was repeatedly washed more than 6 times, and cultivated in the SWM-3 medium. Each (mono)clonal culture of *H. circularisquama* was started from a single cell. In general, the pipette-washing protocol eliminates (contaminated) bacteria that may be co-existing with the algal cells, and has been most effective for establishing axenic microalgal culture. However, *H. circularisquama* cultures have still included bacteria extracellularly and intracellularly. After isolation, all algal cultures were maintained in the modified SWM-3 culture medium, under a 14:10 h light:dark photo-cycle at a light intensity of 180 µmol photon m^{-2} s^{-1} at a temperature of 22°C.

Phylogenetic analysis of 16S rDNA. Nucleotide sequences of 16S rDNA of the intracellular and extracellular bacteria associated with *Heterocapsa circularisquama* strains were determined. Five *H. circularisquama* strains were cultivated in modified SWM-3 medium (1 l) up to an algal cell density of 5×10^4 cells ml^{-1}, and the algal cells containing intracellular bacteria were pelleted by centrifugation at 2600 × *g* for 15 min, followed by washing twice with fresh SWM-3 saline. The supernatant was centrifuged again at 28000 × *g* for 15 min, and the extracellular bacteria were thus harvested. Both algal and extracellular bacterial cell pellets were stored at −70°C until the following experiments.

The algal cell pellets containing intracellular bacteria were pulverized in liquid nitrogen with a ceramic blender. Grinding in liquid nitrogen minimized the degradation of bacterial DNA by algal nucleases. We did not perform any special steps to separate the intracellular bacteria from algal cell debris. Genomic DNAs of the intracellular and extracellular bacteria were extracted and purified using a DNA extraction kit (Sepa Gene, Sanko Junyaku).

The 16S rDNA fragments (ca. 1450 bp) of bacteria were amplified by polymerase chain reaction (PCR). Reaction mixtures (final volume, 100 µl) contained 200 µM of dNTPs, 0.5 units of Ex Taq polymerase (Takara Bio), and 0.2 µM each of oligonucleotide primers 6F and 1492R (Table 1). These primers specifically bind to eubacterial 16S rDNA. Genomic DNA of bacteria was added at a final concentration of 1 to 10 ng $µl^{-1}$. Thermal cycling was performed using a Program Temp Control System PC-700 (Astec) under the following conditions: denaturation at 95°C for 1 min, annealing at 57°C for 2 min, and extension at 72°C for 2 min, for a total of 30 cycles. The PCR amplicons were separated by 1.5% agarose gel electrophoresis, and about 1450 bp of DNA bands (16S rDNA fragments) were excised and purified by phenol-chloroform extraction, chloroform extraction followed by ethanol precipitation. Finally, the DNA fragments were resuspended in 10 µl of sterile distilled water.

The purified bacterial 16S rDNA fragments were cloned into *Escherichia coli* using a commercially prepared vector with a TA cloning kit (Invitrogen) according to the manufacturer's protocol. Transformation products were incubated for 18 h at 37°C on LB agar plate with 50 µg ml^{-1} of ampicillin and 40 µg ml^{-1} of X-gal. More than 25 white colonies per sample were picked up with sterile toothpicks, and more than 250 transformants were obtained (intracellular and extracellular bacteria of each of the 5 strains of *Heterocapsa circularisquama*). The recombinant plasmids were isolated from the individual transformants using a Plasmid Miniprep kit (Bio-Rad Laboratories).

Table 1. Oligonucleotides used as primers for amplification and as probes for FISH, and sequencing of 16S rDNA

	Sequence (5′ to 3′)	Target organisms	Target site rRNA position
Probe			
Euca1195R	GGGCATCACAGACCTG	Eukaryote	23S rRNA (1182–1195)
Eub338R	GCTGCCTCCCGTAGGAGT	Eubacteria	16S rRNA (323–338)
Ark40R	TCCGGCAGGATCAACCGGAA	Archaebacteria	16S rRNA (21–40)
G233R	GCTAATCTTTAAGCACGAGG	bac-G	16S rRNA (214–233)
F87R	GCAAGCACAACCTGTTACCC	bac-F	16S rRNA (68–87)
Primer			
M13(-40)F	GTTTTCCCAGTCACGA		
M13R	CAGGAAACAGCTATGAC		
6F	GRAGTTTGATCMTGGC	Eubacteria	16S rRNA (6–24)
27F	AGAGTTTGATCCTGGCTCAG	Eubacteria	16S rRNA (8–27)
519R	GTATTACCGCGGCTGCTG	Eubacteria	16S rRNA (519–536)
735R	GYCGCYTTCGCCACTGGTGT	Eubacteria	16S rRNA (716–735)
926R	CCGTCAATTCMTTTGAGTT	Eubacteria	16S rRNA (926–908)
S14R	CATTGTAGCACGTGTGTAG	Eubacteria	16S rRNA (1241–1223)
1395R	GTACTTGGCCCGRGAAC	Eubacteria	16S rRNA (1395–1379)
1492R	GGTTACCTTGTTACGACTT	Eubacteria	16S rRNA (1510–1492)

Partial sequences (ca. 360 bp) of 16S rDNA clones were determined using a Dye Deoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems) with the 27F sequencing primer (Table 1) and DNA autosequencing system (Model 373A) according to the recommended protocol. The sequences determined were compared with the DDBJ (DNA Data Bank of Japan) database using the program Fasta Search (Altschul et al. 1990).

After alignment and comparing the partial nucleotide sequences of 16S rDNA clones determined in this study, the clones were divided into ribotypes. Several representative clones of each ribotype were sequenced entirely (ca. 1450 bp each). The primers for sequencing M13 (–40)F, M13R, 519R, 735R, 926R, S14R, 1395R are shown in Table 1.

All sequences were analyzed with Check-Chimera Version 2.7 (Ribosomal Database Project) to exclude the possibility of chimera production by PCR (Robison-Cox et al. 1995). A phylogenetic tree including *Heterocapsa circularisquama*-associated bacteria was constructed according to the neighbor-joining algorithmic method (Saitou & Nei 1987), using the ca. 1450 bp sequences of 16S rDNA. Reference sequences in the phylogenetic tree were selected from the GenBank database (Table 2)**.** Bootstrap analysis of up to 1000 replicates was carried out with the computer program Clustal W Version 1.7 (European Bioinformatics Institute) (Thompson et al. 1994).

Fluorescence *in situ* hybridization (FISH). To analyze the bacterial distribution, the intracellular bacteria of *Heterocapsa circularisquama* were stained and observed using the FISH technique with newly designed nucleotide probes (Table 1). The nucleotide probes targeting the intracellular bacteria were designed using the 16S rDNA sequences. In addition, the probes Eub338R for eubacteria (Amann et al. 1990a) and Ark40R for archaebacteria (Giovannoni et al. 1988) were also used as a positive and a negative control, respectively (Table 1). The probe Euca1195R (Giovannoni et al. 1988) was used to detect eukaryotic ribosomal RNA in *H. circularisquama* cells. Algal cells of the 5 *H. circularisquama* strains were fixed with paraformardehyde solution (final concentration of 4%) in phosphate-buffered saline (200 mM sodium phosphate buffer, pH 7.4) for 3 h at room temperature, and then fixed algal cells with the associated bacteria were bound on nuclepore filters (0.2 µm pore size). Each nuclepore filter was put into an Eppendorf tube and washed sequentially with 50, 80 and 100% ethanol for 1 min. Then, 20 µl of hybridization solution (30% formamide, 0.9 M NaCl, 0.1% sodium dodecyl sulfate, 20 mM Tris buffer, pH 7.2) was added to each sample on the nuclepore filter. Following preincubation at 37°C for 30 min, 9 µl of hybridization solution contain-

Table 2. Sequence accession numbers (Acc. no.) for the 16S rDNA clones from intracellular bacteria of *Heterocapsa circularisquama* and for the reference sequences used in hylogenetic analysis

Acc. no.	Species
Intracellular bacteria of *Heterocapsa circularisquama* (clone)	
AB058907	bac-G (Y2)
AB058908	bac-G (UA36)
AB058909	bac-G (A55)
AB058910	bac-G (I 29)
AB058911	bac-G (UB43)
AB058905	bac-F (UB42)
Reference sequences	
M11223	*Agrobacterium tumefaciens*
U71009	*Alcaligenes faecalis*
AF074970	*Bacillus subtilis*
M86695	*Bacteroides distasonis*
M11656	*Bacteriodes fragilis*
M63247	*Buchnera aphidicola* (*Chaitophorus viminalis*)[a]
M63254	*Buchnera aphidicola* (*Myzus persicae*)[a]
M63249	*Buchnera aphidicola* (*Pemphigus betae*)[a]
M63252	*Buchnera aphidicola* (*Rhopalosiphum maidis*)[a]
AB021418	*Comamonas terrigena*
D89798	*Coxiella burnetii*
D84559	*Coxiella* sp.
M58768	*Cytophaga hutchinsonii*
M58769	*Cytophaga latercula*
D12668	*Cytophaga marinoflava*
AF098671	*Desulfovibrio desulfuricans*
M59062	*Erythrobacter longus*
V00348	*Escherichia coli*
M62797	*Flavobacterium aquatile*
AF030380	*Flavobacterium xanthum*
AY006471	*Flexibacter echinicida*
D14023	*Flexibacter maritimus*
M58788	*Flexibacter ruber*
M62795	*Flexibacter sancti*
AF001367	*Gelidibacter algens*
X84979	Gill symbiont of *Codakia orbicularis*
X84980	Gill symbiont of *Lucina pectinata*
M90415	Gill symbiont of *Solemya velum*
L01575	Gill symbiont of *Thyasira flexuosa*
X73395	*Legionella feeleii*
Z49741	*Legionella lyticum*
M59157	*Legionella pneumophila*
M96395	*Nitrosomonas europaea*
M22365	*Oceanospirillum linum*
Z21729	*Photobacterium fischeri*
U14586	*Polaribacter filamentus*
M58775	*Polaribacter glomeratus*
M61002	*Polaribacter irgensii*
M59064	*Pseudomonas aeruginosa*
U62912	*Psychroserpens burtonensis*
M21293	*Rickettsia rickettsii*
Z19081	Symbiont of *Anomalops katoptron*
AB001519	Symbiont of *Haemaphysalis longicornis*
U77480	Symbiont of *Ridgeia piscesae*
U77478	Symbiont of *Riftia pachyptila*
AF165907	Symbiont of vestimentiferan
M21774	*Thermotoga maritima*
AF369642	*Vibrio* AB003
X76334	*Vibrio vulnificus*
M21292	*Wolbachia persica*

[a]A host name is shown in parentheses

ing 2.5 µg of fluorescence isothiocyanate (FITC)-labeled probe was added. The algal cells were hybridized at 37°C for 6 h in a water bath and washed twice with 20 to 40 µl of hybridization solution at 37°C for 20 min.

After hybridization and washing, the nuclepore filter with the algal cells was placed on a filtering device and rinsed with distilled water. Subsequently, the filter was observed under an epifluorescence microscope (Nikon) equipped with the dichroic mirror system for FITC (excitation wavelength, 465 to 495 nm; dichroic mirror, 505 nm). Photomicrographs were taken with color reversal 400 nm film (Fujifilm). Exposure time was 0.5 s for epifluorescence micrographs.

RESULTS

Comparison of 16S rDNA clones

16S rDNA fragments (ca. 1450 bp) of intracellular and extracellular bacteria co-existing with the 5 *Heterocapsa circularisquama* strains were amplified by PCR with primers targeting eubacterial 16S rDNA. The PCR amplicons were cloned into *Escherichia coli*, and a total of 293 clones including eubacterial 16S rDNA fragments was obtained. Partial sequences (ca. 360 bp) of the 16S rDNA clones showed that the bacterial populations from the 5 different strains of *H. circularisquama* were divided into only 2 ribotypes. The pairwise sequence similarities among clones of each ribotype were high and ranged from 99.1 to 100%, even when they were obtained from different *H. circularisquama* clones. All sequence variations of 16S rDNA clones obtained from intracellular (145 clones) and extracellular (148 clones) bacterial populations were aligned in Figs. 2 & 3. One major ribotype (bac-G; Fig. 2) is composed of 273 clones and is similar to *Coxiella burnetii* (average sequence similarity, 93.3%), which belongs to the gamma-proteobacteria subdivision. The other minor ribotype (bac-F), which comprised the remaining 20 clones, was related to *Flexibacter maritimus* (average sequence similarity, 90.5%) from the *Flexibacter-Cytophaga-Bacteroides* group (FCB-group) (Fig. 3). The ribotype bac-G was predominantly detected in all 5 strains of *H. circularisquama* (HY9423, HU9436, HA92-1, HI9428 and HU9433), whereas the ribotype bac-F was recovered from 3 strains only (HA92-1, HI9428 and HU9433; Table 3). As intracellular bacteria, bac-F was detected in only 1 algal strain (HU9433; Table 3).

To compare the extracellular bacterial populations with the intracellular bacterial populations, 148 clones of 16S rDNA amplicons were sequenced from culture supernatants of the 5 algal strains (see 'Materials and methods'). Their partial sequences demonstrated that most (131/148) of the clones from extracellular bacterial populations belonged to the ribotype bac-G, and pairwise similarities between intracellular and extracellular clones attained 99.0% (Fig. 2). The remaining 17 of the 148 clones belonged to the ribotype bac-F (>99.1% similarity; Fig. 3). The ribotype bac-G clones were also obtained from all 5 algal strains in intracellular bacteria; however, the bac-F clones were detected from 3 strains in extracellular bacterial populations (HA92-1, HI9428 and HU9433), with ratios to the total clones being 3 (1/31), 10 (3/31) and 42% (13/31), respectively (Table 3).

Phylogenetic analysis

The entire nucleotide sequences (ca. 1450 bp) of 16S rDNA clones belonging to the ribotype bac-G (clone name: UB43) and the ribotype bac-F (clone name: UB42), which were originally amplified from the culture of *Heterocapsa circularisquama* strain HU9433, were determined. A wide-range phylogenetic tree that included UB43 and UB42 was constructed together with some 16S rRNA genes available from the DDBJ database (Fig. 4). The tree indicated that, as expected, UB43 (bac-G) belonged to the gamma-proteobacteria subdivision and UB42 (bac-F) belonged to the FCB-group. Furthermore, 4 clones of 16S rDNA (Y2, UA36, A55 and I29, originating from the algal strains HY9423, HU9436, HA92-1 and HI9428, respectively), which all belonged to bac-G, were sequenced entirely as well as UB43. Although these 5 clones were originally derived from 5 different strains of *H. circularisquama*, their sequences were highly similar to each other and the pairwise similarities were more than 99.7%. On a phylogenetic tree including the 5 16S rDNA clones of bac-G and several known symbiotic or pathogenic gamma-proteobacteria (Fig. 5), the branch of bac-G

Table 3. Number of 16S rDNA sequence types retrieved from the intracellular bacteria and the extracellular bacteria in 5 *Heterocapsa circularisquama* strains. 16S rDNA clones belonging to the same ribotype, bac-G or bac-F, showed high degrees of similarity (more than 99.0%) to each other

Strain	Intracellular bacterial population		Extracellular bacterial population	
	bac-G	bac-F	bac-G	bac-F
HY9423	26	0	26	0
HU9436	28	0	27	0
HA92-1	31	0	30	1
HI9428	31	0	30	3
HU9433	26	3	18	13
Total (clones)	142	3	131	17

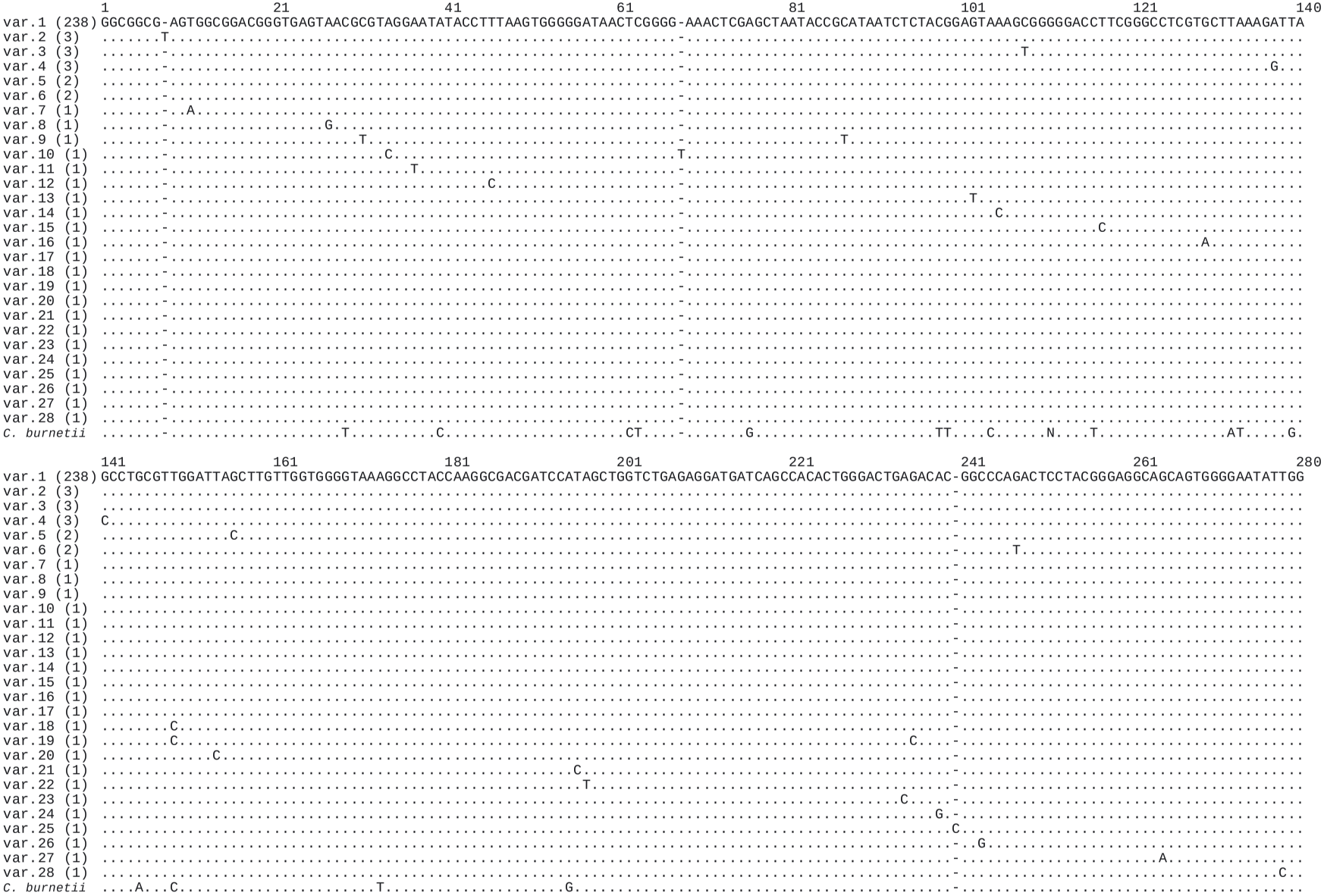

Fig. 2. Alignment of partial sequences (*Escherichia coli* numbering 56 to 356 bp) of 16S rDNA clones belonging to bac-G, and 16S rDNA of *Coxiella burnetii* (D89798). There is minor sequence variation (var. 1 to 28) among total of 273 clones from intracellular and extracellular bacterial populations of 5 *Heterocapsa circularisquama* strains. The number of 16S rDNA clones belonging to each variation is indicated in parentheses. A dot indicates the identical nucleotide as that of the dominant sequence var. 1, and a dash indicates an introduced gap

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                1                  21                 41                 61                 81                101                121                140
var.1 (15)      AAGATTAATACTCCATAGCATTAAATTTTGGCATCGAAATTTAATTAAAGATTTATTGGTAAAAGATGGCTATGCGTCTTATTAGCTAGATGGTAAGGTAACGGCTTACCATGGCAACGATAAGTAGGGGCCCTGAGAGG
val. 2 (1)      .....G.....T.....
val. 3 (1)      .....T.....T.....
val. 4 (1)      .....T.....T.....
val. 5 (1)      .....T.....
val. 6 (1)      .....CT....A..C.—-..G.....TTT.AA.G.....C....G.....A.....C.....G.....T.....
F. maritimus    .A.....G

                141                161                181                201                210
var.1 (15)      GGGATCCCCCACACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGG-AATATTGGT
val. 2 (1)      .....-.....
val. 3 (1)      .....-.....
val. 4 (1)      .....-.....
val. 5 (1)      .....-.....
val. 6 (1)      .....T.....
F. maritimus    .....-.....G
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Fig. 3. Alignment of partial sequences (*Escherichia coli* numbering 154 to 262 bp) of 16S rDNA clones belonging to bac-F, and 16S rDNA of *Flexibacter maritimus* (D14023). There is minor sequence variation (var. 1 to 6) among a total of 20 clones from intracellular and extracellular bacterial populations of 5 *Heterocapsa circularisquama* strains. The number of 16S rDNA clones belonging to each variation is indicated in parentheses. A dot indicates the identical nucleotide as that of the dominant sequence (var. 1), and a dash indicates an introduced gap

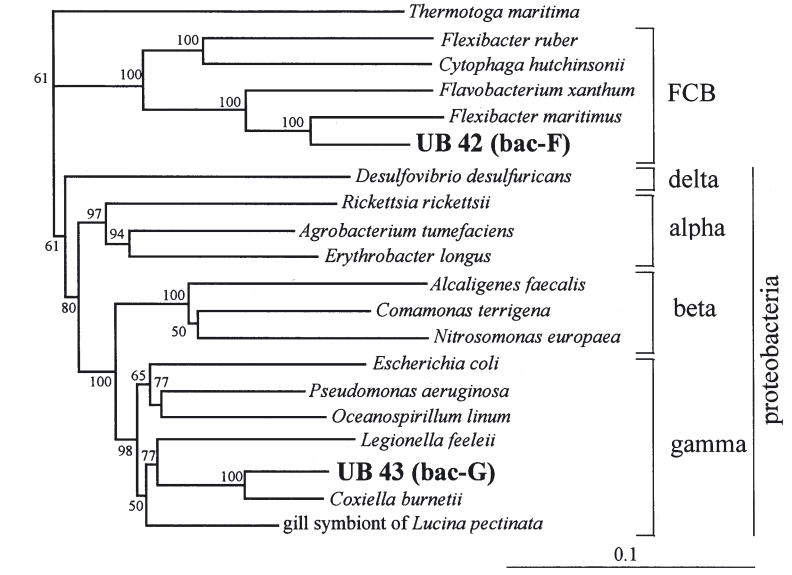

Fig. 4. A tree inferred from full-length nucleotide sequences of 16S rDNA (ca. 1450 bp) showing phylogenetic positions of 2 ribotypes (bac-F and bac-G) from intracellular bacteria of *Heterocapsa circularisquama* cells. Members of well-known proteobacterial subdivisions (alpha, beta, gamma, delta) and *Flexibacter-Cytophaga-Bacteroides* (FCB) group in the DDBJ database are included. The tree was constructed according to the neighbor-joining algorithmic method (Saitou & Nei 1987). Percentages of 1000 bootstrap simulations that supported each branch are indicated at each (neighbor-joining) node. Bootstrap values below 50% are not shown. The scale bar shows 10 nucleotide substitutions per 100 nucleotides. Sequence accession numbers are given in Table 2

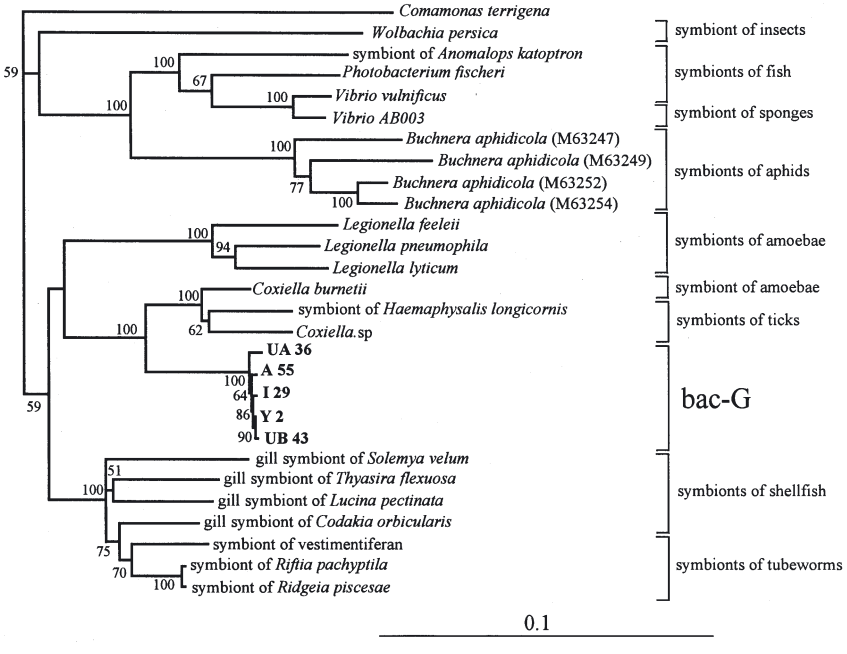

Fig. 5. Phylogenetic tree inferred from the full-length nucleotide sequences of 16S rDNA (ca. 1450 bp) showing the relationship between 16S rDNA clones (Y2, UA36, A55, I29, and UB43) of the ribotype bac-G and several symbiotic gamma-proteobacteria associated with eukaryotes. The tree construction and bootstrap simulation are referred to in Fig. 4 and 'Materials and methods'. Sequence accession numbers are given in Table 2

clones related to a branch of *Coxiella burnetii* and 2 symbionts of ticks, but separated with 100% of bootstrap value. Another small-range phylogenetic tree including UB42 (bac-F) and several members of the FCB-group bac-F (Fig. 6) showed that the ribotype bac-F related to a branch of the genus *Flexibacter*, but is presumably a new species because of its low sequence similarities (90.5% at most) with other members of *Flexibacter*.

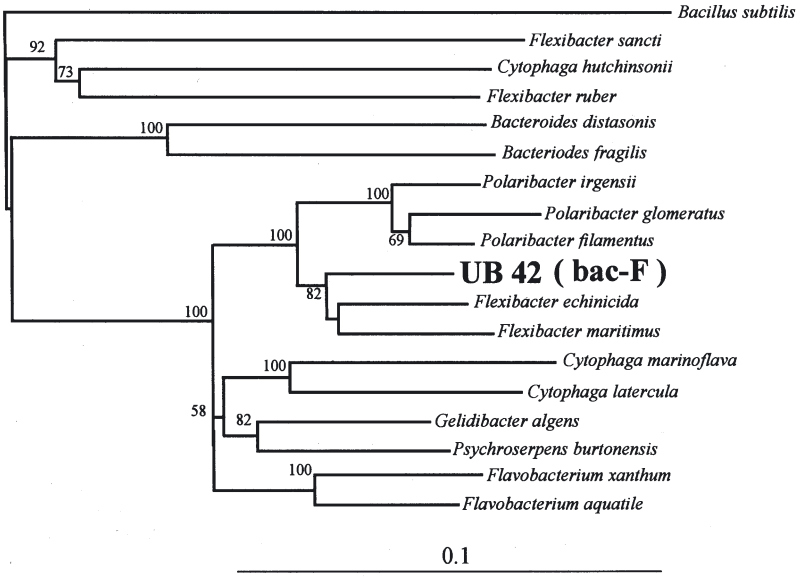

Fig. 6. Phylogenetic tree inferred from the full nucleotide sequences of 16S rDNA (ca. 1450 bp) showing the relationship between a clone of the ribotype bac-F and the members of the *Flexibacter-Cytophaga-Bacteroides* (FCB) group in the DDBJ database. The tree construction and bootstrap simulation are referred to in Fig. 4 and 'Materials and methods'. Sequence accession numbers are given in Table 2

Whole-cell *in situ* hybridization of *Heterocapsa circularisquama*

To investigate the distributions of bac-G-like and bac-F-like bacteria in *Heterocapsa circularisquama* cells, we designed FITC-labeled nucleotide probes targeting the ribosomal RNA of bac-G (probe G233R) and bac-F (probe F87R). These probes were complementary to a characteristic region of the retrieved 16S rRNA corresponding to positions 214 to 233 (G233R) and 68 to 87 (F87R) of *Escherichia coli* (Table 1). The hybridization conditions and specificities of the 2 probes were determined preliminarily against some gamma-proteobacteria and GFC-group bacteria. In addition, a probe for eubacteria (Eub338R), a probe for archaebacteria (Ark40R), and a probe for eukaryotes (Euca1195R) were labeled with FITC. The position of algal nuclei were confirmed after DAPI (4′,6-diamidino-2-phenylindole) staining and indicated with dashed lines in Fig. 7. Epifluorescence microscopy after whole-cell *in situ* hybridization of *H. circularisquama* cells revealed that the probe G233R for bac-G bound to the entire nuclear areas of all 5 algal strains analyzed (Fig. 7a). In contrast, the probe F87R targeting bac-F reacted with several small particles dispersed over the cytoplasm of the algal cells of 3 *H. circularisquama* strains (HA92-1, HI9428 and HU9433; Fig. 7b). The signals by Eub338R (probe for eubacteria) as a positive control overlapped those by G233R and F87R, i.e. the nuclei of all 5 *H. circularisquama* strains and the cytoplasm of the 3 strains. Euca1195R (probe for eukaryotic ribosomes) labeled whole algal cells, and Ark40R (probe for archaebacteria) showed no signals.

The extracellular bacteria, which are composed of bac-G and/or bac-F by 16S rDNA analysis, could not be detected by FISH using any of these probes in all 5 algal strain cultures. However, after the algal culture supernatants of HU9433 including extracellular bacteria were cultivated for 6 h with 10 mg l^{-1} of trypticase peptone, FISH signals of bacteria could be detected by using the F87R probe and the Eub338R probe.

DISCUSSION

Five clonal cultures of *Heterocapsa circularisquama*, which were isolated from different localities on the coast of western Japan, possessed BLPs intracellularly. It is significant to first identify the bacteria when investigating the relationships between intracellular bacteria and *H. circularisquama*. However, it was difficult to isolate and cultivate the bacteria independently from algal cultures, and therefore we analyzed the 16S rDNA sequences of bacteria without cultivation.

Microscopic observation by whole-cell *in situ* hybridization with the probes Ark40R for archaebacterial rRNA and Eub338R for eubacterial rRNA revealed that the intracellular bacteria in *Heterocapsa circularisquama* cells were derived from eubacteria, and not from archaebacteria (Fig. 7). This conclusion is supported by the fact that the PCR primer set for archaebacteria could not amplify any 16S rRNA gene fragments from intracellular bacterial genomes. In contrast, eubacterial 16S rDNA was well amplified by PCR from the bulk DNA of intracellular bacteria in all 5 strains of *H. circularisquama*; it was cloned into *Escherichia coli*, and finally 26, 28, 31, 31 and 29 clones of 16S rDNA were obtained from algal strains HY9423, HI9428, HU9436, HA92-1 and HU9433, respectively. Based on the nucleotide sequence similarity, we divided a total of 145 clones of 16S rDNA into groups (ribotypes). As a result, surprisingly, 145 clones of 16S

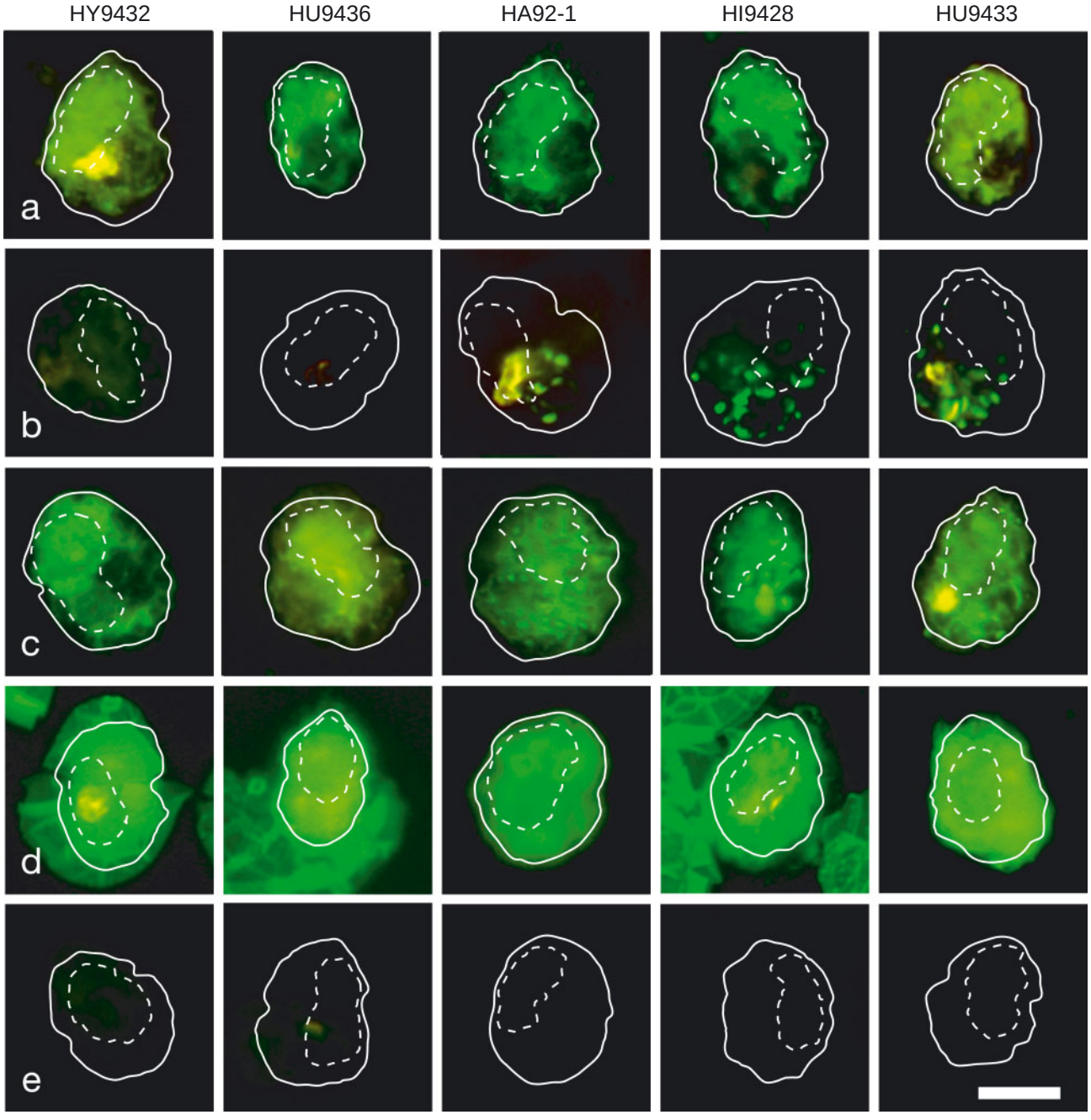

Fig. 7. Whole-cell *in situ* hybridization against bacteria-like particles in *Heterocapsa circularisquama* cells. Five algal strains (HY9423, HU9436, HA92-1, HI9428 and HU9433) are shown. Algal cells were hybridized and stained with 5 FITC-labeled probes: (a) G233R (probe for bac-G), (b) F87R (probe for bac-F), (c) Eub338R (probe for eubacteria), (d) Euca1195R (probe for eukaryotes), and (e) Ark40R (probe for archaebacteria), respectively. White lines in the micrographs show outline of the algal cells, and white dashed lines indicate outlines of nucleus observed after DAPI staining. All photomicrographs were taken at a magnification of 1000× (scale bar = 10 µm)

rDNA contained only 2 ribotypes (bac-G and bac-F), indicating that the intracellular bacterial populations are not so diverse. The ribotype bac-G was particularly dominant (142/145 clones) and was recovered from all 5 algal strains. In contrast, bac-F was obtained from the 3 clones only in the strain HU9433.

Only 2 eubacterial ribosome RNA genes were detected, nevertheless there are many kinds of bacteria around *Heterocapsa circularisquama* cells in natural environments. One plausible reason is that these 2 bacterial populations were artificially generated during isolation of microalgae. In this study, we

used 5 strains of *H. circularisquama* which had been isolated from different bays far apart from each other (Fig. 1) collected on different dates by 2 different scientists. Furthermore, the thorough micropipette-washing protocol was used instead of antibiotics for the establishment of axenic algal culture. Therefore, it is inconceivable that the use of antibiotics or something related to the handling of microalgal culturing caused the poorly diverse bacterial populations in *H. circularisquama* cells. Furthermore, we detected the bacterial particles belonging to bac-G in the *H. circularisquama* cells which are isolated and maintained in other laboratories, using the FISH technique (data not shown).

Some scientists may point out that incorrect gene amplification during PCR could cause bias in bacterial species composition analysis. However, the PCR primers in this study for eubacterial 16S rDNA were widely used in various samples and we could amplify a variety of eubacterial 16S rDNA from lake water, seawater, coastal sands, and microalgal cultures. Consequently, we can conclude that 2 ribotypes of eubacteria (bac-G and bac-F) were generally associating with *Heterocapsa circularisquama*.

The populations of extracellular bacteria in cultures of *Heterocapsa circularisquama* were also investigated using the same protocol as that used for intracellular bacterial analysis. Sequence analysis of 148 clones of 16S rDNA amplicons from 5 different microalgal cultures indicated that the extracellular bacterial populations were also composed of the 2 ribotypes bac-G and bac-F. As expected, the 16S rDNA clones of ribotype bac-G, which was dominant in the intracellular bacterial populations, was recovered more frequently than ribotype bac-F from all 5 *H. circularisquama* cultures. These observations indicate that the genetic population structures of the intracellular and extracellular bacteria were quite similar to each other.

Interestingly, 16S rDNA clones of bac-F could be recovered from the extracellular bacteria of HA92-1 and HI9428 cultures, in which no 16S rDNA clones of bac-F were recovered from the intracellular bacteria. However, on whole-cell *in situ* hybridization targeting ribosomal RNA of bac-F, positive fluorescence was observed in algal cells of HA92-1 and HI9428, as well as of HU9433 (Fig. 7b). Genomic DNA of the intracellular bacteria may be difficult to extract from the algal cells, or some unknown interference such as algal cell components (Wintzingerode et al. 1997) might have inhibited PCR amplification of the 16S rDNA from bac-F in both algal strains (HA92-1 and HI9428). These results suggest that bac-F is an endosymbiotic bacterium that exists specifically in 3 strains of *Heterocapsa circularisquama*: HA92-1, HI9428 and HU9433.

We believe that the extracellular bacteria originated from the intracellular bacteria because of the following 2 reasons. First, to establish the axenic algal cultures, we washed the algal cells again with sterilized SWM-3 medium using micropipettes. This technique usually eliminates the extracellular bacteria. One week after the pipette-washing treatments, no extracellular bacteria existed in any of the algal cultures, while intracellular bacteria were observed by DAPI staining and epifluorescence microscopy. However, after several weeks' cultivation, the extracellular bacteria reappeared in the culture. This fact clearly indicates that the intracellular bacteria was released from algal cells (probably dead cells) and form the extracellular bacterial population as the algal cultivation proceeded. Second, it was difficult to detect extracellular bacteria by the whole-cell *in situ* hybridization technique. From our results, we deduce that the extracellular bacteria contained insufficient amounts of ribosome for FISH detection as the hybridization probes in this study targeted ribosomal RNA. This means that the extracellular bacteria are inactive, probably starved, outside algal cells (Kerkhof & Ward 1993), a presumption which is supported by the fact that the bac-F type bacterial particles in the culture of HU9433 supernatant became detectable by FISH after the incubation with peptone addition.

Although a myriad of bacteria could possibly interact with the microalga in marine environments, only 2 types of bacteria were detected in this study. Most bacteria in natural environments which existed outside the algal cells could be eliminated by micropipette-washing during the isolation step of algal strains. However, the intracellular bacteria (bac-G and bac-F) remained associated with *Heterocapsa circularisquama*.

Several 16S rDNA fragments of ribotype bac-G from different *Heterocapsa circularisquama* strains were sequenced completely. The phylogenetic analysis suggests that the ribotype bac-G belongs to the gamma-proteobacteria subdivision (Figs. 5 & 6). Some endosymbiotic bacteria belonging to the gamma-proteobacteria have been previously identified in the tissues of various marine organisms such as finfish (Haygood & Distel 1993, Aznar et al. 1994), shellfish (Distel & Wood 1992, Distel & Cavanaugh 1994, Sipe et al. 2000), tubeworms (Di Meo et al. 2000), and in the gut of aphids (Munson et al. 1991, Chen et al. 1999). The endosymbiotic bacterial group of amoebae, the genus *Legionella*, is also included in the gamma-proteobacteria subdivision (Springer et al. 1992, Birtles et al. 1996). However, there are no symbiotic bacteria forming a single cluster with the bac-G clones except a kind of human pathogen (*Coxiella burnetii*) and endosymbionts of ticks (Noda et al. 1997). *C. burnetii*

was reported to be a pathogen of mammals, causing Q-fever (Baca & Paretsky 1983), and it seems to survive in amoebae in the natural environment (La Scola & Raoult 2001). Nevertheless, the sequence similarities between bac-G clones and some relatives in the databases were comparatively low (≤94.1%). Accordingly, it is thought that the ribotype bac-G is a novel species/genus of endosymbiotic bacteria (Fig. 5).

There were few sequence variations among the bac-G clones. The pairwise sequence similarities among them were considerably high (at least 99.1%) and some of the nucleotide transpositions in the alignment are most likely to be caused by unpredictable mistakes in PCR amplification. Therefore, it is suggested that the ribotype bac-G was composed of a single species, or of close relatives of bacterial species.

Based on the full-length 16S rDNA sequence, bac-F was also subjected to phylogenetic classification. Endosymbiont bac-F was shown to be related to a branch of *Flexibacter maritimus* and *F. echinicida* in the FCB-group (Fig. 6). However, the pairwise similarity values of bac-F for *F. maritimus* and *F. echinicida* were not significantly high (91.1 and 93.1%, respectively). Therefore, bac-F was also considered to be a novel bacterium not cultured previously. Some bacterial strains of the FCB-group were reported as algal-killing (algicidal) bacteria against marine microalgae (Imai et al. 1993, 1998, Maeda et al. 1998, Yoshinaga et al. 1998, Kondo et al. 1999). Most of the algicidal FCB bacteria that kill certain marine microalgae attach directly, and thereafter lyse the microalgal cells (Imai et al. 1993). Although the present study did not provide any direct evidence to indicate that the bac-F type bacterial population kill and/or get organic nutrients from *Heterocapsa circularisquama*, it is probable that a wide variety of FCB-group bacteria live in close association with microalgae in marine environments.

Murray & Stackebrandt (1995) reported the implementation of the provisional status *Candidatus* for incompletely described prokaryotes. We described the 2 intracellular bacteria bac-G and bac-F in this study by the sequence data. Accordingly, the 2 intracellular symbiotic bacteria should be categorized into the status *Candidatus*.

FITC-labeled DNA probes targeting 16S rRNA of bac-G (G233R probe) and bac-F (F87R probe) were prepared, and whole-cell *in situ* hybridization was performed against 5 strains of *Heterocapsa circularisquama*. Epifluorescence microscopy revealed that the fluorescent signals of the eubacteria-targeting probe (Eub338R) mostly covered the same areas of intracellular bacteria stained with bac-G or bac-F targeting probe (Fig. 7a–c). This observation might support our conclusion that the poor diversity of intracellular bacterial populations by 16S rDNA-PCR analysis was not a result of artifact during PCR and cloning steps. The fluorescent signal of bac-G was observed above the nuclear area of *H. circularisquama* cells, while the signal of bac-F was distributed over the cytoplasm as several small particles (Fig. 7a,b). In a previous study (Maki & Imai 2001a), the intracellular bacteria were not observed in the nuclear area of *H. circularisquama* under electron microscopy. Accordingly, bac-G may localize on the nuclear surface, while bac-F would exist throughout the cytoplasmic space. The microscopic observations also support the possibility that both species of endosymbiotic bacteria shared their habitats in algal cells.

Previously we reported that the intracellular bacteria of *Heterocapsa circularisquama* grow or survive dependently on the alga in different degrees from essential to optional (Maki & Imai 2001a,b). Most intracellular bacteria need living alga for their growth and survival, except the intracellular bacteria of *H. circularisquama* strain HU9433 (Maki & Imai 2001b). Only bac-F of strain HU9433 is culturable on the medium for heterotrophic bacteria, but those of strains HA92-1 and HI9428 are not (data not shown). There may possibly be some differences in the physiological characteristics of bac-F type bacteria associated with different strains of microalgae, in spite of the bacterial species being the same.

The genetic analysis in this study revealed that only 2 species of bacteria (bac-G and bac-F) dominantly occupy the intracellular bacterial populations of *Heterocapsa circularisquama*, although many species of bacteria surround *H. circularisquama* in natural environments. Accordingly, *H. circularisquama* would spontaneously maintain 2 specific bacteria, by supporting their growth and survival (Maki & Imai 2001b). It is suggested that the relationship between *H. circularisquama* and 2 ribotypes of bacteria is considerably close and should be termed as endosymbiosis. The *H. circularisquama* cultures used in this study have been kept in the laboratory for more than 5 yr. Such prolonged cultivation might reduce the bacterial species within the algal cells to only 2 species. There is also the possibility that natural *H. circularisquama* has more than 2 bacterial species as intracellular bacteria. However, the 2 bacterial species can at least be regarded as common symbiotic bacteria for *H. circularisquama* around Japanese embayments. Further investigation on the intracellular and extracellular bacterial populations associated with *H. circularisquama* in natural environments is necessary. The FISH technique is a powerful tool for detecting specific microbial populations in seawater samples (Amann et al. 1990a,b, Rehnstam et al. 1993, Roller et al. 1994), and would also be useful for detecting the endosymbiotic bacteria of *H. circularisquama* in marine environments.

Additionally, detailed mechanisms in the symbiotic interaction between bacteria and *Heterocapsa circularisquama* should be studied further because several scientists have pointed out that bacteria associated with the toxic dinoflagellate *Alexandrium* spp. may influence the production of paralytic shellfish toxin (Hold et al. 2001). Lewis et al. (2001) suggested that intracellular BLPs were observed in some species of *Alexandrium* by electron microscopy, and therefore it would be interesting to discover whether the intracellular bacteria of *H. circularisquama* influence bivalve mortality.

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