

## Methyl halide production by cultures of marine proteobacteria *Erythrobacter* and *Pseudomonas* and isolated bacteria from brackish water

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### Abstract

Bacteria were investigated for the production of methyl chloride, methyl bromide, and methyl iodide from brackish water. Bacteria were isolated and incubated at 25°C, and the concentrations of methyl halides in the gas phase above cultured samples were determined using dynamic headspace gas chromatography/mass spectrometry. Bacterial growth was monitored by measuring the optical density at 600 nm. Bacteria isolated from brackish water were identified by 16 Svedberg unit ribosomal-ribonucleic acid (16S rRNA) gene sequence analysis. Five genetically different strains of bacteria belonging to *Erythrobacter* or *Pseudomonas* produced methyl chloride, methyl bromide, and methyl iodide for several days in the culture. Cultured strains of these genera also released methyl chloride, methyl bromide, and/or methyl iodide. Maximum production rates for methyl halide were observed in the culture of isolated bacteria (HKF-1) belonging to *Erythrobacter*. The production rates for methyl chloride, methyl bromide, and methyl iodide by HKF-1 were  $537 \pm 124$ ,  $67.4 \pm 25.7$ , and  $48.8 \pm 9.8$  pmol L<sup>-1</sup> h<sup>-1</sup>, respectively. Aquatic bacteria could be a new source of methyl halides in marine environments.

Biogenic methyl halides such as methyl chloride (CH<sub>3</sub>Cl), methyl bromide (CH<sub>3</sub>Br), and methyl iodide (CH<sub>3</sub>I) are important halogen carriers from the biosphere to the atmosphere. These compounds release halogens in the troposphere and in the stratosphere by photolysis, and released halogens catalyze ozone depletion (Read et al. 2008). In the ocean, macroalgae produce halogenated organic compounds (reviewed by La Barre et al. 2010) and release volatile halogenated organic compounds, such as bromoform (e.g., *Dictyosiphon foeniculaceus*; Laturnus 1996). Scarratt and Moore (1996) reported the production of CH<sub>3</sub>Cl and CH<sub>3</sub>Br in a culture of marine phytoplankton (*Phaeodactylum tricorutum*, *Thalassiosira weissflogii*, and *Phaeocystis* sp.). *Prochlorococcus* and *Synechococcus* were also reported to produce CH<sub>3</sub>Cl, CH<sub>3</sub>Br, and CH<sub>3</sub>I (Brownell et al. 2010). To date, however, very few marine phytoplankton have been studied—in part because few are actually available in culture. Abiotic reactions such as nucleophilic substitutions of CH<sub>3</sub>Br and CH<sub>3</sub>I with chloride ion are substantial sources of CH<sub>3</sub>Cl in seawater (Elliott and Rowland 1993). Also, some papers have pointed to the photochemical production of CH<sub>3</sub>I (Moore and Zafiriou 1994) and CH<sub>3</sub>Cl (Moore 2008). As regards estimation of the global budget of methyl halides, known sources of CH<sub>3</sub>Cl and CH<sub>3</sub>Br have been insufficient with respect to the global sink (Butler 2000; Schäfer et al. 2007). Therefore, it remains necessary to identify new sources of CH<sub>3</sub>Cl and CH<sub>3</sub>Br; ectomycorrhizal fungi have been reported as a potential source of CH<sub>3</sub>Cl, CH<sub>3</sub>Br, and CH<sub>3</sub>I (Redeker et al. 2004).

In the model of the global atmospheric budget of CH<sub>3</sub>I, five sources (ocean, rice paddies, wetlands, biomass burning, and wood fuel) and a sink (photolysis) of CH<sub>3</sub>I were balanced, and the oceans accounted for 70% of the global source (Bell et al. 2002). As for the estimates of the oceanic CH<sub>3</sub>I emission, however, there remains substantial

uncertainty (Bell et al. 2002). The contribution of the cyanobacterium (*Prochlorococcus marinus*) to CH<sub>3</sub>I production is controversial, and production rates of CH<sub>3</sub>I from *P. marinus* cultures reported in the literature vary by about three orders of magnitude for the cell number-normalized production rates (or by about five orders of magnitude for the fresh weight-normalized production rates; Smythe-Wright et al. 2006; Brownell et al. 2010; Hughes et al. 2011). As regards volatile organic iodine compounds, biogenic marine aggregates have also been studied as oceanic source of CH<sub>3</sub>I, C<sub>2</sub>H<sub>5</sub>I, and C<sub>3</sub>H<sub>7</sub>I, and the results suggested that detrital particles could be hot spots of iodocarbon production in the ocean (Hughes et al. 2008).

Methyl chloride, methyl bromide, and methyl iodide production by phytoplankton have been correlated with enzymatic methylation by S-adenosyl-L-methionine utilizing methyl halide transferase (Manley 2002). In a recent study, screening for the methyl halide transferase genes from plants, fungi, and bacteria has revealed that bacterial methyl halide transferase can produce CH<sub>3</sub>Cl, CH<sub>3</sub>Br, and CH<sub>3</sub>I (Bayer et al. 2009). However, direct evidence of CH<sub>3</sub>Cl and CH<sub>3</sub>Br production by heterotrophic bacteria has not been reported to date. Heterotrophic bacteria have only been reported to produce iodinated methanes such as CH<sub>3</sub>I and CH<sub>2</sub>I<sub>2</sub> (Amachi et al. 2001; Fuse et al. 2003; Schulz and Dickschat 2007).

In this study, we describe the production of CH<sub>3</sub>Cl, CH<sub>3</sub>Br, and CH<sub>3</sub>I in cultures of several species of bacteria collected from a brackish-water environment. We also investigated methyl halide production by culture-collection strains belonging to the marine proteobacteria *Erythrobacter* and *Pseudomonas*.

### Methods

*Isolation, growth conditions, and bacterial strains*—Isolation of bacteria from brackish water collected from August

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2009 to April 2010 (salinity,  $\sim 0.5\%$ ; water temperature, 5–30°C) from Sanaru Lake, Japan (34°42.7'N, 137°41.5'E) was carried out. Brackish-water samples were directly streaked on plates contained 37.4 g of marine broth 2216 (Difco) and 15 g of agar (Wako Pure Chemical Industries) dissolved in 1.0 L of ultrapure water (Milli-Q, Millipore), and samples were incubated in the dark at 25°C. After several days of incubation, single colonies were selected with a sterile needle and restreaked onto the same agar plates. The isolated bacterial strains were preincubated in test tubes. The cells were then collected by centrifugation (12,000 rpm; 3 min), washed three times with fresh medium, and inoculated in 10 mL of marine broth 2216 with potassium iodide (KI; final concentration,  $1 \mu\text{mol L}^{-1}$ ) in 20-mL glass vials and without agitation in the dark at 25°C. After incubation of the cells for several days, the concentration of methyl halides in the vials were measured as described below (see "Measurement of Methyl Halides in Cultured Samples"). Then the strains in which methyl halide concentrations were higher than those of control samples were selected as methyl halide-producing bacteria.

Three strains of  $\alpha$ -proteobacteria (*Erythrobacter aquimaris* JCM12189<sup>T</sup>, *Erythrobacter citreus* JCM21816, and *Erythrobacter longus* JCM6170<sup>T</sup>) and four strains of  $\gamma$ -proteobacteria (*Pseudomonas aeruginosa* NBRC12689<sup>T</sup>, *Pseudomonas stutzeri* DSM13592<sup>T</sup>, *P. stutzeri* DSM50227, and *P. stutzeri* DSM14165<sup>T</sup>) were obtained from Riken Bioresource Center, Saitama, Japan; Japan Collection of Microorganisms (JCM), National Institute of Technology and Evaluation (NITE), Chiba, Japan; NITE Biological Resource Center (NBRC); and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM). The rate of production of methyl halides during growth was measured in seven strains of *Erythrobacter* and *Pseudomonas* and one strain, *Pseudomonas* sp. MT-1, isolated from mud from the Mariana Trench (Tamegai et al. 1997).

*Time course experiment and production rate*—To obtain the time courses of methyl halide concentrations of cultures of isolated bacteria,  $\text{CH}_3\text{Cl}$ ,  $\text{CH}_3\text{Br}$ , and  $\text{CH}_3\text{I}$  in the cultures of isolated strains were measured for 240 h. Bacterial growth was monitored by measuring optical density at 600 nm ( $\text{OD}_{600}$ ) during the culture period using a WPA CO7500 colorimeter (Biochrom). After the measurement of methyl halides in cultured samples (10 mL), the cap of the sealed vial was removed, and optical density was measured with a 10-mm cell. For each measurement of methyl halides (and optical density) at 0, 50, 100, 150, 200, and 240 h, separate vials of cultured samples were prepared. For some samples, cell numbers in the medium were also estimated using a plate count method. We spread the diluted culture sample on an agar plate and counted the number of formed colonies. To determine rates of methyl halide production by bacteria in picomol or nanomol per liter of culture per hour, the methyl halide concentrations in the cultured samples were also measured. The production rates were calculated on the basis of the increase in the methyl halide concentrations of cultured samples at 120, 240, or 360 h ( $n = 3$ ). To assess the production of  $^{13}\text{CH}_3\text{Br}$  and  $^{13}\text{CH}_3\text{Cl}$  from  $^{13}\text{CH}_3\text{I}$  by nucleophilic substitution of

the  $\text{I}^-$  ion for  $\text{Cl}^-$  or  $\text{Br}^-$ , that is, by a process known as halide exchange (Schäfer et al. 2007), we also carried out an incubation experiment involving  $^{13}\text{CH}_3\text{I}$  addition to the medium (final concentration,  $10 \text{ nmol L}^{-1}$ ).

*Measurement of methyl halides in cultured samples*—Biogenic gas concentrations were measured according to a modified method reported by Kurihara et al. (2010). Briefly, the cultured bacterial samples in glass vials sealed with silicone-polytetrafluoroethylene septa (Gerstel K.K.) were measured using an automated dynamic headspace extraction system (DHS, Gerstel K.K.). The dynamic headspace system was used to purge the gas phase above each sample (10 mL) with ultrapure helium, and the extracted gases (e.g., methyl halides) were preconcentrated in a trap column of a glass tube containing Tenax TA, which was maintained at room temperature. Compounds were released from the trap column by heating the apparatus to 200°C, and the gases were introduced into the capillary column (DB-624; length, 20 m; inner diameter, 0.18 mm; film thickness,  $1 \mu\text{m}$ ; Agilent Technologies) after cryofocusing at  $-150^\circ\text{C}$  using liquid nitrogen. Compounds were measured with a gas chromatograph (6890N, Agilent Technologies) mass spectrometer (5975C, Agilent). The trace gases were quantified according to the peak area of the calibration standards. To calibrate the concentrations of trace gases, we introduced standards of at least at four concentrations each that spanned the range of trace gas concentrations in the samples. To monitor and correct for sensitivity drift, a liquid standard of 2-iodopropane ( $[\text{CD}_3]_2\text{CDI}$ , Sigma-Aldrich) was added as an internal standard to each sample automatically with a glass syringe using a DHS system (Gerstel K.K.) just before measurement. Standards of  $\text{CH}_3\text{I}$  (99.5% pure),  $\text{CH}_2\text{Br}_2$  (99% pure),  $\text{CHBr}_3$  (99% pure),  $\text{CH}_2\text{ClI}$  (97% pure),  $\text{CH}_2\text{BrI}$  (97% pure, lot 09211BJ), and  $(\text{CD}_3)_2\text{CDI}$  (98% pure) were purchased from Sigma-Aldrich. The standard of  $^{13}\text{CH}_3\text{I}$  (99% pure) was purchased from ISOTECH<sup>®</sup>. The standard of  $\text{CH}_3\text{Cl}$  (99.9% pure) and  $\text{CH}_3\text{Br}$  (99.9% pure) was purchased from Supelco (Bellefonte). These reagents were used without further purification. Pesticide residue-grade solvents and chemicals were obtained from Wako Pure Chemical Industries. Aqueous working standards were prepared by diluting suitable aliquots of standards (dissolved in methanol) with culture medium used for the bacterial growth experiment. The calibration standards in the bacterial medium were added to the vials and analyzed. The sample vial was thermostated and agitated while the headspace was purged with a controlled flow of inert gas, providing reproducible extraction of trace gases by this DHS system. Calibrations were carried out approximately once per week. The instrumental blanks were also measured prior to sample measurement. The correlation coefficients of the calibration curves were  $> 0.96$  for all compounds. The detection limits ( $3\sigma$ ,  $n = 10$ ) of  $\text{CH}_3\text{Cl}$ ,  $\text{CH}_3\text{Br}$ ,  $\text{CH}_3\text{I}$ ,  $\text{CH}_2\text{Br}_2$ ,  $\text{CHBr}_3$ ,  $\text{CH}_2\text{ClI}$ ,  $\text{CH}_2\text{BrI}$ , and  $(\text{CD}_3)_2\text{CDI}$  in this study were 762, 19.1, 17.9, 2.0, 2.1, 1.7, 1.2, and  $1.4 \text{ pmol L}^{-1}$ , respectively. The boiling point of  $\text{CH}_3\text{Cl}$  ( $-24^\circ\text{C}$ ) is lower than those of other compounds (from  $3.5^\circ\text{C}$  for  $\text{CH}_3\text{Br}$  to  $149^\circ\text{C}$  for  $\text{CHBr}_3$ ). In our

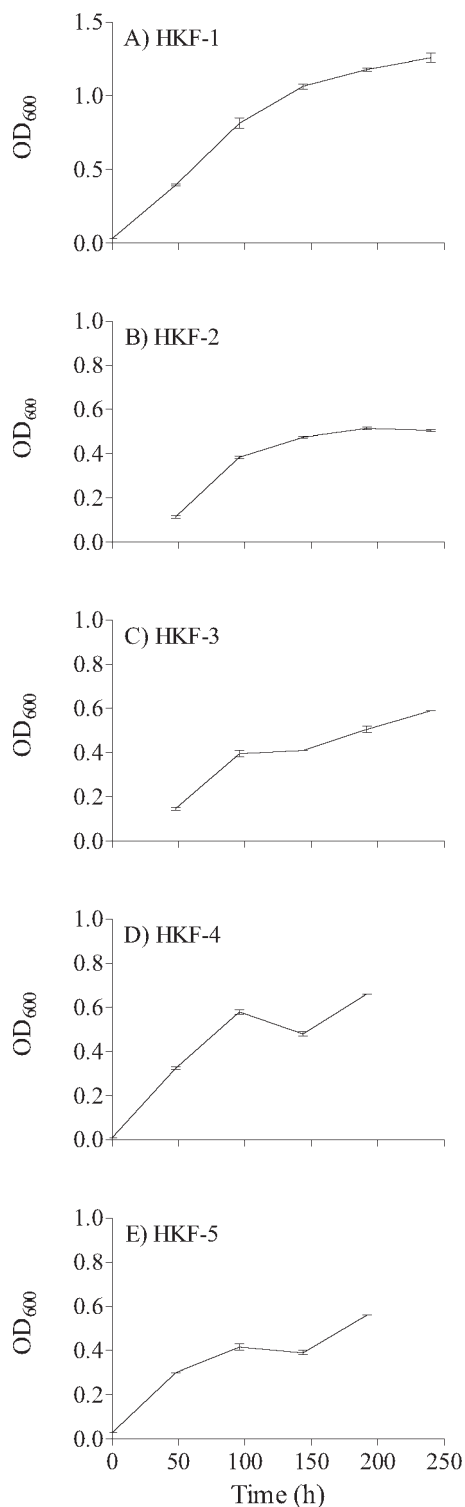


Fig. 1. Time course of OD<sub>600</sub> in strains HKF-1–HKF-5 grown in marine broth 2216 medium. (A) strain HKF-1, (B) strain HKF-2, (C) strain HKF-3, (D) strain HKF-4, (E) strain HKF-5. The line and the error bars indicate the mean and range from each analysis, respectively ( $n = 2$ ).

measurement using the DHS method, trace gases were preconcentrated on an adsorbent trap containing Tenax TA at 20°C. Therefore, the trap efficiency of very volatile compounds such as CH<sub>3</sub>Cl was low, and the detection limit for CH<sub>3</sub>Cl was relatively high. The relative standard deviation of replicate analyses ( $n = 10$ ) of CH<sub>3</sub>Cl, CH<sub>3</sub>Br, CH<sub>3</sub>I, CH<sub>2</sub>Br<sub>2</sub>, CHBr<sub>3</sub>, CH<sub>2</sub>ClI, CH<sub>2</sub>BrI, and (CD<sub>3</sub>)<sub>2</sub>CDI in this study were 24.7%, 16.3%, 10.5%, 2.9%, 4.1%, 5.1%, 2.9%, and 8.8%, respectively.

**Molecular biological studies**—Chromosomal deoxyribonucleic acid (DNA) was extracted from each strain using a FastPure DNA Kit (Takara Bio). The polymerase chain reaction (PCR) for the amplification of the 16 Svedberg unit ribosomal-ribonucleic acid (16S rRNA) gene was performed using oligonucleotides Eubac27F (AGAGTTT-GATCCTGGCTCAG) and Eubac1492R (GGTACCTT-GTTACGACTT) (Lane 1991). The resulting DNA fragment was purified by Illustra Glass Fiber matrix PCR DNA and the Gel Band Purification Kit (GE Healthcare UK) and sequenced directly. The determined sequences were deposited in DNA Data Bank of Japan, European Molecular Biology Laboratory, and GenBank with the appropriate accession numbers (HKF-1: AB633199; HKF-2: AB633200; HKF-3: AB633201; HKF-4: AB633202; and HKF-5: AB633203). DNA sequencing was carried out by Bio Matrix Research. The PCR analysis performed using a MyCycler (Bio-Rad) and AmpliTaq Gold DNA polymerase (Applied Biosystems). Oligo-DNAs for the PCR primers were synthesized by Sigma Genosys. The 16S rRNA gene sequences were aligned using the Clustal W program (Thompson et al. 1994). A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei 1987).

**Data analysis**—The statistical analysis was carried out by replacement of concentrations under the detection limit with concentrations at half the detection limit. Student's *t*-test with Welch's correction was performed using the GraphPad Prism5 software, version 5.01 (GraphPad Software), to analyze methyl halide concentrations between time 0 and 120, 240, or 360 h of culture.

## Results

**Survey of methyl halide-producing bacteria**—Based on our isolation and methyl halide measurements of the cultured samples of isolated bacteria from brackish water, we selected nine isolated bacteria that produced CH<sub>3</sub>Cl, CH<sub>3</sub>Br, or CH<sub>3</sub>I (9 of 88 single colonies). Then we identified five genetically different strains (HKF-1–HKF-5) producing methyl halide from among these nine isolated bacteria. Strain HKF-1 formed orange or red colonies on the agar plates, whereas colonies of strains HKF-2–HKF-5 were white. The OD<sub>600</sub> time courses of the cultured samples of strains HKF-1–HKF-5 indicated that all strains increased over a time span of approximately 240 h (Fig. 1A–E). The highest OD<sub>600</sub> (1.26) was observed in the culture of HKF-1 (Fig. 1A). The maximum OD<sub>600</sub> of the other strains varied from 0.51 to 0.66 (Fig. 1B–E). The time courses of CH<sub>3</sub>Cl, CH<sub>3</sub>Br, and CH<sub>3</sub>I concentrations of the cultured samples of

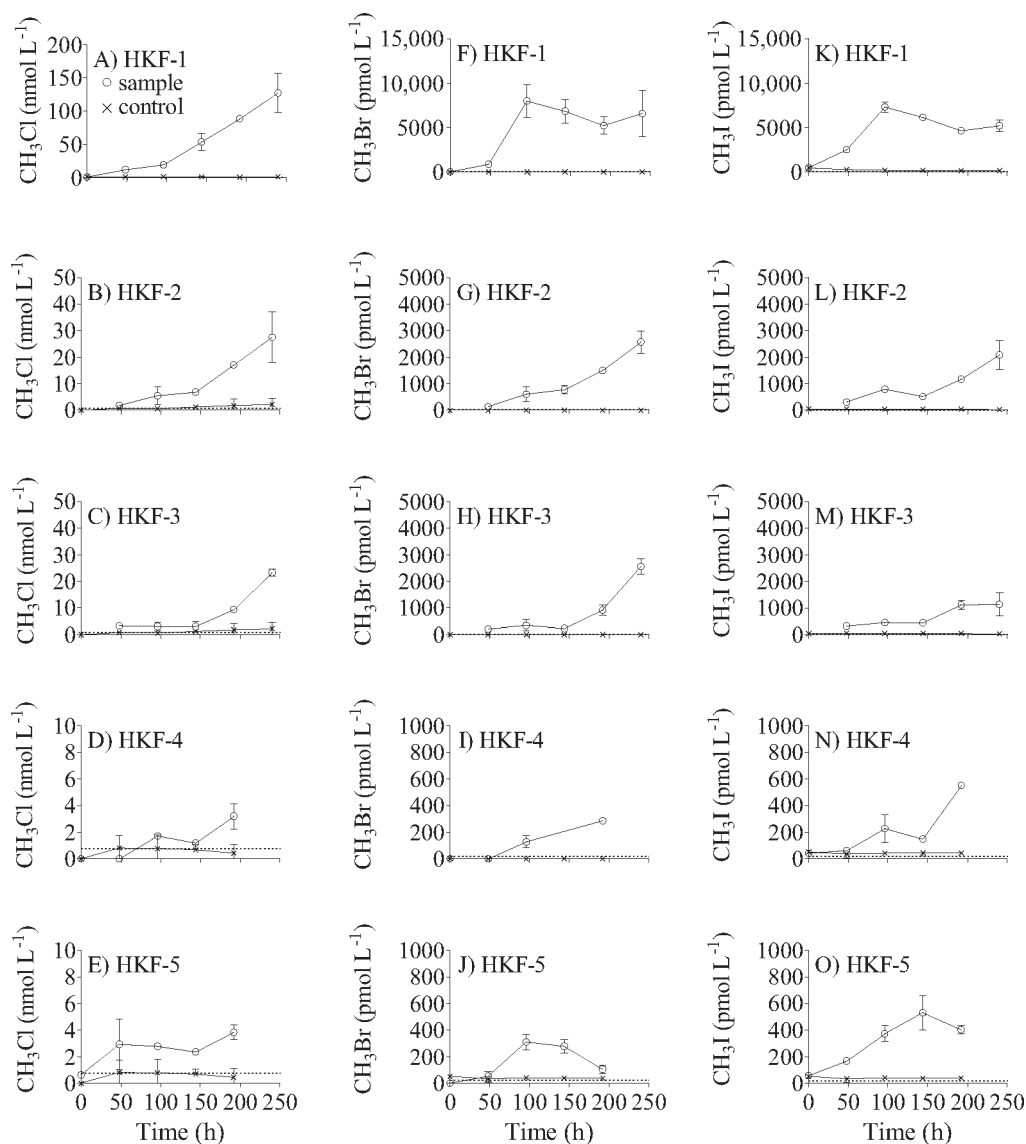


Fig. 2. Time course of methyl halide production by strains HKF-1–HKF-5 grown in marine broth 2216 medium. (A, F, K) strain HKF-1; (B, G, L) strain HKF-2; (C, H, M) strain HKF-3; (D, I, N) strain HKF-4; (E, J, O) strain HKF-5. Concentration of (A–E)  $\text{CH}_3\text{Cl}$ ; (F–J)  $\text{CH}_3\text{Br}$ ; (K–O)  $\text{CH}_3\text{I}$ . The circles and the error bars indicate the mean and range from each analysis, respectively ( $n = 2$ ). Dashed lines indicate the detection limits.

strains HKF-1–HKF-5 are shown in Fig. 2. The results indicated that the concentrations of methyl halides had increased in these five strains as they grew from the exponential phase to the stationary phase. In this study, the highest concentrations of  $\text{CH}_3\text{Cl}$ ,  $\text{CH}_3\text{Br}$ , and  $\text{CH}_3\text{I}$  were observed in the culture of strain HKF-1. The maximum concentrations of methyl halides in the HKF-1 culture were 3- to 40-fold higher than that of the other four strains. No other halomethanes (i.e.,  $\text{CH}_2\text{Br}_2$ ,  $\text{CH}_2\text{ClI}$ ,  $\text{CH}_2\text{BrI}$ , and  $\text{CHBr}_3$ ) were detected in the cultures of these five strains. Production of  $\text{CH}_3\text{Cl}$  via conversion of  $\text{CH}_3\text{I}$  ( $10 \text{ nmol L}^{-1}$ ) to  $\text{CH}_3\text{Cl}$  by nucleophilic substitution with a  $\text{Cl}^-$  ion in seawater at  $25^\circ\text{C}$  was estimated as  $4.87 \text{ nmol L}^{-1}$  for 10 d and  $7.54 \text{ nmol L}^{-1}$  for 21 d, using the rate constants given in Elliott and Rowland (1993). These values were over the limit of detection

in our study ( $\text{CH}_3\text{Cl}$ :  $0.76 \text{ nmol L}^{-1}$ ); however, neither the level of  $^{13}\text{CH}_3\text{Cl}$  nor that of  $^{13}\text{CH}_3\text{Br}$  increased during the incubation period of 504 h (21 d) in the culture medium to which  $^{13}\text{CH}_3\text{I}$  had been added ( $10 \text{ nmol L}^{-1}$ ; data not shown). These results showed that production of  $\text{CH}_3\text{Cl}$  or  $\text{CH}_3\text{Br}$  by halide exchange of  $\text{CH}_3\text{I}$  with a  $\text{Cl}^-$  or a  $\text{Br}^-$  ion may not contribute substantially to the production of  $\text{CH}_3\text{Cl}$  (or  $\text{CH}_3\text{Br}$ ) observed in the culture medium.

**Phylogenetic analysis**—The 16S rRNA gene of strains HKF-1–HKF-5 were sequenced and compared to those of other bacteria (Fig. 3). The 16S rRNA gene sequence comparisons revealed that strain HKF-1 belonged to the *Erythrobacter* (close to *Citromicrobium* sp.) group and that strains HKF-2–HKF-5 belonged to *Pseudomonas*.

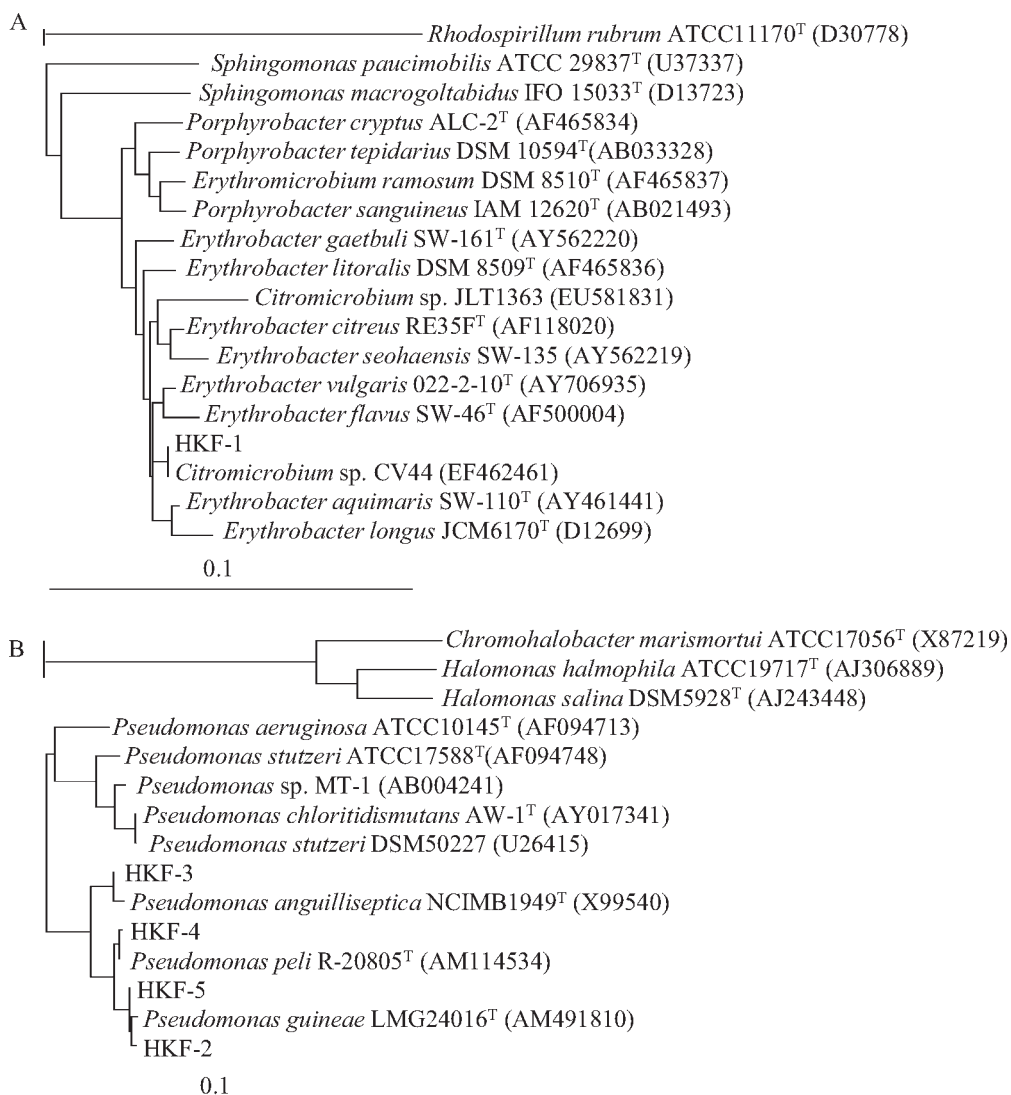


Fig. 3. Neighbor-joining tree based on partial 16S rRNA gene sequences showing the diversity of bacteria capable of producing methyl halides. (A) The phylogenetic positions of strain HKF-1 within the proteobacteria  $\alpha$  subgroup and (B) of strains HKF-2–HKF-5 within the proteobacteria  $\gamma$  subgroup are indicated. *Rhodospirillum rubrum*, *Chromohalobacter marismortui*, *Halomonas halmophila*, and *Halomonas salina* were used as the out-group. The scale bar corresponds to 1% sequence divergence.

*Production of methyl chloride, methyl bromide, and methyl iodide by marine bacteria from a culture collection*—Based on the detection of methyl halides in the cultures of five isolated bacteria related to *Erythrobacter* or *Pseudomonas*, we examined and demonstrated methyl halide production by *Erythrobacter* or *Pseudomonas* marine bacteria from culture collections (Figs. 4–6). The production of CH<sub>3</sub>Cl, CH<sub>3</sub>Br, and CH<sub>3</sub>I by HKF-1–HKF-5 is also shown in Figs. 4–6, respectively. Significant increases in CH<sub>3</sub>Cl were observed in the cultures of HKF-1, HKF-3–HKF-5, and *P. stutzeri* DSM50227 ( $\gamma$ -proteobacteria; Fig. 4A–C,E). Two cultures of  $\alpha$ -proteobacteria, *E. aquimaris* and *E. longus*, produced CH<sub>3</sub>Br (Fig. 5D). In the case of CH<sub>3</sub>I, the cultures of both  $\alpha$ -proteobacteria *E. aquimaris* and  $\gamma$ -proteobacteria *P. aeruginosa* and *P. stutzeri* (DSM50227) produced CH<sub>3</sub>I during growth (Fig. 6D,E). No other halomethanes (i.e., CH<sub>2</sub>Br<sub>2</sub>, CH<sub>2</sub>ClI, CH<sub>2</sub>BrI, or CHBr<sub>3</sub>) were increased in

these cultures of marine bacteria of *Erythrobacter* or *Pseudomonas* from culture collections. This result was similar to those for bacteria isolated from brackish water.

## Discussion

*Methyl halide production by bacteria*—Our results revealed marine bacterial CH<sub>3</sub>Cl, CH<sub>3</sub>Br, and CH<sub>3</sub>I production. Although in a previous paper, it was reported that bacterial methyl halide transferase could produce methyl halide-containing CH<sub>3</sub>Cl, CH<sub>3</sub>Br, or CH<sub>3</sub>I (Bayer et al. 2009), the direct measurement of bacterial production of CH<sub>3</sub>Cl and CH<sub>3</sub>Br had not yet been reported. To date, only CH<sub>3</sub>I production by bacteria has been reported (Amachi et al. 2001; Fuse et al. 2003; Schulz and Dickschat 2007). As shown in Figs. 1 and 2, the concentrations of CH<sub>3</sub>Cl, CH<sub>3</sub>Br, and CH<sub>3</sub>I increased in the cultured samples

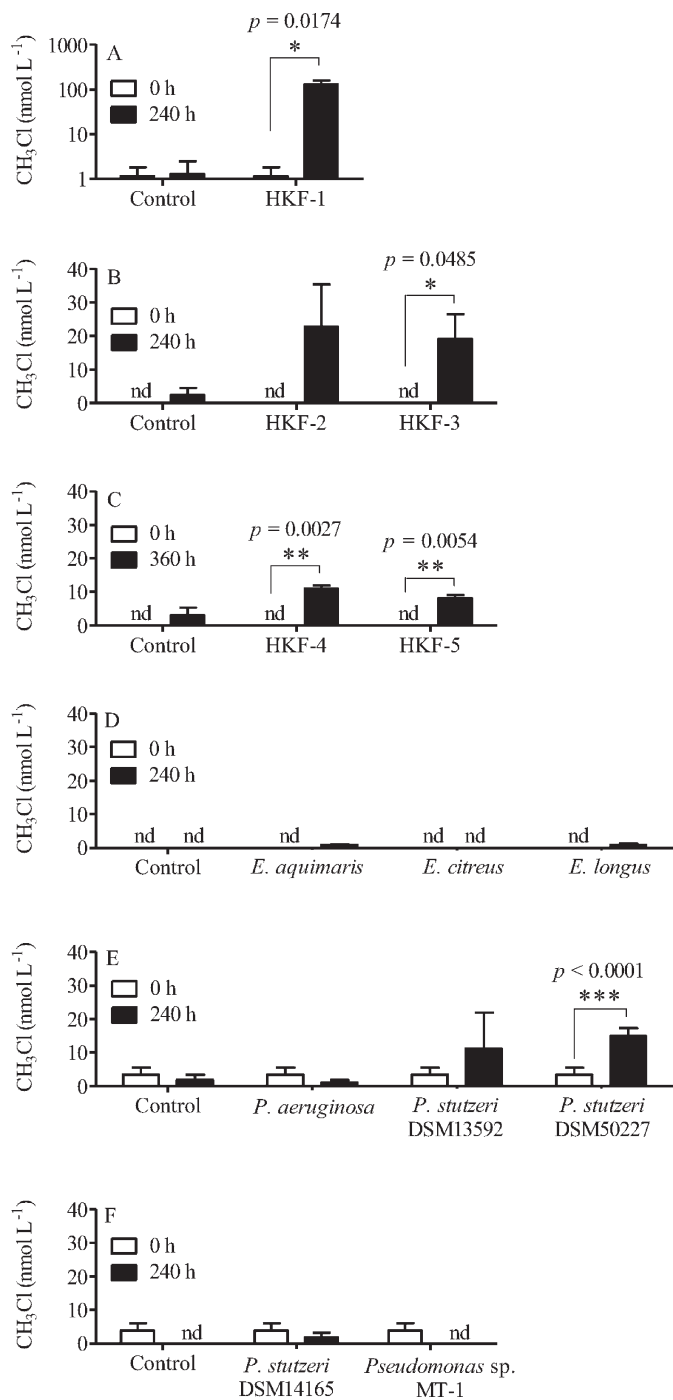


Fig. 4. The production of  $\text{CH}_3\text{Cl}$  in nanomol per liter of cultured samples of strains HKF-1–HKF-5, *Erythrobacter*, and *Pseudomonas*. Bars represent means  $\pm$  SD ( $n = 3$ ). Student's *t*-test with Welch's correction was performed for the statistical analysis of methyl chloride concentrations starting at time 0–240 or 360 h of culture. Asterisks indicate significant differences (\*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ ) in comparison to the 0-h baseline. nd = not detected.

of five isolated strains (HKF-1–HKF-5). Of these isolated bacteria, strain HKF-1 showed the highest productivity of  $\text{CH}_3\text{Cl}$ ,  $\text{CH}_3\text{Br}$ , and  $\text{CH}_3\text{I}$ . The relationships between the growth phase and  $\text{CH}_3\text{Cl}$ ,  $\text{CH}_3\text{Br}$ , and  $\text{CH}_3\text{I}$  production

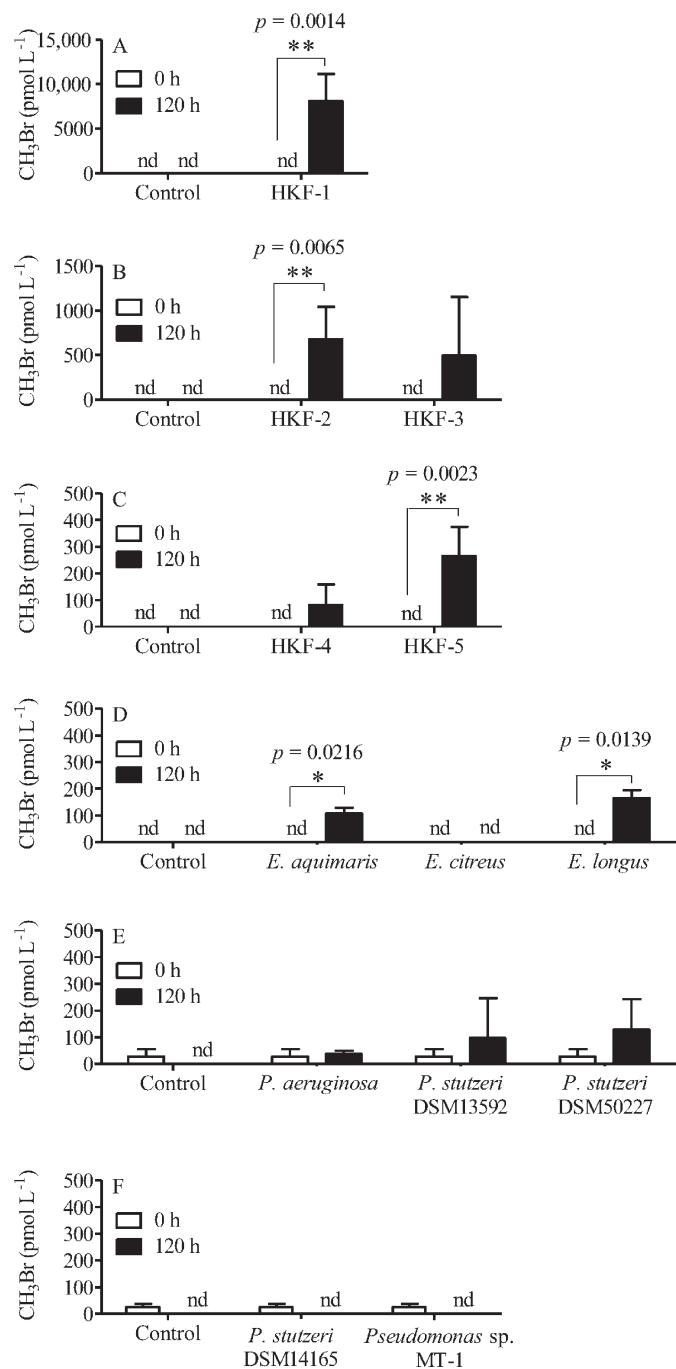


Fig. 5. The production of  $\text{CH}_3\text{Br}$  in picomol per liter of cultured samples of strains HKF-1–HKF-5, *Erythrobacter*, and *Pseudomonas*. Bars represent means  $\pm$  SD ( $n = 3$ ). Student's *t*-test with Welch's correction was performed for the statistical analysis of methyl bromide concentrations from time 0–120 h of culture. Asterisks indicate significant differences (\*:  $p < 0.05$ , \*\*:  $p < 0.01$ ) in comparison to the 0-h baseline. nd = not detected.

for the five isolated strains were not identical (Figs. 1, 2). In most cases, methyl halides were increased after bacterial growth ( $\geq 50$  h) during the incubation period (0–240 h; Figs. 1, 2). Strains HKF-3–HKF-5 and *P. stutzeri* cultures produced similar levels of  $\text{CH}_3\text{Cl}$ , whereas the culture of strain HKF-1 produced higher levels of  $\text{CH}_3\text{Cl}$  (Fig. 4).

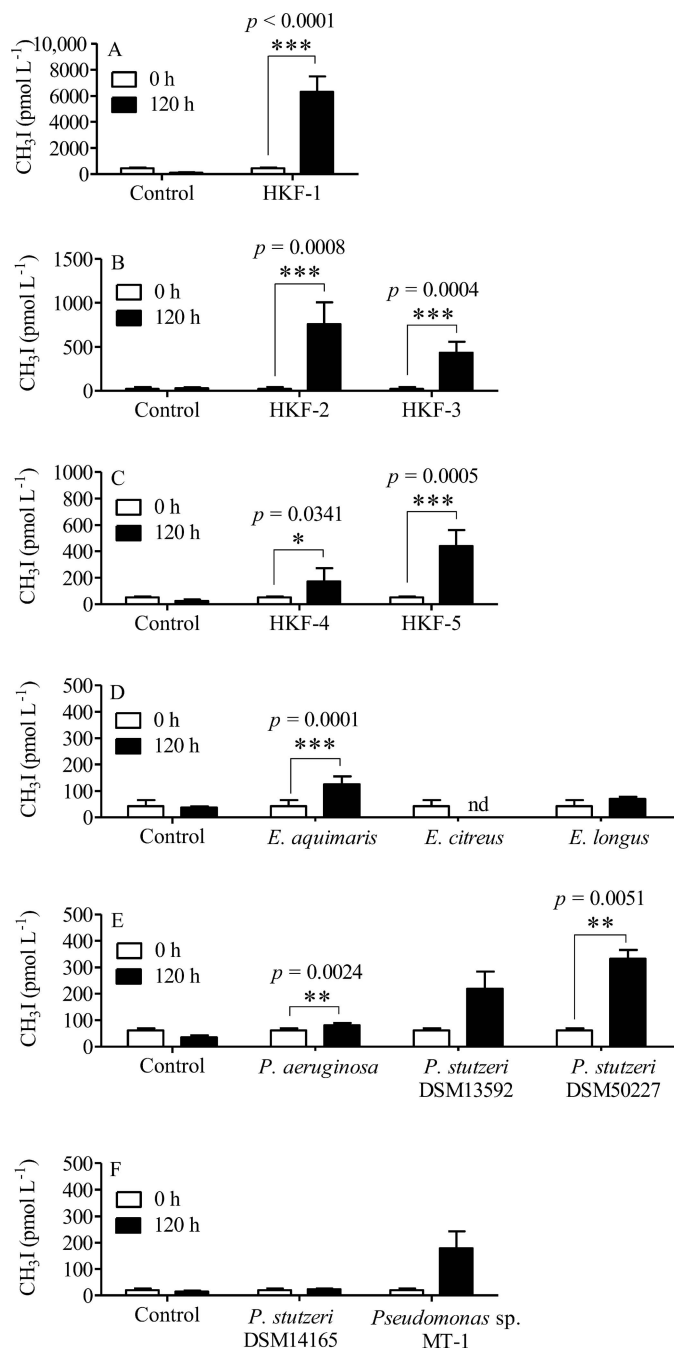


Fig. 6. The production of  $\text{CH}_3\text{I}$  in picomol per liter of cultured samples of strains HKF-1–HKF-5, *Erythrobacter*, and *Pseudomonas*. Bars represent means  $\pm$  SD ( $n = 3$ ). Student's *t*-test with Welch's correction was performed for the statistical analysis of methyl iodide concentrations from time 0–120 h of culture. Asterisks indicate significant differences (\*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ ) in comparison to the 0-h baseline. nd = not detected.

Cultures of strains *E. aquimaris* and *E. longus* produced  $\text{CH}_3\text{Br}$ , whereas statistically significant production of  $\text{CH}_3\text{Br}$  was not observed in the cultures of *Pseudomonas* strains (Fig. 5). Significant increases in  $\text{CH}_3\text{I}$  were measured in the five cultures of isolated bacteria and in the cultures of strains *E. aquimaris*, *P. aeruginosa*, and *P. stutzeri* DSM50227

(Fig. 6). Fuse et al. (2003) reported that *Roseovarius* spp. ( $\alpha$ -proteobacteria, iodide-oxidizing bacteria) produce  $\text{CH}_3\text{I}$ . The concentrations of the halides  $\text{Cl}^-$ ,  $\text{Br}^-$ , and  $\text{I}^-$  in the cultures of the present study were  $487$ ,  $0.61$ , and  $9.4 \times 10^{-4}$  mmol  $\text{L}^{-1}$ , respectively. The values for  $\text{Cl}^-$  and  $\text{Br}^-$  are similar to their concentrations in seawater, and the value for  $\text{I}^-$  was higher than its concentration in seawater. The result was significant in that the bacterial production of the methyl halides  $\text{CH}_3\text{Cl}$  and  $\text{CH}_3\text{Br}$  was observed in cultures at realistic concentrations of the corresponding halides  $\text{Cl}^-$  and  $\text{Br}^-$  in seawater.

**Phylogenetic analysis**—The 16S rRNA gene sequence analyses placed the HKF-1 strain within the *Erythrobacter* lineage of the  $\alpha$ -proteobacteria and the HKF-2–HKF-5 strains within the *Pseudomonas* lineage of the  $\gamma$ -proteobacteria (Fig. 3). The maximum production rates for  $\text{CH}_3\text{Cl}$ ,  $\text{CH}_3\text{Br}$ , and  $\text{CH}_3\text{I}$  by isolated bacteria (HKF-1) were  $537 \pm 124$ ,  $67.4 \pm 25.7$ , and  $48.8 \pm 9.8$  pmol  $\text{L}^{-1} \text{h}^{-1}$ , respectively. Strain HKF-1 belonged to *Erythrobacter* and was genetically related to *E. aquimaris* and *E. longus* (Fig. 3); *E. aquimaris* produced  $\text{CH}_3\text{Br}$  ( $0.81 \pm 0.19$  pmol  $\text{L}^{-1} \text{h}^{-1}$ ) and  $\text{CH}_3\text{I}$  ( $0.69 \pm 0.31$  pmol  $\text{L}^{-1} \text{h}^{-1}$ ), and *E. longus* produced  $\text{CH}_3\text{Br}$  ( $1.29 \pm 0.25$  pmol  $\text{L}^{-1} \text{h}^{-1}$ ). As regards production rates, those of the culture collections were approximately one to two orders of magnitude lower than those of the isolated bacteria HKF-1 in this study. These results indicate the diversity of methyl halide production among genetically similar species. The isolated strains of HKF-2–HKF-5 belonging to *Pseudomonas* also produced methyl halides (Figs. 3–6). Statistically significant methyl halide production was also observed in the culture collection of *P. stutzeri* DSM50227 ( $\text{CH}_3\text{Cl}$  and  $\text{CH}_3\text{I}$ ) and *P. aeruginosa* ( $\text{CH}_3\text{I}$ ). Methyl halide production rates of *P. stutzeri* DSM50227 and *P. aeruginosa* were similar to those of strains HKF-2–HKF-5, although a difference in methyl halide production among HKF-1 and the *Erythrobacter* species was observed. These results suggest that the capacity for methyl halide production is widespread among marine proteobacteria, although the methyl halide production in each organism is different in degree.

**Comparison of methyl halide production between bacteria and other organisms**—Some phytoplankton (e.g., *P. tricornutum*, *T. weissflogii*, and *Phaeocystis* sp. [Scarratt and Moore 1996] and *P. marinus* [Brownell et al. 2010]) are known to produce methyl halides, but the contribution of the cyanobacterium (*P. marinus*) to  $\text{CH}_3\text{I}$  production is controversial. The ratios of production rates ( $\text{CH}_3\text{Cl}:\text{CH}_3\text{Br}:\text{CH}_3\text{I}$ ) were similar among HKF-1 (1.0:0.13:0.091), HKF-5 (1.0:0.10:0.15), and *P. marinus* (1.0:0.13:0.054; Brownell et al. 2010). The production rates of  $\text{CH}_3\text{Cl}$  have been shown to be about 10-fold higher than those of  $\text{CH}_3\text{Br}$  in the phytoplankton culture (*P. tricornutum*, *T. weissflogii*, and *Phaeocystis* sp.; Scarratt and Moore 1996). The ratio of the three halide concentrations ( $\text{Cl}^-:\text{Br}^-:\text{I}^-$ ) in the culture medium was  $1:1.3 \times 10^{-3}:1.9 \times 10^{-6}$ . The amounts of methyl halide produced ( $2.6 \times 10^{-5}\%$  for  $\text{Cl}^-$ ,  $1.3 \times 10^{-3}\%$  for  $\text{Br}^-$ , and  $7.8 \times 10^{-1}\%$  for  $\text{I}^-$ ) were minute in comparison with the abundance of halide ions in the culture medium. Therefore, it seemed likely that changes in the relative concentrations

of the three halide ions in the medium would not be significant during the culture experiments. These results (present results see also results of Scarratt and Moore 1996; Brownell et al. 2010) suggest that there might be a similarity among these organisms in terms of the methylation ratio of halogen. Methyl chloride, methyl bromide, and methyl iodide production by phytoplankton have been correlated with enzymatic methylation by S-adenosyl-L-methionine utilizing methyl halide transferase (Manley 2002).

*Production of methyl halides by bacteria in an aquatic environment*—Our results demonstrated that both  $\alpha$ -proteobacteria and  $\gamma$ -proteobacteria were able to produce  $\text{CH}_3\text{Cl}$ ,  $\text{CH}_3\text{Br}$ , and  $\text{CH}_3\text{I}$ . Amachi et al. (2001) and Fuse et al. (2003) incubated strains of terrestrial and marine bacteria, and both reported  $\text{CH}_3\text{I}$  production. Our results provide further evidence for the bacterial production of methyl halides in the environment, especially in a brackish and marine environment. The cell number ( $3 \times 10^9$  cells  $\text{mL}^{-1}$  at  $\text{OD}_{600} = 0.5$  in HKF-1 culture) in the cultures of the present study were three orders of magnitude higher than those in the sea ( $0.5\text{--}5 \times 10^6$  cells  $\text{mL}^{-1}$ ; Ducklow 2000). Normalized concentrations by cell number of  $\text{CH}_3\text{Cl}$  (130 pmol  $\text{L}^{-1}$ ),  $\text{CH}_3\text{Br}$  (8.1 pmol  $\text{L}^{-1}$ ), and  $\text{CH}_3\text{I}$  (6.3 pmol  $\text{L}^{-1}$ ) in seawater were calculated on the basis of the maximum concentrations of  $\text{CH}_3\text{Cl}$  (130 nmol  $\text{L}^{-1}$ ),  $\text{CH}_3\text{Br}$  (8100 pmol  $\text{L}^{-1}$ ), and  $\text{CH}_3\text{I}$  (6310 pmol  $\text{L}^{-1}$ ) in the culture of HKF-1, respectively. A wide variety of bacteria have been described as degrading methyl halides; some bacteria have even been shown to grow on methyl halides as a carbon source (Goodwin et al. 1998; McAnulla et al. 2001). Methyl halide-degrading bacteria have been isolated from a range of environments, such as soils, lake sediments, and seawater samples; while most are  $\alpha$ -proteobacteria, some are  $\gamma$ -proteobacteria (Schäfer et al. 2007). The present results suggested that bacteria could contribute to not only degradation but also production in the methyl halide budget in marine environments.

*Erythrobacter* sp. strains were isolated from the upper ocean, and aerobic anoxygenic photoheterotrophs account for a significant fraction of the marine microbial community in the open ocean (Béjà et al. 2002; Koblížek et al. 2003). The results of the present study, taken together with the isolation of *Erythrobacter* sp. in the upper ocean (Koblížek et al. 2003), showed that aquatic bacteria could be a new source of methyl halides in marine environments, although it should be noted that there is a range of methyl halide production rates, even among proteobacteria, as described above.

#### Acknowledgments

We thank K. Tsujino for his help with the sampling. We also appreciate the constructive comments and helpful suggestions of two anonymous reviewers. This study was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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*Associate editor: Wade H. Jeffrey*

*Received: 27 May 2011*

*Accepted: 19 October 2011*

*Amended: 24 October 2011*