PHYLOGENETIC AND MICROECOLOGICAL STUDIES ON METHANOGENIC SYMBIONTS IN TERMITES

（シロアリ腸内共生メタン細菌に関する系統学的、微生物生態学的研究）

NAOYA SHINZATO

PHYLOGENETIC AND MICROECOLOGICAL STUDIES ON METHANOGENIC SYMBIONTS IN TERMITES

NAOYA SHINZATO

School of Arts and Science

University of Tokyo

December, 1998

CONTENTS

PART III

PHYLOGENETIC RELATIONSHIPS BETWEEN METHANOGENIC SYMBIONTS AND THEIR XYLOPHAGOUS HOST INSECTS

GENERAL INTRODUCTION

Almost all termites harbor methanogenic archaea in hindgut and emit methane. Methane from termite hindgut is mainly formed by the reaction $nCO_2 + 4nH_2 \rightarrow nCH_4 + 2nH_2O$. Methanogens as well as acetogens and sulfate-reducing bacteria in termite hindgut play a role as a "H2 sink". Their H_2-consuming processes by methanogens help anaerobic lignocellulose decomposition in the hindgut.

Termites belong to the order Isoptera, which includes over 2000 described species whose biology, behavior, and nutritional ecology are remarkably diverse. Their populations, particularly in tropical and subtropical regions are so large, that their numbers can exceed 6000 m^{-2} and their biomass density often surpasses that of grazing mammalian (12, 37). It has been estimated that termites from all ecological regions together consume about 3-7 x 10^{15} g of lignocellulose annually and mineralize a significant portion of it (12, 30). Termites are one of the important decomposers of plant in our biosphere.

Termites are divided into two groups (33, 34). So-called lower termites (families Masto-, Kalo-, Hodo-, Rhino-, and Serritermitidae) and Higher termites (family Termitidae). The lower termites harbor a dense and diverse population of bacteria and cellulose-digesting, flagellate protozoa in their alimentary tract (27). The higher termites comprising three fourth of all the termite species, also harbor a dense and diverse array of gut bacteria, but they typically lack protozoa (27).

In the lower termites, the hydrolysis of cellulose is supported by the cellulose-digestion of some anaerobic flagellates (28). They endocytose particles of wood or cellulose into food vacuoles and ferment the polysaccharide components. While, in the higher termites, the hydrolysis of cellulose is performed by cellulases produced by termite themselves (55). The circumstances in the hindgut of termites are basically anaerobic with an E'_0 usually in the range of -230 to -270 mV (2, 3). This means that the

lignocellulose ingested by termites is anaerobiocally hydrolyzed.

The anoxic degradation of lignocellulose occurs by mixed microbial community, with distinct physiological group participating in successive stages of the decomposition. The first step is polymer-hydrolysis of polysaccharides to soluble monomers and dimmers by symbiotic protozoa or enzymes secreted by termites. The second is fermentation of the products to smaller molecules, e.g. organic acids and gases such as H_2 and/or CO_2. The third stage is additional fermentation of organic acids with three or more carbons to acetate + H_2 ± CO_2 by acetogenic proton-reducing bacteria. The final step is supported by H_2 sink organisms, which consume the protons and electrons liberated in the intermediate steps. In gastrointestinal tracts, such as bovine rumen (64) or the gut of termites, the concentration of terminal electron acceptors other than CO_2 is usually limited, this terminal H_2-consumption is carried out by methanogens. And the favorable thermodynamics of this process help to pull whole anaerobic decomposition to completion (54).

On the other hand, the role of termites in the global budget of methane and, in particular, their contribution to the recent increase of tropospheric concentrations of this "greenhouse gas" have been controversially discussed in the last decade. In 1975, Breznak (6) have reported the methane emission rate from three species of lower termites *Reticulitermes flavipes*, *Coptotermes formosanus*, and *Cryptotermes brevis* ranging from 0.3 to 73.1 nmole CH_4 hr^{-1} g fresh wt^{-1}. Zimmerman et al. has suggested that termites are the major global source of methane in the atmosphere (67). They estimated a total methane emission from termites by laboratory and field studies with *Reticulitermes tibialis*, *Gnathamitermes perplexus* and an unidentified species of *Nasutitermes*, and concluded that total emission is from termites 150 Tg CH_4 yr^{-1}. It was corresponding to approximately 30% of the global atmospheric emission of methane. And they added that an enlargement of tropical termite populations due to deforestation resulted in the recent increase of tropospheric concentration of methane. After the report many researchers have measured methane emission from termites in North America (50, 68), Africa (56),

Australia (22) and Amazonia (40). However, most of the field measurement conducted with intact termite nests revealed far lower emission rate. The discrepancy could be explained by internal methane consumption in the nests, or by difference of the termite species investigated. The assumption that termite abundance increases after deforestation has been also criticized by Collins and Wood (12). They reported the decrease of higher termite densities in deforested areas, at least on the long term. The contribution of termites to atmospheric methane now appears to account for less than 5 % of global methane emissions (i.e. 20 Tg yr^{-1}; ref. 40).

In contrast to the ecological works, microbiological studies on the methanogenic bacteria and methane production in termite hindgut are relatively poor. The first observation of the methanogenic bacteria in termite hindgut has been done on the endosymbiotic methanogens in symbiotic protozoa. For many years pure culturing of symbiotic protozoa in termites have not been available. Yamin (65) succeeded in obtaining axenic cultures of *Trichomitopsis termopsidis* from *Zootermopsis angusticollis*. Nutritional and growth characteristics of *T. termopsidis* were studied by Odelson and Breznak (45, 46). In these works, they realized that the axenic cultures produce small amount of methane during the growth. However, strangely, they could not observe any F420-autofluorescent cells, which is expected for methanogen, by epifluorescence microscopic examination.

Lee et al. (38) exploited the autofluorescence of F420 and F350, the metabolic cofactors present in methanogens, by epifluorescence microscopy to visualize methanogens in hindgut contents of *Z. angusticollis*. Small, rod-shaped methanogens resembling *Methanobrevibacter* sp. were found to be associated with three flagellated protozoa, *T. termopsidis*, *Tricercomitus termopsidis*, and *Hexamastix termopsidis*. They also showed that the methanogens housed in the cytoplasm of *T. termopsidis* by electronmicroscopic observation with thin sections of the protozoa. In *Z. angusticollis*, free-living methanogen, i.e. non-endosymbiotic methanogen, were not found in the

hindgut fluid.

In a follow-up study, Messer and Lee (41) found that methanogenesis in Z. *angusticollis* appeared to be limited by reductant availability (presumably H_2), as incubation of termites in atmospheres containing 25% H_2 doubled exposed to hyperbaric oxygen which eliminated many of the protozoa from hindguts. By using various treatments to selectively eliminate or affect certain members of the gut flora of Z. *angusticollis*, Messer and Lee concluded that large protozoa of the genus *Trichonympha* evolved most of the H_2 in the hindgut ecosystem, but that methanogenic bacteria symbiotic with *T. termopsidis* produced most of the methane.

However, these microecological studies were made on a few lower termite species. In many termites, including higher termites which possess no symbiotic flagellates in the hindgut, methane production mechanisms are still unclear. In addition, a large number of methanogens attached on the hindgut epithelium were observed in some higher and lower termites (Yamaoka, personal communication). The contribution of these free-living methanogens has to be also determined. Most of the microecological features of the symbiotic methanogens in termites remains unknown. To gain a better understanding of this symbiosis, more fundamental characteristics have to be determined.

Purpose of the study

The main purposes of this study are:

(1) To clarify the details of the methanogenesis of termites, e.g. quantity of methane emission from termites or microecological feature of methanogens in termite hindgut.

(2) To estimate the phylogenetic diversity of the symbiotic methanogens and, if they diverged, to clarify the relationships between the phylogenies and their *in situ* niches in termite hindgut.

(3) To make clear the phylogenetic relationships between host termites and symbiotic methanogens and to discuss the transmission and/or infection mode of the symbionts.

In order to attain these purposes, the following aspects were investigated on some termite species distributed around Japan Archipelago.

(a) Methane emission of intact termites was measured. The termite species used were *Neotermes koshunensis* (Kalotermitinae), *Hodotermopsis japonica* (Termopsinae), *Reticulitermes speratus* (Rhinotermitinae), *Coptotermes formosanus* (Rhinotermitinae), *Odontotermes formosanus* (Macrotermitinae), and *Nasutitermes takasagoensis* (Nasutitermitinae). Although methane emission rate of *C. formosanus* sampled in North America has been estimated (6), no examination on methane emission or methanogen observation in the other termites have been made so far. Symbiotic methanogens in hindgut contents were observed by epifluorescence microscopy to detect the autofluorescence of specific cofactor F420, addition to the above termites, *R. miyatakei*, *R. flaviceps*, and *Pericapritermes nitobei* (Termitinae) were used. The correlation of methane emission rates of each termite species, and distribution of symbiotic methanogens in hindgut were discussed (Part I).

(b) Methanogen communities in the hindgut of the lower termite *R. speratus* were phylogenetically surveyed using small-subunit ribosomal RNA (SSU rRNA) gene sequence. *R. speratus* was collected from 6 sampling points of 4 regions, Tokyo, Kobe, Yamaguchi, and Okinawa in Japan Archipelago. Molecular phylogeny of methanogenic symbiont in the termite was determined, and their diversity and *in situ* localization were discussed (Part II).

(C) Methanogenic symbionts of 7 termite species in Japan Archipelago consisted of 4 lower and 3 higher termite species, and one Australian lower termite *Mastotermes darwiniensis* were also phylogenetically examined. The Japanese termite species used were *Reticulitermes kanmonensis*, *C. formosanus*, *N. koshunensis*, *H. japonica*, *P. nitobei*, *N. takasagoensis*, and *O. formosanus*. And then, methanogen composition and geographical distribution of respective species were compared, and the transmission and infection of methanogens to the next termite generation were discussed (Part III).

PART I

METHANE PRODUCTION BY MICROBIAL SYMBIONTS IN LOWER AND HIGHER TERMITES OF THE RYUKYU ARCHIPELAGO

ABSTRACT

Methane emission rate of the 6 termite species including higher and lower termites collected in Ryukyu Archipelago was measured in laboratory. The methane emission rate of the termites depended on termite species except for one lower termite *Neotermes koshunensis*. The methane emission rates of *N. koshunensis* was colony-dependent, and no methane emission was detected in some of the colonies.

Short rod-shaped methanogens were observed to associate with some species of flagellates in the hindgut of the lower termites by epifluorescence microscopy. Methanogens adhering to the hindgut epithelium were also seen abundantly. In the colonies of *N. koshunensis* without methane emission, no methanogens were observed in the hindgut. Electronmicroscopic observation showed that two unique filamentous methanogens existed in the hindgut of the higher termite *Nasutitermes takasagoensis*, each methanogen showed the ultrastructural features of genus *Methanothrix* and *Methanospirillum* respectively.

Analyses of volatile fatty acids in the hindgut fluid of *N. koshunensis* revealed that acetic acid is a major component in the volatile fatty acids of the termite. The acetic acid concentration of *N. koshunensis* treated with a methanogenesis inhibitor, 2-bromoethanesulfonate, was 1.6-fold higher than the control. It was suggested that, in the colonies without methane emission, H_2 may be converted into acetic acid instead of methane by acetogens.

INTRODUCTION

Methane emission from termites have been accepted to contribute to the total global methane concentration because of their large world population and the high ability to produce methane. In 1982, Zimmerman and coworkers (67) have calculated a total methane emission of 150Tg CH_4 $year^{-1}$ from termites corresponding to approximately 30% of the global production of methane. Since the sensational report, the role of termites in the global budget of methane and, in particular, their contribution to the recent increase of tropospheric concentrations of this "green house gas" have been controversially discussed in the last decade (22, 50, 56, 68). At the present time, the contribution of termites to atmospheric methane appears to account for <5% of global methane emission (40). To conclude the termite contribution on atmospheric methane, reliable estimation of termite abundance on the earth is necessary, and sufficient number of termite species have to be examined.

On the other hand, microbiological studies on the methanogenic bacteria and methane production in termite hindgut is fewer than the ecological works. Lee and coworkers (38) have examined methanogen-localization in hindgut of the lower termite *Zootermopsis angusticollis*. They reported that short rod-shaped methanogens are found in some flagellated protozoa based on the observation of hindgut contents by epifluorescence microscopy and of sections of the flagellates by electronmicroscopy. Messer and Lee (41) have concluded that large cellulolytic flagellates emit H_2 and CO_2, and small flagellates associated with methanogen converted them into methane by selective elimination of the components in the hindgut of *Z. angusticollis*. However, these microecological studies were made on a few lower termite species. In many termites, including higher termites which possess no symbiotic flagellates in the hindgut, methane production mechanisms are still unclear. In addition, a large amount of methanogens attached on the hindgut epithelium were observed in some higher and lower termites (Yamaoka, personal

communication). The contribution of these free-living methanogens has to be also determined.

The Ryukyu Archipelago belong to sub-tropical zone, where biota is remarkably diverse as compared with the other region of Japan. From the area, 16 species of termites which belong to 4 families, 6 sub-families among known 7 families and 15 sub-families have been reported. The methane emission from these termites have not been reported except for one *Coptotermes* species, and no microbiological knowledge on methanogens have been reported so far.

In this study, methane emission rates of the termites collected in the Ryukyu Archipelago were measured, and the *in situ* localization of methanogens was observed to discuss the methane production scheme in these termites.

MATERIALS AND METHODS

Termites

Nine termite species consisted of 6 lower and 3 higher termites were collected in the forests in the Ryukyu Archipelago with their nest wood, and maintained in laboratory with nest wood at 25°C until use. Termite species and the sampling points were listed in Table 1.1. Workers of the higher termites and pseudergates of the lower termites were used in this study.

Quantification of methane production

Methane emission from intact termite was measured by gas chromatography. Four lower termites *N. koshunensis*, *H. japonica*, *R. speratus*, and *C. formosanus*, and 2 higher termites *O. formosanus*, and *N. takasagoensis* were examined. Termites were sealed in a 7 ml glass culture tube with a Teflon/silicone serum cap, and the gap between the glass tube and the serum cap was sealed with silicone grease. Number of termites used in one independent measurement was determined by their wet weight, and were as follows; *N. koshunensis*, 20; *H. japonica*, 10; *R. speratus* and *C. formosanus* , 60; *O. formosanus* and *N. takasagoensis*, 30. After 2 hr incubation at room temperature (15-20°C) in the dark, 100 µl of headspace gas was taken with a precision syringe. All measurements were made in triplicate. Methane in headspace gas was measured at 60°C using a Shimadzu gas chromatograph GC-7A with a Porapak N column equipped with a flame ionization detector and a Shimadzu chromatopac CR6A integrating recorder. The results were normalized as methane production rates per termite and to the rates per termite wet weight with external standard (100 or 500 ppm).

Epifluorescencemicroscopy

In situ localization of symbiotic methanogens in hindgut was examined by F420

autofluorescence detected by epifluorescence microscopy (29). Digestive tracts of the termite from the midgut to the colon were taken with a pair of fine-tipped forceps, and the paunch region was pierced with a needle for sampling of the contents. The contents were resuspended into 5% formaldehyde in Solution U (60). The hindgut wall was dissected with a laser blade in a drop of same fixative on a microscopic slideglass. An Olympus model BH2 microscope (Olympus, Japan) with suitable filter systems (29) was used for phase contrast and epifluorescence microscopy.

Electron microscopy

To obtain ultrastructural information of the filamentous methanogens observed by epifluorescence microscopy in *N. takasagoensis*, negatively stained gut contents were observed by electronmicroscopy. For electron microscopy, methanogens attaching on hindgut wall were prepared from 10 individuals of the termite as follows; after squeezing out of gut contents, methanogens attaching on the hindgut wall were separated from the epithelial surface by vigorous shaking of the sample in 1 ml of distilled water with a vortex mixer. The bacterial cells were collected by centrifugation, and negatively stained with 1% neutral potassium phosphotungustate. The stained cells were mounted on formbar-coated copper meshes and observed under a transmission electron microscope model JEM-2000EX (JEOL Ltd., Japan) operated at an accelerating voltage of 100 kV.

Analysis of VFAs in hindgut fluid

Volatile fatty acids (VFAs) in the hindgut fluid of the termite were analyzed by gas chromatography to compare the concentrations and the compositions of VFAs in the hindgut fluid of *N. koshunensis*. VFAs analysis in termite hindgut was done according to Odelson and Breznak (47). Fresh hindgut fluid in the paunch was issued by piercing with a needle, and then the eluate was sampled with a micro-pipette. Seventy µl of the sample a total was collected in a test tube on ice. The sample was centrifuged at 13,000 rpm for 1

hr at 2°C, and the supernatant was used for gas chromatography. Representative C_2 to C_4 VFAs, i.e. acetic acid, propionic acid, butyric acid, isobutyric acid, isovaleric acid, and valeric acid were used as standards. Extracellular pool size of the hindgut was estimated as follows; 10 µl of the hindgut fluid sampled as described above was resuspended in 10 µl of 2 mM phosphate-buffered saline, and it was aspirated into a hematocrit capillary tube. Both ends of the tube were plugged with paraffin, and the tube was centrifuged at 13,000 rpm for 1 hr at 2°C. A ratio of the supernatant fluid volume to the pellet volume calculated was used for estimation of the VFAs concentrations in the termite hindgut.

BES treatment

To consider the effect of the interruption of the flow from H_2 to methane on VFA concentration in the hindgut, methanogenesis was artificially inhibited with a methanogenesis specific inhibitor 2-bromoethane sulfonate (BES) basically according to Messer and Lee (41), and then the VFAs were analyzed. Fifty individuals of the termites were fed with a filter paper soaked with 60 µg BES per mg filter paper for a week. Control group was fed with a filter paper soaked with distilled water.

RESULTS

Methane production from termites

Methane emission was observed in all species of the termites examined in this study (Table 1.2). In *N. koshunensis*, however, methane emission was not detected from termites in 6 out of 15 colonies examined (Table 1.4). The lower termite *H. japonica* showed the highest methane emission rate (34.9 nmol individual^{-1} hr^{-1}) and the lowest one was observed in the higher termite *O. formosanus* (0.2 nmol individual^{-1} hr^{-1}). The average rate of all 6 termite species was 6.4 nmol individual^{-1} hr^{-1}.

In situ localization of methanogens

In situ localization of methanogens in hindgut was examined in 8 termite species listed in Table 1.3. Epifluorescence microscopy showed the presence of autofluorescent methanogens in the hindgut of the termites. Short rod-shaped methanogens were found in the cells of the specific flagellates in the lower termites. Estimated genera of the flagellates were listed in Table 1.3. In *N. koshunensis*, 3 flagellate species were observed to be associated with methangens. Particularly, *Foaina* sp. and *Trichomitopsis* sp. possessed methanogens without exception (Fig. 1.1B and 1.2B, respectively). *Foaina* sp. was often found at the posterior part of *Stephanonympha* sp. (Fig. 1.1A). *Trichomitopsis* sp. was swarming freely in the hindgut fluid (Fig. 1.2A). On the other hand, number of methanogens associated with *Devescovinia* sp. was not high and varied depending on individual cells (data not shown). In addition, epifluorescent methanogens were occasionally observed to be adhering on the stalk surface of the large flagellate *Rostronympha* sp., which were found on the epithelium of the paunch and on food flocks present in the paunch lumen (data not shown). The only flagellate found in *C. formosanus* was tentatively identified as *Spirotrichonympha* sp. (Fig. 1.3A and B).

Among 3 species of genus *Reticulitermes*, flagellates with endosymbiotic methanogen

were not observed.except for one flagellate *Microjoenia* sp. found in *R. speratus*. Instead, majority of methanogens were observed to be adhering on the epithelium of the posterior paunch and anterior colon in *R. speratus*, *R. miyatakei*, *R. flaviceps* and *C. formosanus* (Fig. 1.4-11). Bacterial trichomes and food flocks present in the paunch lumen were often found completely covered with methanogens (Fig. 1.5, 10). In *N. takasagoensis* and *P. nitobei* which lacked flagellates within the hindguts, filamentous autofluorescent bacteria were mainly adhering on the epithelium of the posterior paunch and anterior colon (Fig. 1.12B and 13B). In hindgut of *O. formosanus*, only a small number of short rod-shaped autofluorescent methanogens were observed (data not shown).

Ultrastructure of filamentous methanogens

Four morphologically different filamentous bacteria were distinguished by the electronmicroscopic observation of the bacteria peeled from the hindgut epithelium. The first type of the bacteria was about 10 μm in length and 0.4 μm in width, flat ended, possessed flagella stretched from both ends of the cell and sheath-like structure (Fig. 1.14a and b). The second one was similar to the first type except that flagella were not observed (Fig. 1.15a and b). The third type was approximately 6.2 μm in length and 0.5 μm in width, round ended, possessed polar tufted flagella and sheath-like structure (Fig. 1.16a and b). The last was resembled to the third type, but the cell was remarkably long (more than 50 μm, Fig. 1.17a-c).

VFAs in termite hindgut

Analysis of VFAs in the hindgut fluid of *N. koshunensis* showed that acetic acid was the most major fatty acids in this termite hindgut and the estimated acetic acid concentration in the hindgut was 178.4 mM in the methane emitting colony, and 233.3 mM in the no-methane emitting one (Table 1.8). A little amount of butyric acid was also

detected, but the other VFAs examined were not detected. Beside acetic acid, a fatty acid eluting with the retention time 2.87 min showed extensively high concentration (Fig. 1.19).

After one week treatment with BES, F420 autofluorescent methanogens were nealy completly eliminated from the hindgut (Table 1.7). The treatment also resulted in the increase of hindgut acetic acid concentration up to 1.6-fold greater than the before treatment (from 92.3 to 152.4 mM; Table 1.9 and Fig. 1.20).

DISCUSSION

Methane emission rate of the termites used in this study depended on the termite species as reported (5). In *N. koshunensis*, however, methane emission rates depended on each colony (discussed below). The methane emission rate of *R. speratus* was comparable to that of *Reticulitermes* species *R. flavipes* (47) and *R. tibilias* (67). On the other hand, methane emission rate of some other termites was inconsistent with the previous reports (6). *C. formosanus* had emission rates up to 500-fold higher than values in a previous report: 0.3 nmol CH_4 hr^{-1} g fresh wt^{-1}, in ref. (6). The fungus-growing termite *O. formosanus* showed smallest methane emission rate in this study. Generally, in fungus-growing termites and soil-feeding termites, methanogenesis is thought to be major H_2 sink in the hindgut. The reason of the difference is unknown, but their diet may be one of the reason. Termites which feed on dead plant matter with a carbon to nitrogen ratio much higher than their own tissues have to balance their C and N inputs. Methanogenesis in termites seems to be major C-eliminating process, which is affected by C/N ratio of diet (25). Indeed, when feeding on cellulose filter paper, rates of methane emission by *R. flavipes* increased from 73.1 to 1340 nmol hr^{-1} per g fresh wt (6). Similar result was obtained for *N. takasagoensis* in this study (Table 1.6). To conclude the contribution of termite to global methane emission, more extensive and detailed studies may be needed.

In *N. koshunensis*, although some of the methanogens were non-endosymbiotic, e.g. freely-swarming or epithelium-adhering methanogens, most of the methanogens were observed to be endosymbiotic to the flagellates. The flagellate associated with methanogen, *Foaina* sp. was occasionally observed to attach on the large cellulolytic flagellate *Stephanonympha* sp.. This association may be explained by the hypothesis proposed by Messer and Lee (41). They studied the H_2 flow in the hindgut ecosystem of the lower termite *Z. angusticollis*, and concluded that large cellulolytic flagellate *Trichonympha* sp. emit H_2 and CO_2, and then methanogen-associating small flagellate *Trichomitopsis* sp.

mainly convert them into methane. This inter-species H_2 transfer may occur between *Stephanonympha* and *Foaina*.

In the species of family Rhinotermitidae, i.e. *C. formosanus*, *R. speratus*, *R. miyatakei*, and *R. flaviceps*, the majorities of methanogens were non-endosymbiotic, which attached on the epithelium of the posterior paunch and anterior colon. In these termites, the non-endosymbiotic methanogens appeared to mainly contribute to the methane production. In *R. speratus*, some of the methanogens were found on filamentous bacteria or bactrial trichomes. The similar observation was reported in *R. flavipes* (35). The relationship between these bacteria and methanogens was little known. However morphotypes of the methanogens observed in the lower termites were almost all short-rods, the observations consistent with the previous reports (38, 41): in the higher termites *N. takasagoensis* and *P. nitobei*, most of the methanogens seemed to be filamentous and situated close to the epithelium of the posterior portion of the paunch and of the anterior portion of the colon. By electronmicroscopic observation of the gut-wall-attaching bacteria of *N. takasagoensis*, the type-1 filamentous bacterium (Fig. 1.14a and b) was identified as *Methanothrix*-morphotype based on the following characteristics (66): cells are rod-shaped with flat ends; forming very long and flexible filaments; non-motile; individual cells held together by a sheath-like structure with regular striations; and cells are separated by multilayered 'cell spacer' plugs. The second type of the bacterium (Fig. 1.15a and b) was identified as *Methanospirillum* morphotype based on the following characteristics (21): cells are curved rods; forming long and wavy filaments; motile by means of polar tufted flagella; individual cells held together by a sheath-like structure with surface striations; and cells are joined by a 'cell spacer'.

In *N. koshunensis*, wide variation in the methane emission rate was observed among colonies, and no methane emission were detected in some of the colonies (Table 1.4). These differences seemed to be colony-dependent, because no methane emission were detected from the individuals collected from colonies with no-methane emission even after

1 month laboratory incubation (Table 1.5). In the hindguts of *N. koshunensis* without methane emission, no autofluorescent methanogens were recognized in the flagellates. No correlation between methane emission and termite wet weight was observed (Fig. 1.18). Because the species live on one piece of wood, the possibility that plant species of their nest wood affect on their methane emissions, were examined. However, the plant species may not responsible to the colony dependent methane emission observed in *N. koshunensis*, since both types of colonies, i.e. with and without methane emission were found in different colonies of the same nest wood species (Table 1.4).

VFAs in termite hindgut have been detailed be examined by Odelson and Breznak (47). They showed that acetic acid is the major component of VFAs in termite hindgut, and suggested that most of the respiratory substrate required by a termite is provided as acetic acid. VFA analysis of *N. koshunensis* also showed that acetic acid was the most abundant VFA in this termite hindgut. This was relatively higher than that reported for other termites (57.6 mM in *Incistermes*; 66.2 mM in *Zootermopsis*; 80.6 mM in *Reticulitermes*, ref. 47). The acetic acid concentration of the termites in no-methane emitting colony was 1.3-fold higher than that of methane emitting ones (Table 1.8). Differences in VFA concentration and composition especially of the longer chain fatty acid with the retention time more than 2.8 min between methane emitting colony and no-methane emitting one suggest that the termites in these colonies possess different composition of microflora each other (Fig. 1.19). Acetic acid in termite hindgut was also produced from H_2 and CO_2 by homoacetogens (8). Indeed, generally, CO_2-reducing acetogenesis is higher than methanogenesis as the H_2 sink in wood feeding termites (4). Messer and Lee (41) have mentioned that it might be useful for the host termite to use H_2 and CO_2 in the hindgut for acetic acid synthesis but not for methane production. If it is true, in the no-methane emitting colonies, H_2 and CO_2 may flow to the acetic acid synthesis instead of methane production. Though more studies are needed to examine the relations between methane emission and VFAs, this hypothesis was supported by the analysis of VFAs in BES

treated termites. The inhibitor increased acetic acid concentration up to 1.6-fold higher than that of control group (Table 1.9). On the other hand, some differences were also observed in VFAs between natural no-methane emitting colony and artificially inhibited no-methane emitting one (Fig. 1.19 and 20). This also suggests that the lack of methanogens from hindgut microflora is not the only reason for the differences. The natural no-methane emitting colonies may establish a unique microflora in the hindgut. However, more studies are needed to explain the methane production mechanism in the hindguts of these termites.

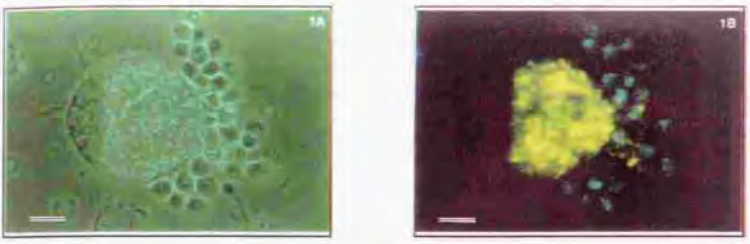


Fig. 1.1. (A) Phase-contrast light micrograph of the flagellate *Stephanonympha* sp. with *Foaina* sp. in the hindgut fluid of *N. koshunensis*. (B) Epifluorescence micrograph of the same field as in (A). Methanogens are visible as blue-green autofluorescent rods associating to *Foaina* sp.. Standard bar represents 15 μm. Yellow fluorescence are emitted from ingested wood particles.

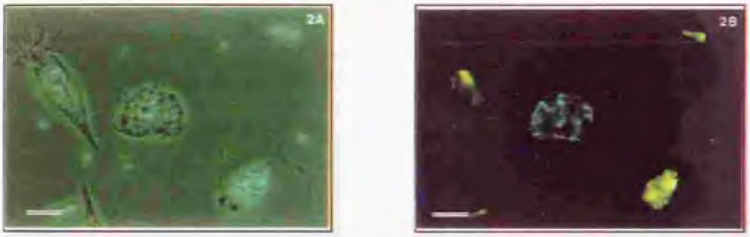


Fig. 1.2. (A) Phase-contrast light micrograph of the flagellate *Trichomitopsis* sp. in the hindgut fluid of *N. koshunensis*. (B) Epifluorescence micrograph of the same field as in (A). Methanogens are visible as blue-green autofluorescent rods associating to *Trichomitopsis* sp. Standard bar represents 15 μm.

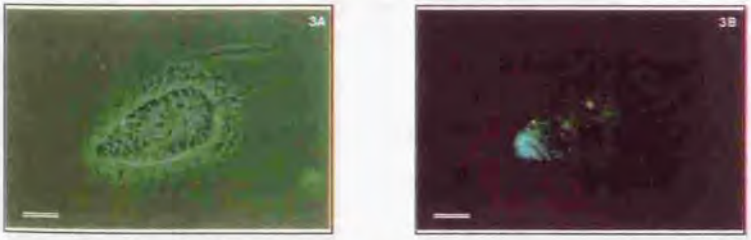
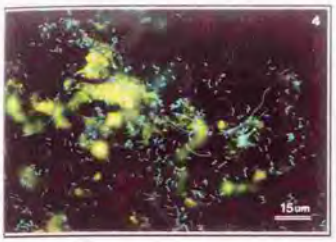


Fig. 1.3. (A) Phase-contrast light micrograph of the flagellate *Spirotrichonympha* sp. in the hindgut fluid of *C. formosanus*. (B) Epifluorescence micrograph of the same field as in (A). Methanogens are visible as blue-green autofluorescent rods associating to *Spirotrichonympha* sp. Standard bar represents 15 μm.



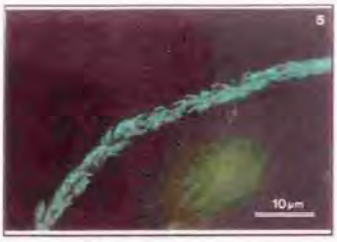
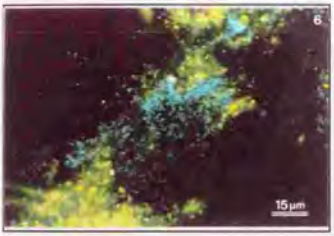


Fig. 1.4. Epifluorescence micrograph of the hindgut epithelium of *R. speratus*. Methanogens are visible as blue-green autofluorescent short-rods attaching on the gut epithelium.
Fig. 1.5. Epifluorescence micrograph of the hindgut epithelium of *R. speratus*. Methanogens are attaching to a bacterial trichome.



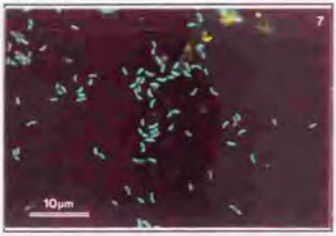
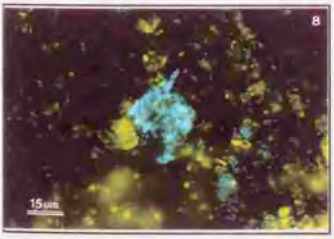


Fig. 1.6. Epifluorescence micrograph of the hindgut epithelium of *R. miyatakei*. Methanogens are visible as blue-green autofluorescent short-rods attaching on the gut epithelium.
Fig. 1.7. Epifluorescence micrograph of methanogens in the hindgut fluid of *R. miyatakei*.



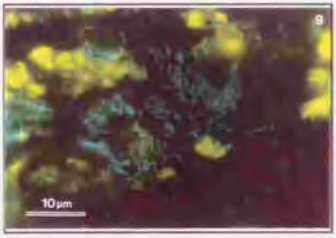
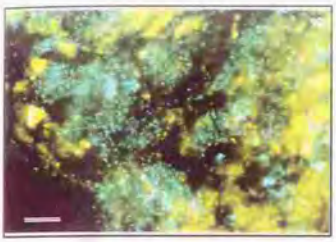


Fig. 1.8. Epifluorescence micrograph of the hindgut epithelium of *R. flaviceps*. Methanogens are visible as blue-green autofluorescent short-rods attaching on the gut epithelium.
Fig. 1.9. Epifluorescence micrograph of the hindgut epithelium of *R. flaviceps*. Methanogens are visible as blue-green autofluorescent short-rods attaching on the gut epithelium.



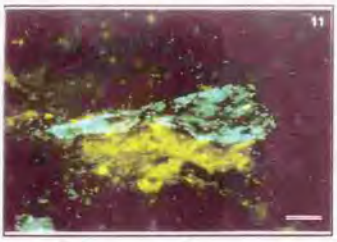


Fig. 1.10. Epifluorescence micrograph of the hindgut epithelium of *C. formosanus*. Methanogens are visible as blue-green autofluorescent short-rods attaching on the gut epithelium. Standard bar represents 15 μm.

Fig. 1.11. Epifluorescence micrograph of the hindgut epithelium of *C. formosanus*. Methanogens are visible as blue-green autofluorescent short-rods attaching on the gut epithelium. Standard bar represents 15 μm.

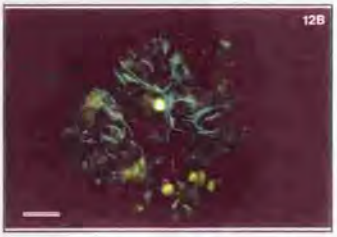


Fig. 1.12. (A) Phase-contrast light micrograph of the hindgut epithelium of *N. takasagoensis*. (B) Epifluorescence micrograph of the same field. Methanogens are visible as blue-green autofluorescence rods attaching on the gut epithelium. Standard bar represents 15μm.

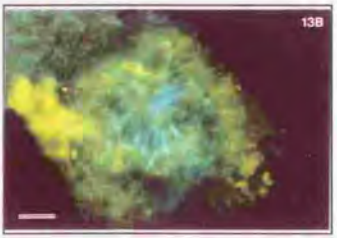


Fig. 1.13. (A) Phase-contrast light micrograph of the hindgut of epithelium of *N. takasagoensis*. (B) Epifluorescence micrograph of the same field. Methanogens are visible as blue-green autofluorescent rods attaching on the gut epithelium. Standard bar represents 15μm.

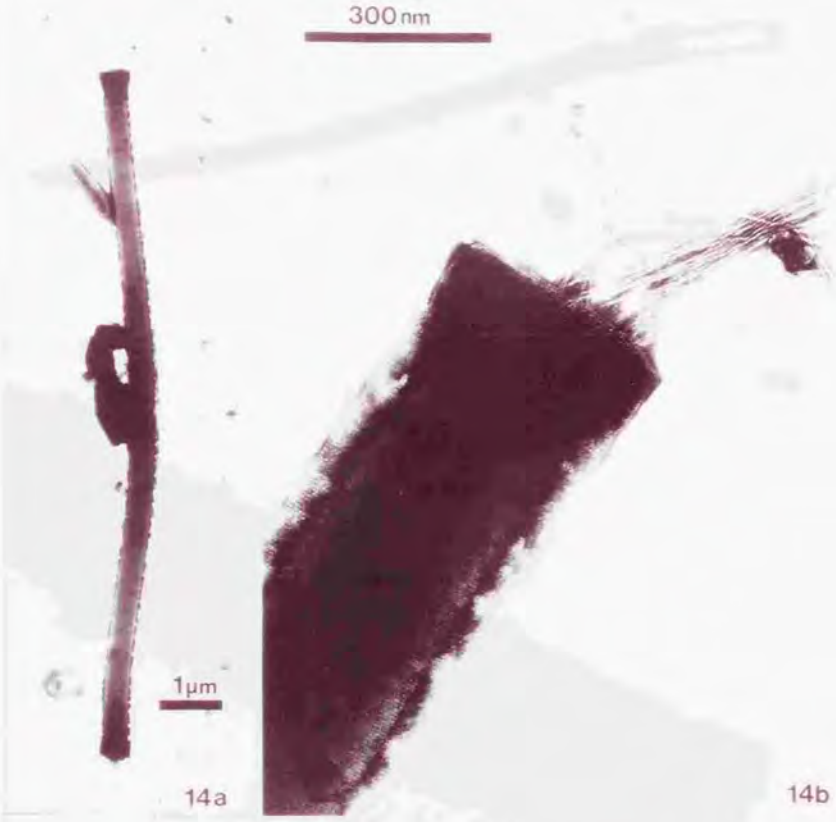


Fig. 1.14a, b. Trasmission electron micrographs of *Methanospirillum*-morphotype methanogens in the hindgut epithelium of *N. takasagoensis*.

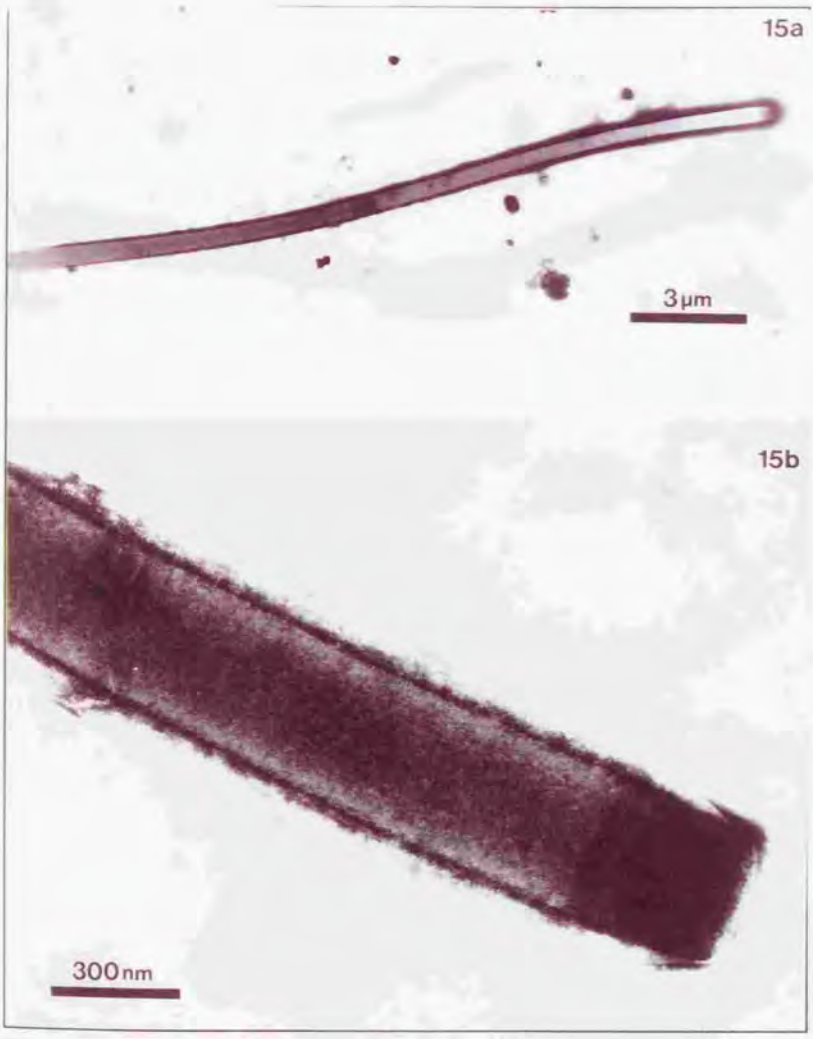


Fig. 1.15a, b. Transmission electron micrographs of *Methanothrix*-morphotype methanogen in the hindgut epithelium of *N. takasagoensis*.

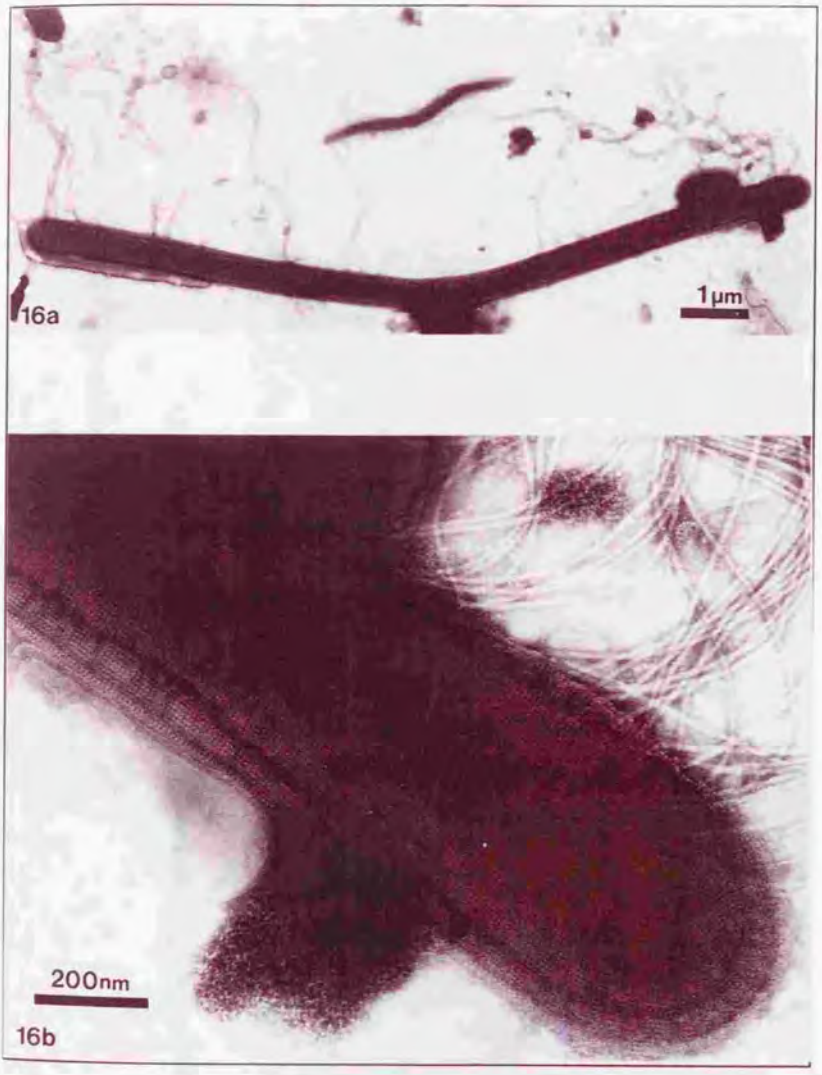


Fig. 1.16a, b. Transmission electron micrographs of filamentous bacteria in the hindgut epithelium of *N. takasagoensis*. Characteristics of methanogens were observed.

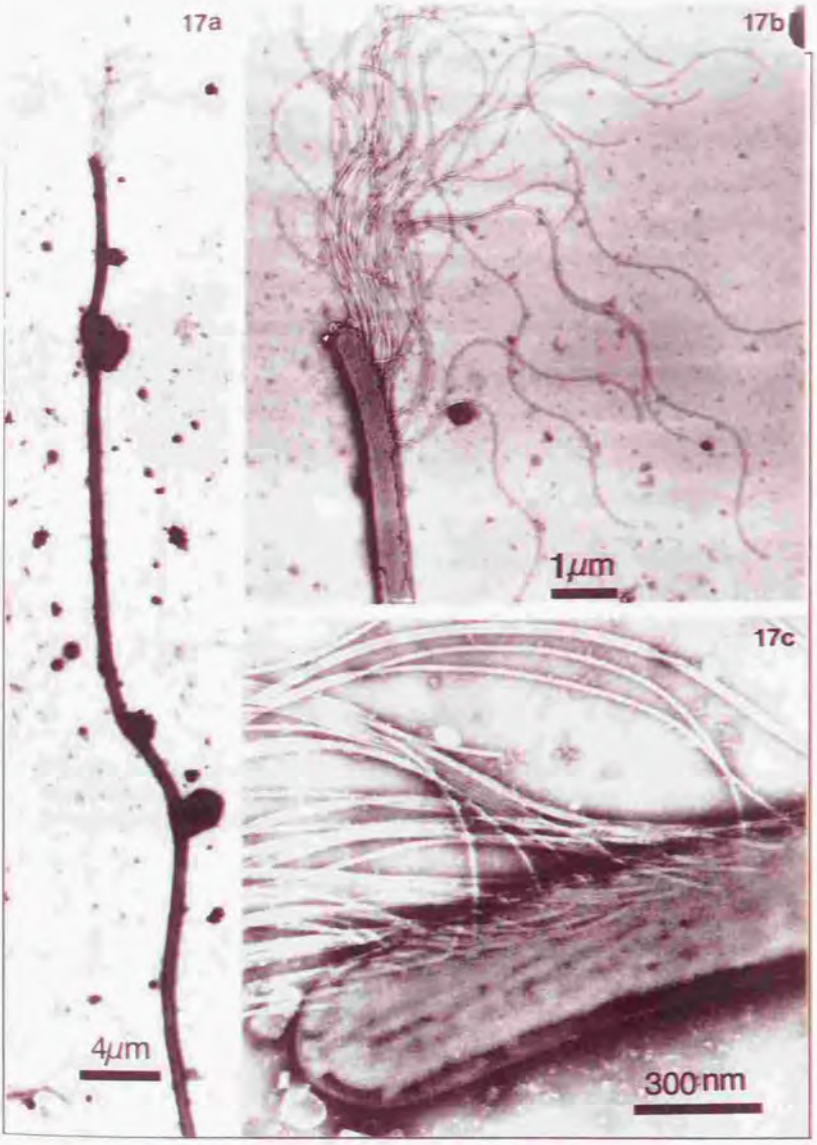


Fig. 1.17a, b, c. Transmission electron micrographs of filametous bacteria in the hindgut epithelium of *N. takasagoensis*. Characteristics of methanogens were observed.

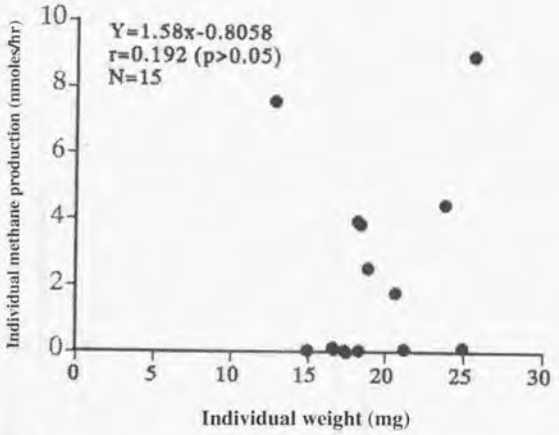


Fig. 1.18. Scatterplot of methane emission vs individual weight of *N. koshunensis*. Correlation factor of r = 0.192 was obtained from the plot for 15 individuals indicating no significant correlation between individual weight and methane.

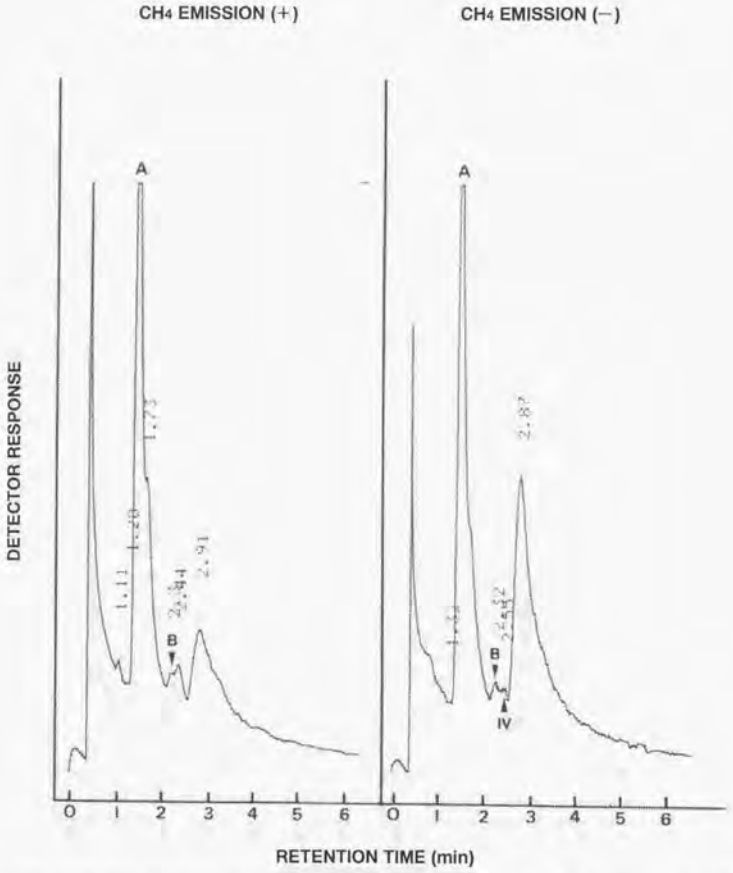


Fig. 1.19. Gas chromatograms of samples of hindgut fluid from *N. koshunensis* with or without methane emission. Symbols: A, acetic acid; B, butyric acid; IV, iso-valeric acid.

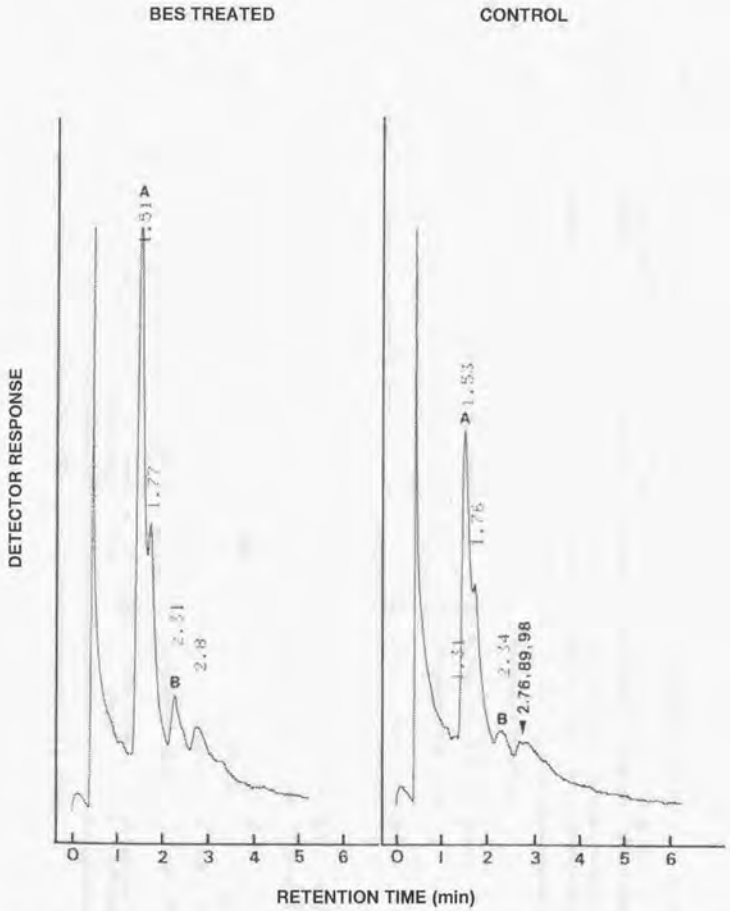


Fig. 1.20. Gas chromatograms of samples of hindgut fluid from *N. koshunensis* which was treated with BES for 7 days. Symbols: A, acetic acid; B, butyric acid.

Table 1.1. List of termites used in this study and brief notes on their life styles

Family	Subfamily	Species	Location	Methanogenesis	Microscopy	Life style	Food and Habitat
Kalotermitidae	Kalotermitinae	*Neotermes koshunensis*	Okinawa Is. Ishigaki Is.	examined	examined	One piece	Soft-wood. Dead portion of living trees, stumps and fallen trees.
Termopsidae	Termopsinae	*Hodotermopaia japonica*	Amami-oshima Is.	examined	examined	One piece	Damp-wood. Stumps and fallen trees.
Rhinotermitidae	Rhinotermitinae	*Reticulitermes speratus*	Okinawa Is.	examined	examined	Intermediate	Euryphagous, Acerose tree. Decayed wood in contact with damp soil.
		Reticulitermes miyatakei	Amami-oshima Is.	not examined	examined	Intermediate	Euryphagous, Acerose tree. Decayed wood in contact with damp soil.
		Reticulitermes flaviceps	Amami-oshima Is.	not examined	examined	Intermediate	Euryphagous, Acerose tree. Decayed wood in contact with damp soil.
		Coptotermes formosanus	Okinawa Is.	examined	examined	Intermediate	Euryphagous, Acerose tree. Subterranean carton nests or wood-work of ground.
Termitidae	Macrotermitinae	*Odontotermes formosanus*	Okinawa Is.	examined	examined	Separate	Euryphagous, Fungi-growing. Subterranean fungus comb.
	Nasutitermitinae	*Nasutitermes takasagoensis*	Iriomote Is.	examined	examined	Separate	Euryphagous, Debris or grass. Arboreal carton nests
	Termitinae	*Pericapritermes nitobei*	Iriomote Is.	not examined	examined	Separate	Euryphagous, Debris or grass. Subterranean nests.

Table 1.2. Methane emission rate of live termites

Termite species	Fresh weight (mg/individual)	CH_4 emission in (nmol/individual/hr)	CH_4 emission in (nmol/g fresh wt/hr)
N. koshunensis	19.6 ± 0.5	2.19 ± 2.95	117.6 ± 169.1
H. japonica	54.1 ± 2.3	34.90 ± 1.57	645.2 ± 6.1
R. speratus	1.8 ± 0.1	0.38 ± 0.05	213.9 ± 34.5
C. formosanus	3.8 ± 0.2	0.50 ± 0.07	133.3 ± 22.7
O. formosanus	5.1 ± 0.6	0.20 ± 0.03	39.9 ± 3.8
N. takasagoensis	5.7 ± 0.9	0.49 ± 0.20	87.3 ± 33.2

Table 1.3. Symbiotic flagellates associated with methanogens

Termite species	Flagellate associated with methanogen	Occurrence of free-living methanogen	Morphotype of free-living methanogen
N. koshunensis	*Trichomitopsis* sp.	+	short-rod
	Devescovinia sp.		
	Foaina sp.		
R. speratus	*Microjoenia* sp.	+	short-rod and filamentous (rarely)
R. miyatakei	none	+	short-rod
R. flaviceps	none	+	short-rod
C. formosanus	*Spirotrichonympha* sp.	+	short-rod
O. formosanus	—	+	short-rod
N. takasagoensis	—	+	filamentous and short-rod
P. nitobei	—	+	filamentous

Table 1.4. Methane production and nest wood plants of *N. koshunensis*

Colony No.	Plant species	CH_4 emission in (nmol/individual/hr)	Methanogen
1	*Rhus succedanea*	N. D.	−
2		<0.2	−
3		4.4 ± 0.4	+
4		<0.2	−
5		N. E.	−
6	*Cinnamomum japonicum*	1.8 ± 0.3	+
7		N. D.	−
8		N. E.	+
9		N. E.	+
10	*Casuarina equisetifolia*	3.8 ± 0.4	+
11	*Bischofia javanica*	8.9 ± 0.4	+
12		3.9 ± 0.2	+
13	*Michelia compressa* var. *formosana*	2.5 ± 0.6	+
14	*Wendlandia formosana*	7.5 ± 0.7	+
15	*Glochidion zeylanicum*	N. D.	−
16	*Castanopsis sieboldii* var. *lutchuensis*	N. D.	−
17	*Symplocos prunifolia*	N. D.	−
18	*Schefflera octophylla*	N. D.	−
19	*Ficus virgata*	N. E.	−

N. D. : Not detected

N. E. : Not examined

Table 1.5. Methane emission rates of *N. koshunensis* after laboratory incubation for a month

Colony	Diet	Methane emission (nmol/individual/hr)	
		Before incubation	After incubation
1	Filter paper	N. D.	N. D.
2	Filter paper	3.82 ± 0.39	4.04 ± 0.33

Table 1.6. Effect of diet on methane emission of *N. koshunensis*

Diet	Time	fresh wt (mg/individual)	CH_4 emission in (nmol/individual/hr)
Wood (nest)	—	5.5 ± 0.1	0.38 ± 0.05
Filter paper	1 month	5.7 ± 0.4	0.86 ± 0.19

Table 1.7. Effect of BES treatment on methane emission of *N. koshunensis* and *N. takasagoensis*

Addition	*N. koshunensis*		*N. takasagoensis*	
	methane emission (nmol/individual/hr)	Methanogen	methane emission (nmol/individual/hr)	Methanogen
None	3.91 ± 0.21	+	1.10 ± 0.06	+
BES	0.14 ± 0.02	—	0.09 ± 0.01	—

Table 1.8. Extracellular pool size of acetic acid in the hindgut fluid of *N. koshunensis* with or without methane emission

Methane emission	Acetic acid (mM)
Detected	178.4
Not detected	233.3

Table 1.9. The effect of BES treatment on acetic acid concentration in the hindgut fluid of *N. koshunensis*

Treatment	Methanogenesis	Acetic acid (mM)
None	+	92.3
+ BES	−	152.4

PART II

MOLECULAR PHYLOGENY AND *IN SITU* LOCALIZATION OF METHANOGENIC SYMBIONTS LIVING IN HINDGUT OF THE LOWER TERMITE *RETICLITERMES SPERUTUS*

ABSTRACT

Small-subunit ribosomal RNA (SSU rRNA) genes of methanogens living in the lower termite *Reticulitermes speratus* (Kolbe) collected in Japan were amplified from the DNA extracted from the termite hindguts. They were cloned, and their sequences were determined.

Phylogenetic analysis of the sequences of 60 clones revealed that most of them (56 clones) clustered in the genus *Methanobrevibacter* in the order Methanobacteriales with some divergence. Another clone showed a sequence related to *Methanocorpusculum parvum* in the order Methanomicrobiales. The remaining three clones were classified in the order Thermoplasmales but were distinct from known members in this order.

Fluorescence *in situ* hybridization analysis using oligonucleotide probes designed to specifically detect the cloned sequences showed that members of *Methanobrevibacter* are present in the hindgut probably on the gut epithelium.

INTRODUCTION

Symbiotic methanogens of some lower termites are present in two different niches in termite hindgut: endosymbiotic methanogens are associating with some species of flagellates and non-endosymbiotic ones are adhering to hindgut surface (38, 57).

Recently, two novel species of methanogens *Methanobrevibacter cuticularis* and *Methanobrevibacter curvatus* were isolated from the American lower termite *Reticulitermes flavipes*. These methanogens were observed to colonize on the epithelial surface of the hindgut instead of to associate with flagellates as reported so far (35).

On the other hand, phylogenetic study of the flagellate associated methanogens in lower termites has not been made so far. The endosymbiotic methanogens associated with flagellated protozoa have been reported from some lower termites, which were thought to be major agent of methanogenesis in these termite hindgut (38, 41, 57). Methanogen associated flagellate *Trichomitopsis termopsidis* has been cultured from the lower termite *Zootermopsis angusticollis* (65). The cultured flagellate showed methane emission in the medium, but their methanogenic symbionts could not be detected with epifluorescence microscopy. In addition, Odelson and Breznak (46) have reported that the endosymbiotic methanogens were failed to be cultivated from the culture of *T. termopsidis*. These results may indicate some physiological adaptation derived from their mutualistic relationships.

Though no flagellate associated with methanogens has been reported in *R. flavipes*, in the same *Reticulitermes* species *R. speratus* in Japan Archipelago, some flagellate associated with methanogens were observed in the previous observation (Part I). In the present investigation, the symbiotic methanogen community in *R. speratus* which possessed flagellates associated with methanogen was phylogenetically surveyed by PCR analysis of SSU rRNA genes and examined their in situ localization by fluorescence *in situ* hybridization (FISH) analysis.

MATERIALS AND METHODS

Termites

Six colonies of the lower termite *Reticulitermes speratus* (Kolbe) (order Isoptera, family Rhinotermitidae) were collected with their nest wood from forests in Tokyo, Kobe, Yamaguchi and Okinawa Is. in Japan (Fig. 2.1). One colony was collected from each sampling point except Tokyo area, where three colonies were sampled. The termites were maintained in the laboratory at 25°C until use. The contents of termite containers were periodically moistened by spraying the surface of infested wood with distilled water.

DNA extraction

Ten individuals of pseudergates (so-called workers) from each colony were employed for the total DNA extraction. After surface sterilization with 70% ethanol, digestive tracts of the termites were pulled out with a pair of fine-tipped forceps. The gut tissues and the contents were crushed with a Teflon homogenizer in 1 ml of extraction buffer consisting of 100 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.1% SDS. The mixture was frozen in liquid nitrogen and thawed at 57°C five times, and then treated with 0.5 mg ml^{-1} proteinase K (Nakarai tesque, Japan) at 57°C for 16 hr. The solution was extracted with phenol and chloroform several times and the DNA was precipitated with ethanol according to the standard protocol (52). The DNA was electrophoresed on 0.8% agarose gel to remove inhibitory elements for PCR. High molecular weight genomic DNA was excised from the gel and recovered with a Gene Clean II kit (BIO 101 Inc., USA).

PCR amplification

PCR primers were designed to amplify a part of archaebacterial SSU rRNA gene (about 0.5 k base pairs). The primers used were ME855F (5'-TTAAAGGAATTGGCGG GGGA-3') and ME1354R (5'-TGACGGGCGGTGTGTGCAAG-3'), the numbers

represent nucleotide positions in the counterpart of *Escherichia coli*. To facilitate the addition of adenine at the 3'-end in the PCR reaction, 5'-end of the primers were designed to be thymine.

PCR reaction was performed in a final volume of 50 µl. Each reaction mixture contained 1 µl of template DNA solution, 5 µl of 10 x PCR buffer (100 mM Tris-HCl, 500 mM KCl, 25 mM $MgCl_2$, 1% TritonX-100, pH9.0), 0.1 mg ml^{-1} of BSA, 1 µl of dNTPs mixture (50 µM each), 20 pmol each of the primers, 1 unit of Taq DNA polymerase (Toyobo, Japan) and sterile Millipore water to make it 50 µl. Reaction was performed in a thermal cycler, GeneAmp 2400 (Perkin-Elmer) with the thermal program which comprised 40 cycles of denaturation at 94°C for 30 sec, primer annealing at 60°C for 30 sec, and primer extension at 72°C for 90 sec, followed by an additional extension at 72°C for 5 min. The PCR products were analyzed by electrophoresis on 1.5% agarose gel. The band of about 0.5 kbp was cut out from the gel and purified with a Gene Clean II kit.

Cloning and sequencing of amplified DNA

The amplified DNA fragments were ligated with a ddT-tailed vector as described by Holton and Graham (26). Plasmid DNA of pBluescript II SK$^+$ (Toyobo, Japan) was digested with *Eco*RV, and the blunt-ended vector was tailed with dideoxythymidine triphosphate using terminal nucleotidyl transferase kit (Boehringer Mannheim). The PCR products were ligated into the plasmid vector with a Ligation kit version 2 (Takara, Japan) and the *E. coli* strain JM105 was transformed with the ligate. The cloned fragments were sub-cloned into M13 mp18 and 19 phage vectors. The nucleotide sequences were determined by the dideoxynucleotide chain termination method (53) using T7 DNA polymerase and fluorescent labeled universal primers on an A. L. F. DNA Sequencer II

(Pharmacia Biotech).

Phylogenetic analysis

The sequences were examined to test the possibility of chimeric artifacts with CHECK-CHIMERA program in the Ribosomal database project (39). The sequences were aligned with previously reported SSU rDNA sequences from the nucleotide sequence databases GenBank, EMBL, and RDP by using the program package CLUSTAL W (59). To obtain the reliable phylogenetic relationships, only the nucleotide positions unambiguously aligned were used for the following analysis. The programs in the software package PHYLIP version 3.572 (J. Felsenstein and the University of Washington) were employed for the phylogenetic analysis. Phylogenetic trees were constructed from neighbor-joining distance matrix methods (51). Numbers of nucleotide substitution were calculated according to Kimura's two-parameter method (31), taking transition and transversion rates into account. Substitution rates were analyzed by neighbor-joining method to construct phylogenetic tree. Bootstrap confidence interval (20) on each branching pattern was calculated from 1000 repetitions of resampling. The aligned sequences were also analyzed by maximum-parsimony and maximum-likelihood methods to check the tree topology.

Enrichment cultivation of methanogens

Solution U of Trager (60) was modified and employed for enrichment cultivation of methanogen. The medium contained 37 mM NaCl; 18.7 mM $NaHCO_3$; 5.1 mM Sodium Citrate; 13.1 mM KH_2PO_4; 0.75 mM $CaCl_2$; 0.4 mM $MgSO_4$, 0.25% sodium acetate, 0.25% sodium formate, 0.2% yeast extract (Difco), 0.2% trypticase (Difco), 1 mg ml^{-1} resazurin, and 3.2 mM glutathione (reduced form). The antibiotics, vancomycin and kanamycin, 1 mg ml^{-1} each, were supplemented to the medium to repress the growth of eubacteria. Five ml each of the medium without $NaHCO_3$, $CaCl_2$ and the antibiotics was

dispensed into each 18 x 180 mm tube with butyl rubber stopper (Sanshin industrial Co., Ltd., Japan). Oxygen in the medium and headspace of the tubes was removed by degassing with a vacuum pump, and the head space was filled with H_2 and CO_2 mixed gas (4:1) with a high-pressure gassing manifold (1). The $NaHCO_3$, $CaCl_2$ and the antibiotics were added to the autoclaved medium from sterile stock solutions. The pH was adjusted to 7.0 with NaOH solution prior to inoculation.

A termite digestive tract placed in a drop of the medium on a sterile petri-dish was pierced with a needle equipped with a syringe, then issued contents from the hindgut were quickly aspirated into the syringe and inoculated into the medium. The inoculated tubes were incubated vertically at 30°C without shaking.

Probe construction

Oligonucleotide probes were designed to bind to the SSU rRNAs of the methanogens detected in this study at the nucleotide positions from 1,109 to 1,129 and from 1,174 to 1,196 (Fig. 2.4). Two archaea-specific probes, ARC344 5'-GCGCCTGCTGCGCCCGT-3' and ARC915 5'-GTGCTCCCCGCCAATTCCT-3' described by Stahl et al. (58) were used as positive control. The clone type-specific probes were labeled with tetramethylrhodamine isothiocyanate (TRITC, Sigma), and archaea-specific ones were conjugated with fluorescein isothiocyanate (FITC, Sigma). Oligonucleotide probes were labeled with fluorescent dyes as described by Delong et al. (14). 200 µg of oligonucleotide with an amino group at the 5'-terminal was reacted with 200 µg of dye in 0.5 M carbonate buffer (pH 9.0) at room temperature for 16 hr. The oligonucleotide was separated from unreacted dye by passing the solution through a Sephadex G-50 column, and was electrophoresed on 20% acrylamide gel under undenatured conditions to separate from unlabeled one. Probes were recovered from the gel and desalted by Sephadex G-50 column chromatography. The eluate was dried in vacuum and dissolved in autoclaved deionized water.

In situ hybridization

The worker of the members of colony RS1 sampled from Tokyo area was used in this analysis. Samples were prepared basically according to Stahl et al. (58). The sample of symbiotic flagellates and methanogenic bacteria present in the hindgut were prepared as follows. A digestive tracts separated from the termite body was dissected into small pieces, and they were immersed in 1 ml of PBS. The hindgut contents including symbiotic flagellates were released by gentl shaking with a forceps and the washed hindgut pieces were transferred into a new 1.5 ml test tube containing 1 ml of PBS. The symbiotic flagellates released in the PBS were collected by centrifugation at 500 rpm for 1 min and fixed in 1% paraformaldehyde in PBS at 4°C for 30 min. The test tube containing the washed hindgut pieces was vigorously shaken to peel the methanogens off the gut epithelium. The hindgut contents including peeled methanogens were collected by centrifugation at 16,000 rpm for 10 min and fixed in 1% paraformaldehyde in PBS at 4°C for 30 min. These samples of symbiotic flagellates and released methanogens were washed with PBS twice and suspended in PBS. Equal volume of 100% ethanol was added to the suspension. The samples were attached to Teflon-coated slide glasses (Cell-Line, USA) by drying in the air. The slides were pre-coated with gelatin (Type 20, Sigma). The slides were dehydrated in a graded ethanol series for 3 min each in 50, 80, and 100% ethanol. To improve the accessibility of the probes to target rRNA, the slides were treated with proteinase K (1 μg ml^{-1}) in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 37°C for 20 min, and then in 25 mM acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 10 min, to decrease non-specific probe binding. The slides were dehydrated with the ethanol series again. Eight μl of hybridization buffer and 50 ng of each fluorescent oligonucleotide probe were applied to each slide, and the slides were incubated in a moisture chamber at 45°C for 3 hr. The hybridization buffer consisted of 0.9 M NaCl, 50 mM sodium phosphate (pH 7.0), 5 mM EDTA, 0.1 % SDS, 0.5 mg

ml^{-1} poly (A), and 10 x Denhardt's solution (16). The hybridization mixture was removed by flushing the slides with washing buffer which consist of 20% formamide, 2 x SSC (0.3 M NaCl, 30 mM sodium citrate, pH7.0), and 0.1 % SDS. The slides were immersed in 20 ml of the washing buffer at hybridization temperature for 20 min. Then the slides were rinsed with distilled water and dried in the air. The samples were mounted in 50% glycerol in 0.9 M NaCl and sealed with clear nail manicure.

Fluorescence detection and photomicrography

An Olympus model BX60 microscope equipped with a BX-FLA epifluorescence microscopic system and a filter set U-MWBV was used for detection of F420 autofluorescence. A laser scanning confocal imaging system model MRC-600 (BIO-RAD) with a krypton-argon mixed gas laser generator was used for fluorescence in situ hybridization analysis. FITC and TRITC double stained samples were scanned at two wave lengths simultaneously to prevent the decrease of the signals caused by repetitious exposure and to minimize the focus shearing. Photomicrographs were taken with Ektachrome 400 (Kodak) by using a film recorder model FR-2000 (Nippon Avionics, Japan) attached to the microscope.

RESULTS

Phylogeny of symbiotic methanogens of R. speratus

High molecular weight DNA prepared from the termite hindguts was used as the template. A part of SSU rRNA gene sequence was amplified by PCR and cloned into *E. coli*. Ten clones were isolated for each colony of the termite and the sequences of 60 clones were determined in total. Clones which had evolutionary distances within 0.02 substitutions (sequence identity >98%) were grouped together. As the result, the clones were classified into 5 types: type-1A, 1B, 1C, 1D, type-2, and type-3 (Table 2.1). The sequences of the clones were aligned with previously reported SSU rDNA sequences of selected species in *Euryarchaeota*, and 505 unambiguously aligned positions were used in the phylogenetic analysis.

The phylogenetic tree shown in Fig. 2.2 was constructed by the neighbor-joining method. The topology of the tree was consistent with those derived from maximum-likelihood and maximum-parsimony methods (not shown). Type-1, 2, and 3 were separated from each other more than 0.1 evolutionary distances. Validity of the clustering was also confirmed by the high bootstrap values (>92%, 100 repetitions). The clones belonging to type-1A to 1D made a cluster in the order Methanobacteriales.

Detailed phylogenetic relationships among the sub-types of type-1 and the members of the genus *Methanobrevibacter* were analyzed separately (Fig. 2.3). Leadbetter and Breznak (35) have recently isolated *M. cuticularis* and *M. curvatus* from *R. flavipes*. Ohkuma et al. (49) have reported the amplification and cloning of SSU rDNA sequence of methanogens from *R. speratus*. These sequences were included in the analysis. The sequence of the clone M4 amplified from *R. speratus* reported by Ohkuma et al. was found to be type-1B. Absolute and mean distance matrix among the members of Methanobrevibacteriaceae was shown in Table 2.2. In Fig. 2.3, type-1A, 1B, and 1C form a monophyletic group. The tree topology was similar to those derived from the other

methods (maximum-parsimony and maximum-likelihood).

The type-2 clone fell in the order Methanomicrobiales (Fig. 2.2). This clone was closely related to *Methanocorpusculum parvum* with 95.3% sequence identity. The clustering of the clone and *M. parvum* was strongly supported by bootstrap value of 100%. *M. labreanum* str. Z (C. R. Woese, unpublished) that was completely identical to *M. parvum* in the compared sequence positions (910-1,407, *E. coli* numbering) excluding unknown bases, was also the closest neighbor of the type-2 clone. The SSU rDNA sequences of the other known species in the genus could not be found in the databases (GenBank, EMBL, DDBJ, and RDP). The second closest neighbor was the environmental PCR clone CY-46 which was detected from cyanide-degrading consortium isolated from anaerobic waste treatment reactor (9), and the similarity between CY-46 and type-2 clone was 94.9%.

In the present study, three clones of type-3 were obtained from *R. speratus* hindgut microflora by using the primer pair designed to amplify SSU rRNA gene of archaebacteria. The closest neighbors of these clones were *Thermoplasma*-relatives; *Picrophilus oshimae*, with 82.5% identity, and the second closest one was *Thermoplasma acidophilum* showing the similarity of 81.3%. Type-3 clones formed a monophyletic clade with species of Thermoplasmales, which was supported by relatively high boot strap value (90%). Many sequence which are related to *Thermoplasma* have been amplified from various environmental samples and reported (13, 15, 23). Although, these sequences could not be used in the phylogenetic analysis because of the difference of the region determined.

Enrichment cultivation of methanogenic symbionts

Fig. 2.5A shows the fluorescence micrograph of the epithelium of the termite hindgut. There are at least three morphologically different types of autofluorescent microbes on the epithelium: curved short-rods, straight short-rods and filamentous-rods. The hindgut

contents contained free fluorescent cells, curved short-rods and straight short-rods, as well as flagellates with endosymbiotic fluorescent cells when observed by F420 epifluorescence microscopy (Fig. 2.5B and C). Medium was inoculated with the contents of digestive tracts of the termite and incubated at 30°C. After 2 months incubation, cell density of the culture reached about 3.3×10^8 cell per ml. The culture is dominated by fluorescent cells of slightly curved-rods and straight short-rods (Fig. 2.6A). The morphologies were similar to some of the microbes observed on termite hindgut epithelium.

In situ detection of symbiotic methanogens by FISH

We performed whole-cell hybridization with fluorescent oligonucleotide probes to confirm that the amplified and cloned sequences were actually originated from the methanogens living in the hindgut. The hindgut contents of the termite were hybridized with the probes specific to archaea labeled with fluorescein and those specific to cloned sequences labeled with rhodamine. They were inspected with a confocal fluorescence microscope (Fig. 2.7).

Three panels in Fig. 2.7 show prokaryotic cells hybridized with type-1 specific probes. Though wood particles present in the sample emitted fluorescence with a wide spectrum, prokaryotic cells could be morphologically distinguished from the particles as pointed by arrows. The cells hybridized with the archaea-specific probes were all straight-rods, and they frequently existed as two cells attached each other. These morphological features of the cells are consistent with some of observed on the epithelial surface of the hindgut (Fig. 2.5A) and cultured methanogens (Fig. 2.6A). The cells hybridized with the archaea-specific probes were also hybridized with type-1-specific probes (1109TY1 and 1174TY1), whereas type-2 (1109TY2 and 1174TY2, Fig. 2.7D-F) and type-3 (1109TY3 and 1174TY3, results not shown) failed to hybridize with the archaea present in the hindgut. These type-specific probes differ from one another in three to five nucleotide

positions as shown in Fig. 2.4. The results showed that the *in situ* hybridization was performed with sufficient strigency to distinguish the differences in the SSU rRNA sequences.

R. speratus used in this experiment harbored 5 species of flagellates associated with methanogens. Particularly, in 3 species out of 5, *Pyrosonympha* (*Dinenympha*) *parva*, *P. rugosa*, and *P. leidyi*, which belong to the order Oxymonadida, were frequently observed to harbor endosymbiotic methanogens (Fig. 2.5B, C, and D). Identification of the flagellates were done based on the morphological features described by Koidzumi (32). The endosymbiotic cells were detected by the hybridization with archaea-specific probes (Fig. 2.7G, H, and I). However, no type-specific probes hybridized with these endosymbiotic prokaryotes in the flagellates.

The cells of the enrichment culture were also analyzed with these probes. Most of the cells were hybridized with archaea-specific probes and also with type-1 specific probes (Fig. 2.6B, and C). Whereas, the other type-specific probes did not hybridize with the cells. Accordingly, most of the cultured archaea are methanogens of type-1.

DISCUSSION

The SSU rRNA gene sequences of symbiotic methanogens of *R. speratus* were analyzed. The most of the cloned sequences were in the genus *Methanobrevibacter*. *Methanobrevibacter*-related methanogens were detected from all colonies used in this study. *Methanobrevibacter*-related methanogens seem to be most abundant in *R. speratus*. The methanogens previously isolated from *R. flavipes*, i.e., *M. cuticularis* and *M. curvatus* are also in the genus *Methanobrevibacter* (35). *Methanobrevibacter*-related methanogens may be the major phylum of symbiotic methanogens in termites.

Ohkuma et al.(49) have previously analyzed the symbiotic methanogens in *R. speratus* hindgut and found only one type of the sequences in *R. speratus*. On the contrary I obtained rather diverse sequences within the order of Methanobacteriales. Previously, Leadbetter and Breznak (35) have isolated two species, *M. curvatus* and *M. cuticularis*, from *R. flavipes*. Their results are compatible with those of this study. In the early stage of the experiments, only one type of the clones in type-1D were isolated from *R. speratus* (data not shown). These sequences were amplified using a pair of primers different from those used in this work. The primer sequence seems to be an important factor for studying the diversity of microorganism.

In the neighbor-joining tree shown in Fig. 2.3, *M. ruminantium* is the earliest branching species in the genus and the other known species forms an independent cluster. The branching order of the tree is different from the maximum-likelihood tree constructed by Leadbetter and Breznak (35). In their maximum-likelihood tree, *M. cuticularis* clustered with *M. arboriphilicus* and it formed a monophyletic clade with the sister group consisted of *M. ruminantium* and *M. smithii*, whereas *M. curvatus* formed a deep branch separated from the other species of *Methanobrevibacter*. Although, tree topology was similar to that constructed by neighbor-joining method by those authors. The phylogenetic trees in this study constructed by the methods other than neighbor-joining, e.g.

maximum-likelihood and maximum-parsimony, were identical in their topologies to that shown in Fig. 2.3.

Judging from the sequence analysis, the symbiont type-3 seemed to belong to the order Thermoplasmales. The possibility of chimeric artifact of these clones were evaluated by CHECK-CHIMERA program on the RDP. The highest similarity values were 0.450 and 0.547 against *Thermoplasma acidophilum* (M20822, GenBank) and *Methanolobus tindarius* (M59135, GenBank), respectively. Accordingly, type-3 clones are not likely to be chimelic. The archaebacterial signature sequences of SSU rRNA arranged by C. R. Woese (62), i.e. 25 base positions in the sequence from 912 to 1,393 base positions in *E. coli* numbering, were conserved in the clones of type-3 except for one C-U transition at the base position 1,086. These results suggest that the symbiont type-3 is a *Thermoplasma*-related novel archaeon. Known *Thermoplasma* species grow in acidic and hot environment. Symbiont type-3 must be a neutrophilic and mesophilic archaeon, because physical conditions of *Reticulitermes* hindgut are nearly neutral in pH (44) and of course at ambient temperature.

So far, only one clone of type-2 have been obtained. No positive signal could be detected with type-2 specific probes. Significance of the type-2 species in termite hindgut is going to be elucidated.

The endosymbiotic associations between methanogens and flagellated protozoa have been reported in some lower termites based on epifluorescence microscopic observations (38, 57). *R. speratus* also possessed several species of flagellates associated with fluorescent cells. Particularly the 3 species of the order Oxymonadida, *P. leidyi*, *P. rugosa*, and *P. parva* were frequently observed to be associated with fluorescent microbes (Fig. 2.5B, C, and D, respectively). However, these observations were inconsistent with the previous reports on *R. speratus* sampled in the Ryukyu Archipelago (57). The FISH analysis revealed that the endosymbiotic microbes in the flagellates were hybridized with the archaea-specific probes, but were not hybridized with any type-specific probes (Fig.

2.7G, H, I, and data not shown). On the contrary, both the methanogens in the hindgut content (Fig. 2.7B) and in the culture (Fig. 2.6C) were hybridized with type-1 specific probes. It suggests that the methanogens which found in the hindgut fluid and endosymbiotic ones which associate with flagellates are phylogenetically different. However, there are several other possibilities at the moment. The chromosomal DNA of the endosymbiotic archaea could not be isolated by the method used in this report. The sequence of the endosymbiont could not be amplified by the primer pairs used. Possibly as the consequence, the probe and/or the conditions used for hybridization was not suitable for detecting endosymbiont. Characterization of the fluorescent cells of archaea present in flagellates remain to be elucidated.

Although I have tried the hybridization with gut epithelium, the positive signal could not be detected with any of the probes used in this work. Mainly because of the strong interfering fluorescence from the sample, it was difficult to distinguish the probe fluorescence from non-specific one. Accordingly, the cells released from the gut were used for the hybridization analysis. There were at least three types of autofluorescent microbes on the gut epithelium judging from their morphology (Fig. 2.5A). Two types, i.e., straight short-rods and curved short-rods, could by released from the gut epithelium. Only straight short-rods detected in the gut fluid sample by the hybridization analysis (Fig. 2.6C). The straight short-rod cells detected by the type-1 probe is likely to be the microbes observed on the gut epithelium. Phylogeny of the fluorescent cells of curved-rods or filamentous-rods found on the gut epithelium is not clear yet.

The cloned sequences of type-1, type-2 and type-3 were obtained from the sample of RS1, which is used for the hybridization experiments. However, no hybridization signal could not be detected with type-2 and type-3 probes. The fluorescent cells found on the gut epithelium with curved-rods or filamentous-rods may be these species. Alternatively, the conditions used for the hybridization analysis may be unsuitable for these species.

In summary, SSU rRNA gene analyses showed that methanogen community in the

hindgut of *R. speratus* consisted mainly of *Methanobrevibacter*-related methanogens, which had some divergence. And FISH analyses revealed that the *Methanobrevibacter*-related methanogens are in the hindgut probably on the epithelium.

Fig. 2.1. Collection of the colonies of *R. speratus* in the Japan Archipelago. Solid triangles indicate sampling points of the study. The scale bar indicates 400 km.

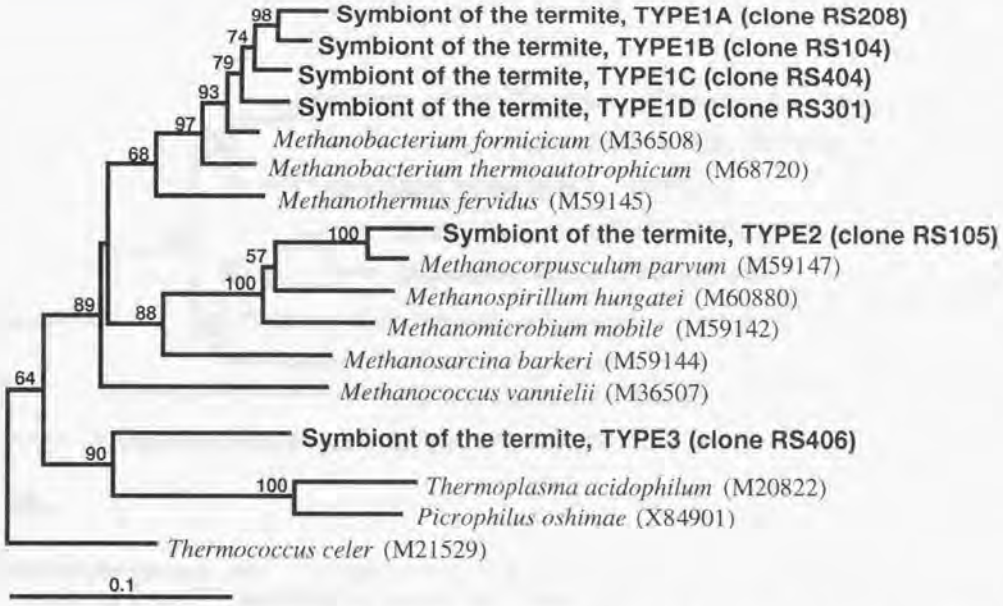


Fig. 2.2. Phylogenetic positions of the PCR clones obtained from *R. sperauts* hindgut contents within the members of Euryarchaeota. Type clones of respective groups of the clones were used for the tree construction and is indicated in parentheses. *T. celer* was used as the outgroup. The scale bar represents 0.1 substitutions per nucleotide position. Figures indicate bootstrap values.

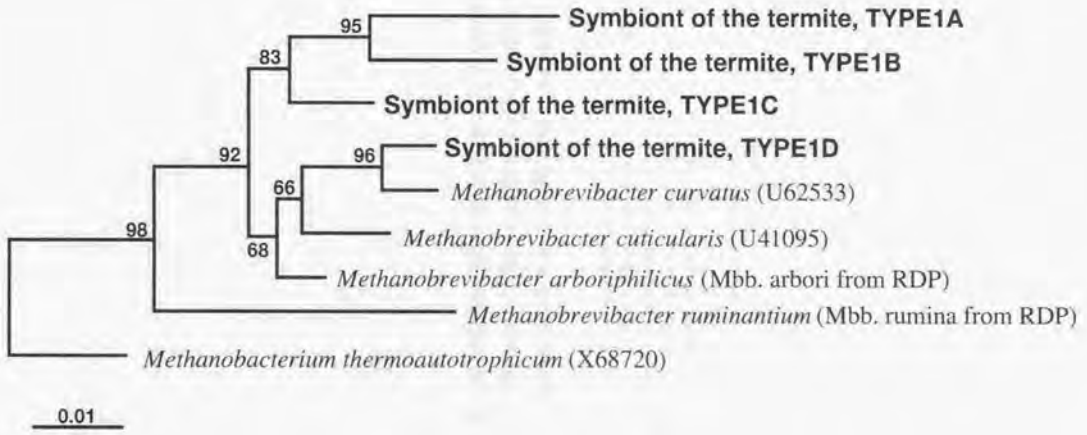


Fig. 2.3. Detailed phylogenetic analysis of type-1 clones within the members of the genus *Methanobrevibacter*. The tree was inferred from 477 unambiguously aligned nucleotide sequences of the SSU rRNA using the neighbor-joining method. The topology was tested by 1,000 repetitions of bootstrap analysis. *M. thermoautotrophicum* was used as the outgroup. The scale bar represents 0.01 substitutions per nucleotide position.

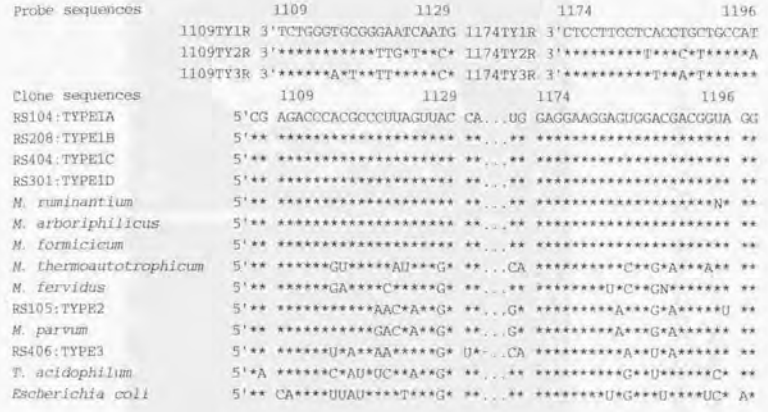


Fig. 2.4. Alignment of the oligonucleotide probe sequences and the corresponding SSU rRNA sequences of termite symbiont clones, methanogens, and *E. coli*. Only nucleotides which are different from these of the type-1 sequence are shown.

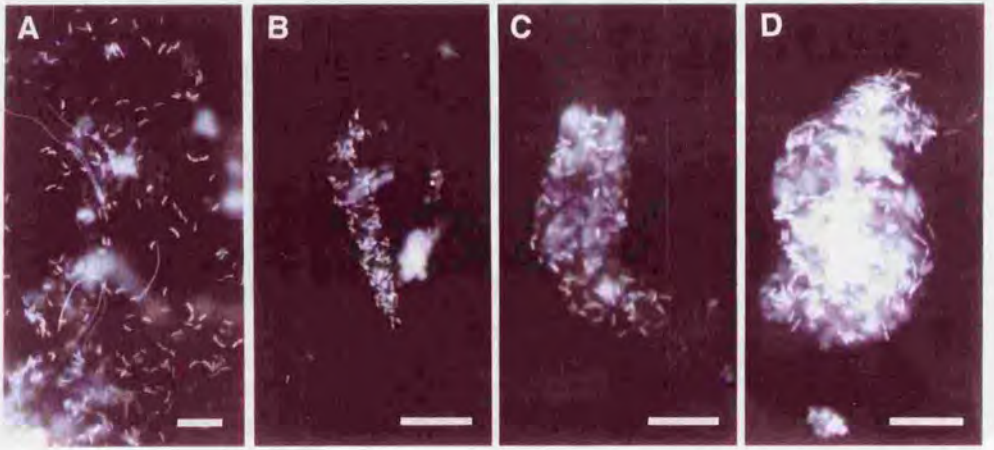


Fig. 2.5. F420 epifluorescence microscopic images of methanogens in the hindgut of *R. speratus*. (A) Epithelial surface of the hindgut. (B-D) Symbiotic flagellates, *P. parva*, *P. rugosa*, *P. leidyi*, respectively. Scale bar represent 10 μm.

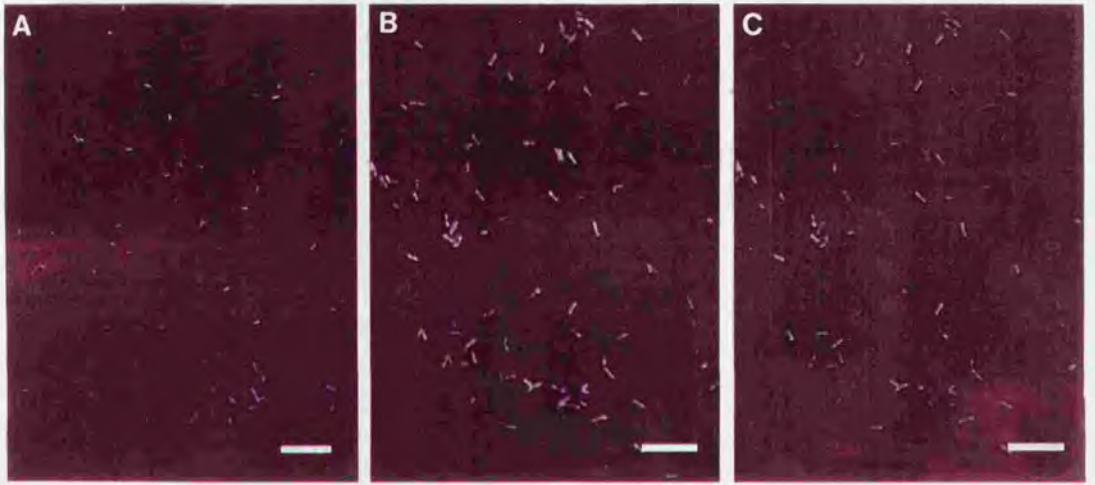


Fig. 2.6. (A) Morphology of the cells of cultivated from *R. speratus* gut content photographed by F420 epifluorescence microscopy. (B) and (C) The cells of the enrichment culture simultaneously hybridized with fluorescein-labeled archaea-specific probes and rhodamine-labeled type-1-specific probes. (B) An image taken for archaea-specific probes, ARC344F and ARC915F. (C) The same field as in (B) showing the image of type-1-specific probes, 1109TY1R and 1174TY1R. Scale bars represent 10 μm.

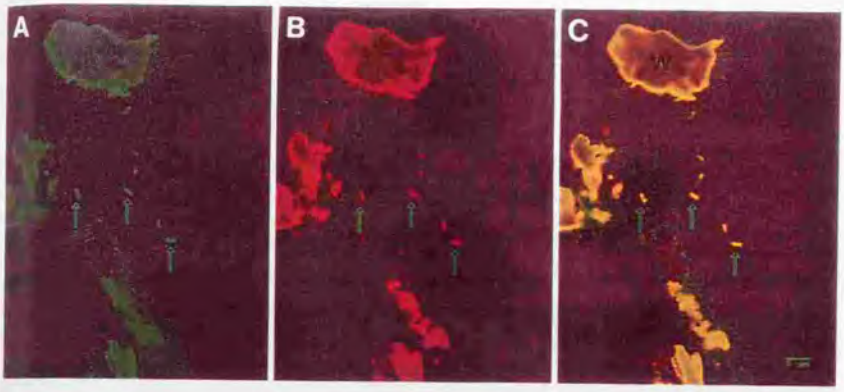


Fig. 2.7A-C. Whole-cell *in situ* hybridization of hindgut contents of *R. speratus* with fluorescein-labeled and rhodamin-labeled probes. (A) A image taken for fluorescein (FITC)-labeled archaea-specific probes, ARC-344F-ARC915F. (B) The same field as in (A) showing the image of rhodamine (TRITC) fluorescence of type-1-specific probes, 1109TY1R-1174TY1R. (C) Merged field of (A) and (B). Scale bars represent 5 μm.

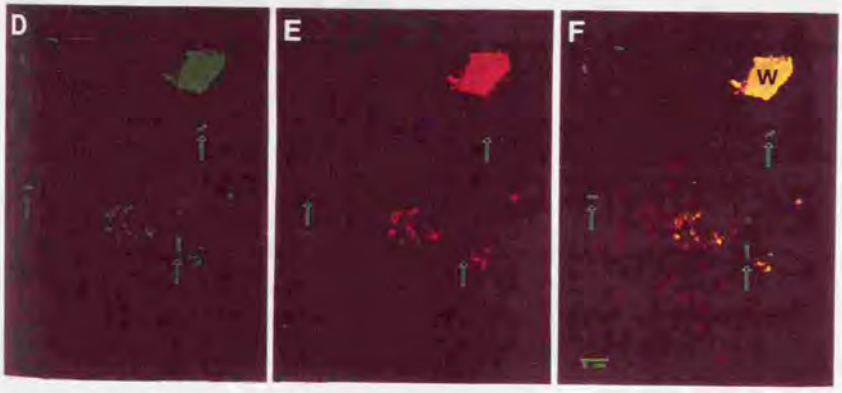


Fig. 2.7D-F. Whole-cell *in situ* hybridization of hindgut contents of *R. speratus*.
(D) A image taken for FITC-labeled archaea-specific probes. (E) The same field as in (D) showing the image of the fluorescence from TRITC-labeled type-2-specific probes, 1109TY2R-1174TY2R. (F) Merged field of (D) and (E). Scale bars represent 5 μm.

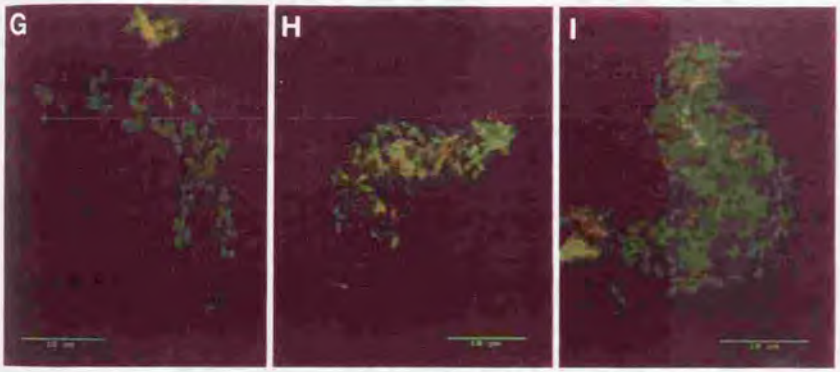


Fig. 2.7G-I. Whole-cell *in situ* hybridization of the methanogen-associating flagellates of *R. speratus*. Symbiotic flagellates of *R. speratus*, *P. parva* (G), *P. rugosa* (H), and *P. leidyi* (I) simultaneously stained with the archaea specific and the type-1-specific probes.Only the merged fields are shown. Scale bars represent 10 μm.

Table 2.1. Number of clones in each type

Area	Colony	Symbiotic type					
		1A	1B	1C	1D	2	3
Tokyo	RS1	4	4	0	0	1	1
	RS2	5	5	0	0	0	0
	RS4	1	6	1	0	0	2
	(M4)*		(1)				
Kobe	RS5	8	2	0	0	0	0
Yamaguchi	RS3	7	0	0	3	0	0
Okinawa	RS6	6	4	0	0	0	0
Total		31	21	1	3	1	3

*Sequence of the clone M4 amplified from hindgut DNA of *R. speratus* was reported by Ohkuma et al. (49). The clone M4 was grouped in type-1B.

Table. 2.2. Absolute and mean distance matrix from the comparison of SSU rRNA gene sequences of *R. speratus* methanogenic symbiont clones and other members of the genus *Methanobrevibacter*.

Organism	Absolute and mean distances								
	1	2	3	4	5	6	7	8	9
1. Symbiont of *Reticulitermes speratus* type 1A	–	0.033	0.041	0.054	0.054	0.054	0.045	0.081	0.039
2. Symbiont of *Reticulitermes speratus* type 1B	16	–	0.031	0.046	0.042	0.046	0.031	0.081	0.008
3. Symbiont of *Reticulitermes speratus* type 1C	20	15	–	0.035	0.033	0.035	0.027	0.067	0.039
4. Symbiont of *Reticulitermes speratus* type 1D	26	22	17	–	0.027	0.012	0.031	0.069	0.052
5. *Methanobrevibacter cuticularis*	26	20	16	13	–	0.027	0.019	0.071	0.050
6. *Methanobrevibacter curvatus*	26	22	17	6	13	–	0.031	0.071	0.050
7. *Methanobrevibacter arboriphilicus*	22	15	13	15	9	15	–	0.062	0.050
8. *Methanobrevibacter ruminantium*	39	39	32	33	34	34	30	–	0.039
9. Symbiont of *Reticulitermes speratus* clone M4*	19	4	19	25	24	24	19	43	–

* Sequence of the clone M4 amplified from hindgut DNA of *R. speratus* was reported by Ohkuma et al. (49). The clone M4 can be grouped in type 1B.

PART III

PHYLOGENETIC RELATIONSHIPS BETWEEN METHANOGENIC SYMBIONTS AND THEIR XYLOPHAGOUS HOST INSECTS

ABSTRACT

SSU rRNA genes of the symbiotic methanogen of 7 lower and higher termites collected in Japan Archipelago, and one Australian lower termite *Mastotermes darwiniensis* were amplified, cloned and phylogenetically analyzed. Most of the clones obtained from the Japanese termites were *Methanobrevibacter*-related methanogens, except for one from the higher termites *Odontotermes formosanus* which affiliated to the order Thermoplasmales. The compositions of symbiotic methanogens in the hindgut of the Japanese termites appeared to be similar to those of the lower termite *Reticulitermes speratus* examined previously. On the other hand, although all of the clones obtained from *M. darwiniensis* were also in *Methanobrevibacter*, these were grouped separately from those of Japanese termites. The difference between the phylogenetic positions of clones isolated from Japanese termites and *M. darwiniensis* suggest the methanogen communities of termites reflect their host localities rather than their phylogenies. The phylogenetic analysis of the methanogen community of soil environment near the termite nest suggest that some of the methanogens in termite hindgut are acquired from the environment.

INTRODUCTION

Species composition of the symbiotic flagellates is usually uniform in a species or a regional population of a host termite. The distinct host-specificity of symbiont compositions in host-intestinal symbiont-assemblage system has been reported (see ref. 19 for helminth community, and ref. 48 for rumen ciliates). And the specificity is thought to be caused by the transmission process of flagellates between termite individuals, together with the close mutualism between these organisms. The flagellates are transmitted between members of a host colony via proctodeal feeding (inter-individual transmission of gut contents from the anus of a donor to the mouth of a receptor). In the stage of colony foundation, the flagellate compositions of the native colonies of an alate pair is succeeded to the new colony members.

Though the transmission scheme has been well investigated, the bacterial microflora in termite digestive tract has been little analyzed. However, it is reasonable to imagine that the bacterial microflora is also transmitted via the proctodeal feeding to the next termite generation. Especially, it seems difficult that the termite hindgut are infected by the new methanogens from the outside environment, because atmospheric oxygen is lethal to them (11). Investigation of the transmission of bacterial microflora is also needed for the better understanding of symbiotic ecosystem in termite hindgut. It has been reported that there are close relation between the phylogeny of the symbiont and that of the host, if there is a distinct mutualism between host and the symbiont (43).

In this study, molecular phylogeny of symbiotic methanogens in 8 termite species belong to 7 subfamily including higher and lower termites was examined, and the transmission of the symbiotic methanogens to the next generation of the termite are discussed.

MATERIALS AND METHODS

Materials

Eight termite species and one xylophagous cockroach were examined in this study. Four lower termites, *Neotermes koshunensis*, *Hodotermopsis japonica*, *Reticulitermes kanmonensis*, *Coptotermes formosanus*, 3 higher termites, *Nasutitermes takasagoensis*, *Pericapritermes nitobei*, *Odontotermes formosanus*, and the xylophagous cockroach *Salganea esakii* were collected with their nest wood from forests in the Japan Archipelago. *Mastotermes darwiniensis* was sampled in Australia and kindly donated by Dr. O. Kitade. The insect materials were maintained in the laboratory at room temperature (15-25°C) until use. The contents of insect containers were periodically moistened by spraying the surface of infested wood with distilled water. The rumen fluid was sampled from rumen (the third stomach) of a cow which fed on hay in Miyazaki city, Japan. The rumen fluid was passed through a cheese cloth to eliminate debris and immediately frozen in liquid nitrogen. The frozen sample was kindly provided by Dr. K. Sakurada. Humus in the copse in Hachioji city was sampled as soil sample. The soil sample was employed for the DNA extraction immediately.

DNA extraction

Total genomic DNA of digestive tracts of the termites and the cockroach were prepared as follows. Ten individuals of worker or pseudergate from each colony of termite, were employed for DNA extraction. After surface sterilization with 70% ethanol, digestive tracts of the termites were pulled out with a pair of fine-tipped forceps. The gut tissues and the contents were crushed with a Teflon homogenizer in 1 ml of extraction buffer consisting of 100 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.1% SDS. The mixture was frozen in liquid nitrogen and thawed at 57°C five times, and then treated with 0.5 mg ml^{-1} proteinase K (Nakarai tesque, Japan) at 57°C for 16 hr. The solution was extracted

with phenol and chloroform several times and the DNA was precipitated with ethanol according to the standard protocol (52).

One ml of rumen fluid filtrated through cheese cloth was centrifuged at 16,000 rpm for 5 min at 4°C. The pellet was resuspended in 1 ml of extraction buffer, and DNA was extracted by the same procedure as the insect materials.

Five hundred g of the soil sample was suspended in 1 L of distilled water. After standing for 10 min, 500 ml of the supernatant was centrifuged at 6,000 rpm for 30 min at 4°C. The pellet was resuspended in 50 ml of the extraction buffer and DNA was prepared by the same procedure described above. The concentrated DNA was electrophoresed on 0.8% agarose gel to remove inhibitory elements for PCR. High molecular weight genomic DNA was excised from the gel and recovered with a Gene Clean II kit (BIO. 101 Inc., USA).

PCR amplification

PCR primers were designed to amplify a part of archaebacterial SSU rRNA gene (approximately 0.5 kbp). The primers used were ME855F (5'-TTAAAGGAATTGGCG GGGGA-3') and ME1354R (5'-TGACGGGCGGTGTGTGCAAG-3'), the numbers represent nucleotide positions in the counterpart of *Escherichia coli*. To facilitate the addition of adenine at the 3' end in the PCR reaction, 5' end of the primers were designed to be tymine.

PCR reaction was performed in a final volume of 50 µl. Each reaction mixture contained 1 µl of template DNA solution, 5 µl of 10x PCR buffer (100 mM Tris-HCl, 500 mM KCl, 25 mM $MgCl_2$, 1% TritonX-100, pH 9.0), 1 µl of 0.1 mg ml^{-1} BSA, 1 µl of dNTPs mixture (each at 50 µM), 20 pmol each of the primers, 1 unit of Taq DNA polymerase (Toyobo, Japan) and sterile Millipore water to make it 50 µl. Reaction was

performed in a thermal cycler, GeneAmp 2400 (Perkin-Elmer) with the thermal program which comprised 40 cycles of denaturation at 94°C for 30 sec, primer annealing at 60°C for 30 sec, and primer extension at 72°C for 90 sec, followed by an additional extension at 72°C for 5 min. The PCR products were analyzed by electrophoresis on 1.5% agarose gel. The band of about 0.5 kbp was cut out from the gel and purified with a Gene Clean II kit.

Cloning and sequencing of amplified DNA

The PCR products were ligated into the plasmid vector with a T-A cloning kit (Invitrogen, Netherlands) and the *E. coli* strain JM105 was transformed with the ligate. The nucleotide sequences were determined by the dideoxynucleotide chain termination method (53) using Dye-Terminator Cycle Sequencing Kit (Applied Biosystems) on an automatic DNA sequencer ABI model 377A (Applied Biosystems).

Phylogenetic analysis

The sequences were examined to test the possibility of chimeric artifacts with CHECK-CHIMERA program in the ribosomal database project (39). The sequences were aligned with previously reported SSU rRNA gene sequences from the nucleotide sequence databases GenBank, EMBL, and RDP by using the program package CLUSTAL W (59). To obtain the reliable phylogenetic relationships, the nucleotide positions suspiciously aligned were excepted from the data set in advance. The programs in the software package PHYLIP version 3.572 (J. Felsenstein and the University of Washington) were employed for the phylogenetic analysis. Phylogenetic trees were constructed from neighbor-joining distance matrix methods (51). Numbers of nucleotide substitution were calculated according to Kimura's two-parameter method (31), taking transition and transversion rates into account. Substitution rate was analyzed by neighbor-joining method to construct phylogenetic tree. Bootstrap confidence interval (20) on each branching pattern was

calculated from 1000 repetitions of resampling. The aligned sequences were also analyzed by maximum parsimony and maximum likelihood methods to check the tree topology.

RESULTS

Phylogeny of methanogens in lower and higher termites

Expected length (approximately 0.5 kbp) of DNA fragments were amplified by PCR using the set of archaeal SSU rRNA gene specific primers for all termites used in this study. The PCR products were cloned into the plasmid vector, and the sequences of 5 clones per termite species were determined. The results of the phylogenetic analysis with members of the *Euryarchaeota* and the cloned sequences of symbiotic methanogens from *R. speratus* showed that all of the clones, except for one obtained from *O. formosanus*, affiliated to the clone type-1A or 1B of *R. speratus* (Part II). Evolutionary distances of these clones from the respective type clones were within 0.02 substitutions per nucleotide position (data not shown). The other clone of *O. formosanus* located near the clone type-3 of *R. speratus* within 0.02 evolutionary distances. In Table 3.1, number of clones affiliated in respective clone type was listed. Though the type-1A sequence was not obtained from *H. japonica*, both type-1A and 1B were detected from all of the other termite species. The total ratio of type-1A and 1B detected from 7 termite species from the Japan Archipelago was 5 : 12.

The phylogenetic relationships of the cloned sequences from the lower termite *M. darwiniensis* within members of the *Euryarchaeota* and the type clones of *R. speratus* were shown in Fig. 3.1. *M. darwiniensis* was the only species sampled in the Australian continental. Inferred from the phylogenetic tree, all of the 5 clones were close to type-1 clones and belonged to genus *Methanobrevibacter*, however, the cloned sequences were separately from other clone sub-types of type-1 found in *R. speratus*.

Phylogeny of methanogens in xylophagous cockroach S. esakii

Archaebacterial SSU rRNA genes were amplified from the extracted DNA from digestive tract of the xylophagous cockroach *S. esakii* in the same manner. Fig.3.2 shows

the phylogenetic positions of the 5 cloned sequences in members of the *Euryarchaeota* and the clones of *R. speratus*. The cloned sequences were affiliated in 2 different clusters distantly related each other. Two clones SE102 and SE103 belonged to genus *Methanobrevibacter* and were grouped with other type-1 clones. However SE102 and 103 were separated from other sub-types of type-1. The remaining 3 clones SE101, SE104, and SE105 were located in family Thermoplasmaceae. These clones branched near type-3 clones but significant diversification was seen.

Phylogeny of methanogens in rumen of cow

In the phylogenetic tree constructed with members of the *Euryarchaeota* and the type clones of *R. speratus*, two clones BR101 and BR105 isolated from rumen fluid of a cow belonged to genus *Methanobrevibacter*, and the remaining 3 clones BR102, BR103, and BR104 were located in family Thermoplasmaceae (Fig. 3.3). The clones BR101 and BR105 located close to *Methanobrevibacter ruminantium* isolated from bovine rumen. These clones were not related to the clone types from 1 to 3 in the termites used in this study.

Phylogeny of archaebacteria in soil environment

The PCR using the archaebacterial SSU rRNA gene specific primers resulted in amplification of 0.5 kbp products from the DNA extracted from humus soil sample. Seven cloned sequences were determined and analyzed. The phylogenetic tree were constructed including members of the *Euryarchaeota* and the type clones of *R. speratus*. The tree showed that the 3 clones XT106, XT108 and XT109 were located in genus *Methanobrevibacter*, and the remaining 4 clones were in family Thermoplasmaceae (Fig. 3.4). In the former 3 clones, XT106 and XT108 were closely related to the clone type-1A of *R. speratus*, and the another clone XT109 located near by the clone type-1B. The clones XT102, 103, and 107 in Thermoplasmaceae formed a cluster within 0.02

substitutions per nucleotide position (data not shown).

DISCUSSION

In part II, I have analyzed the PCR clones of symbiotic methanogens in *R. speratus* collected from Japan Archipelago, most of the clones were type-1A or 1B. In part III, I have analyzed several other species of termite collected from Japan Archipelago. I have found that most of the clones were type-1A and 1B. The types of PCR clones of methanogens in the hindgut of these termites were similar to those of *R. speratus*. Because the higher termites lack symbiotic flagellates from the hindgut microflora (part I, ref. 57), the symbiont type-1A and 1B appeared to be "non-endosymbiotic methanogens", e.g. gut wall-attaching or freely-swarming methanogens, in these termites. Also in the lower termite *R. speratus*, type-1 *Methanobrevibacter*-related methanogens has been found to exist in the hindgut fluid, but not to associate with flagellates (part II).

However there are discrepancies between observation of filamentous methanogens. Morphotypes of methanogens observed in the hindgut of some higher termites were almost "filamentous" as reported previously (57), which is different from the "short-rods" in *R. speratus* (57). Curved or straight short-rod cell morphotypes were characteristic of the genus *Methanobrevibacter* (42). Filamentous morphotype has not been reported in this order. Recently, Leadbetter and Breznak (36) have reported the isolation of a novel species of methanogen *Methanobrevibacter filformis* from *R. flavipes*. Although *M. filiformis* was affiliated in genus *Methanobrevibacter* judging from SSU rRNA gene phylogenetic analysis, morphology of the *M. filiformis* was obviously filamentous. It is still unclear that the filamentous methanogens abundantly observed on the hindgut wall of some higher termites are the *Methanobrevibacter*. Transmission electron microscopic observations of the filamentous methanogens have showed that they had some morphological features of genera *Methanospillilum* and *Methanothrix* (57). The large morphological differences between the two strains of the same genus of methanogens have to be confirmed.

The analyzed PCR clones obtained from the Japanese termites were grouped in type-1A and 1B, irrespective of termite species. It is suggestive that, as long as the non-endosymbiotic methanogens are concerned, methanogen communities of the Japanese termites are resemble to one another. On the other hand, phylogenies of the clones of the Australian lower termite *M. darwiniensis* were slightly different from those of the Japanese termites. Though they were in *Methanobrevibacter*, preliminary observations of the symbiotic methanogens in *M. darwiniensis* has revealed freely-swarming methanogens but no endosymbiotic ones associating with flagellate in the hindgut fluid by epifluorescence microscopic observation, so the cloned sequences seemed to be originated from the non-endosymbiotic methanogens.

M. curvatus and *M. cuticularis* isolated from the American lower termite *R. flavipes* (35) were distinct from type-1 clones. These results suggest that the compositions of methanogen community in termite hindgut are related to the locality rather than the species of their host termites. The working hypothesis is supported by the study on methanogen community of *R. flavipes* (35, 36). The three species of methanogens *M. cuticularis*, *M. curvatus*, and *M. filiformis* were isolated from the hindgut of *R. flavipes*. Although the filamentous methanogen *M. filiformis* was one of the dominant methanogens in *R. flavipes* collected from Woods Hole in Massachusetts, cells of similar morphology were not observed in *R. flavipes* collected from Dansville in Michigan (36).

The methanogen community in termite hindgut seemed to be related to the host locality. Then, what is the factor determine the composition of the community of methanogen in termite hindgut? How are the relations between the methanogen community and the host locality? In the case of symbiotic flagellates of lower termites, flagellate species are known to be characteristic to host termite species. The specificity is thought to be caused by the transmission process of the flagellates via proctodeal feeding. This transmission mode seemed to be applicable to the bacterial community of termite hindgut too, it has not

seen in this experiment, however. If the proctodeal feeding is only the way to acquire the bacterial microflora including methanogen species and lineages of methanogens must reflect the host phylogeny. However, the results obtained from the SSU rRNA gene analyses are inconsistent with this scenario. Another possible scenario is infection from the outside world via feeding action of the host at least partially. In this case, the species and lineages of the bacterial symbionts must relate to the host localities rather than or in addition to their phylogenies.

The SSU rRNA gene analyses of the extracted DNA from humic soil sampled near by nest of *R. speratus* consisted with the later scenario. The analyzed PCR clones included the type-1B and type-1A-related ones. Moreover, the symbiont type-3-related clones were also obtained. The type-3 is unknown species of *Thermoplasma*-related archaea detected from *R. speratus* and *O. formosanus*. Considering these results, it seems reasonable that, although the endosymbiotic methanogens associated with symbiotic flagellates in lower termites are transmitted to their next generation via proctodeal feeding, some of the non-endosymbiotic ones, e.g. gutwall-attaching or freely-swarming methanogens, can be acquired from the outside environment.

Because methanogens are generally extremely sensitive to oxygen, it seems to be difficult for methanogens to infect from the outside exposing to air. However, the two isolated methanogens *M. cuticularis* and *M. curvatus* were shown to have some oxygen tolerance analyzed by the growth experiments within oxgen containing medium and the observation of their *in situ* locations on the gut periphery where it is microoxic (10, 35).

If the infection of methanogen to termites from the outside environment occurs commonly, it may be applicable to other animals which harbor methanogens in their digestive tracts, e.g. xylophagous cockroaches (24) or mammalian herbivores (61). To compare the methanogen communities, xylophagous cockroach *S. esakii* sampled in Yakushima Is. and rumen contents sampled from the cow in Miyazaki city were examined. Though the *Thermoplasma*-related clones were obtained from both *S. esakii* and rumen

contents, the methanogen type-1A or 1B-related clones were not seen. The different compositions of methanogen among *S. esakii*, rumen, and the termites may be caused by the differences of the physicochemical conditions in their digestive tracts.

Table 3.1. Species of termites used in this study and number of clones in each type of symbiotic methanogens

Species	Locality	Colony	Symbiot type						
			1A	1B	1C	1D	2	3	Total
Reticulitermes kanmonensis	Simonoseki c.	RK100	1	4	0	0	0	0	5
Coptotermes formosanus	Okinawa Is.	CF100	1	4	0	0	0	0	5
Neotermes koshunensis	Okinawa Is.	NK100	2	3	0	0	0	0	5
Hodotermopsis japonica	Amamioshima Is.	HJ100	0	5	0	0	0	0	5
Pericapritermes nitobei	Iriomote Is.	PN100	2	3	0	0	0	0	5
Nasutitermes takasagoensis	Iriomote Is.	NT100	3	2	0	0	0	0	5
Odontotermes formosanaus	Okinawa Is.	OF100	1	3	0	0	0	1	5
		Total	10	24	0	0	0	1	35

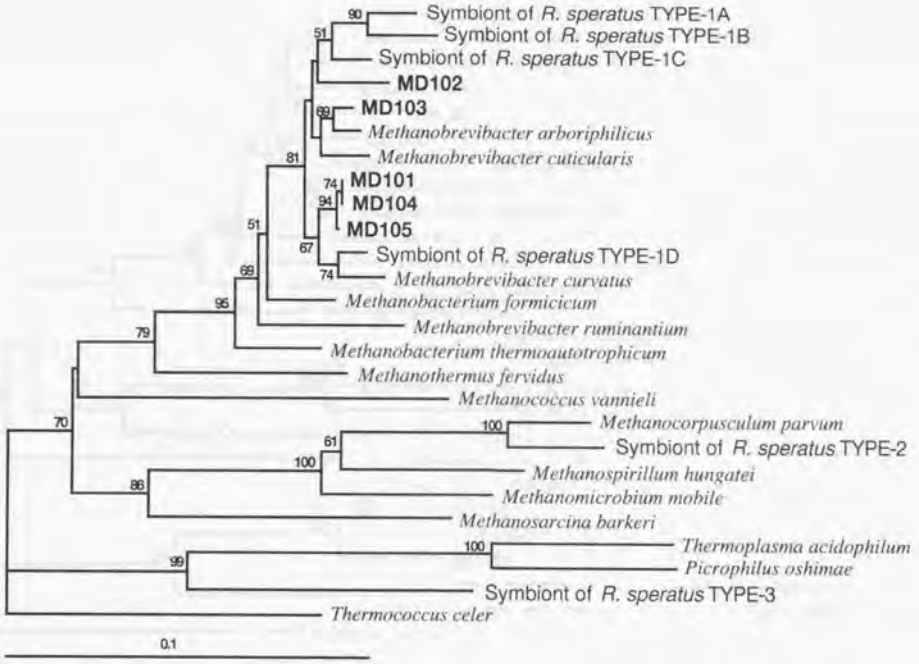


Fig. 3.1. Phylogenetic positions of the clones obtained from the lower termite *Mastotermes darwiniensis* within the members of the *Euryarchaeota*. The phylogenetic tree was constructed from 503 unambiguously aligned nucleotide positions in SSU rRNA gene using the neighbor-joining method. The tree topology was tested by 1000 repetitions of bootstrap analysis.

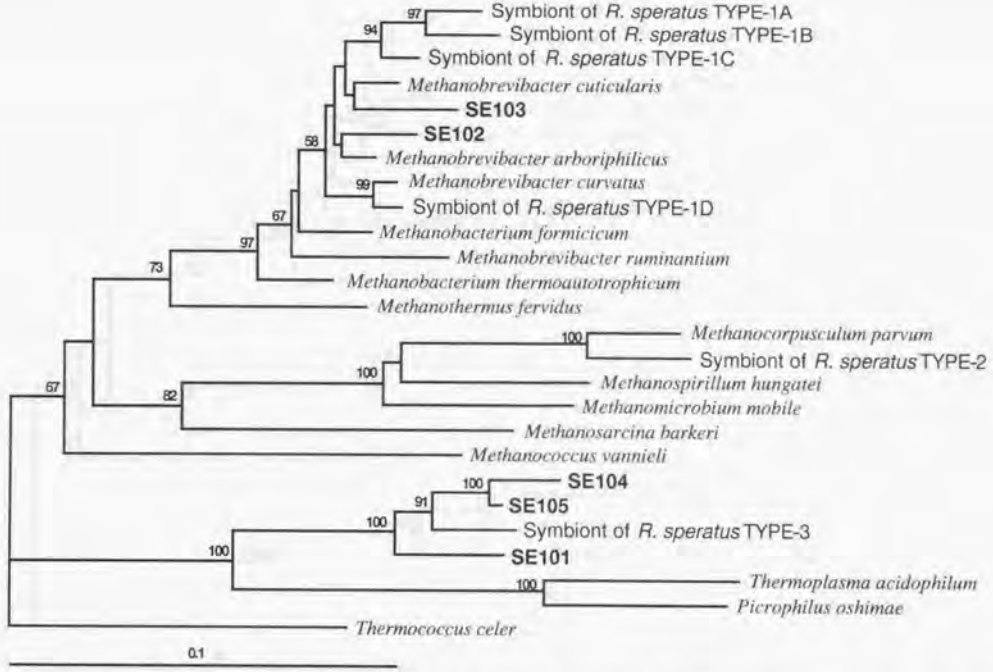


Fig. 3.2. Phylogenetic positions of the clones obtained from the xylophagous cockroach *Salganea esakii* within the members of the *Euryarchaeota*. The phylogenetic tree was constructed from 503 unambiguously aligned nucleotide positions in SSU rRNA gene using the neighbor-joining method. The tree topology was tested by 1000 repetitions of bootstrap analysis.

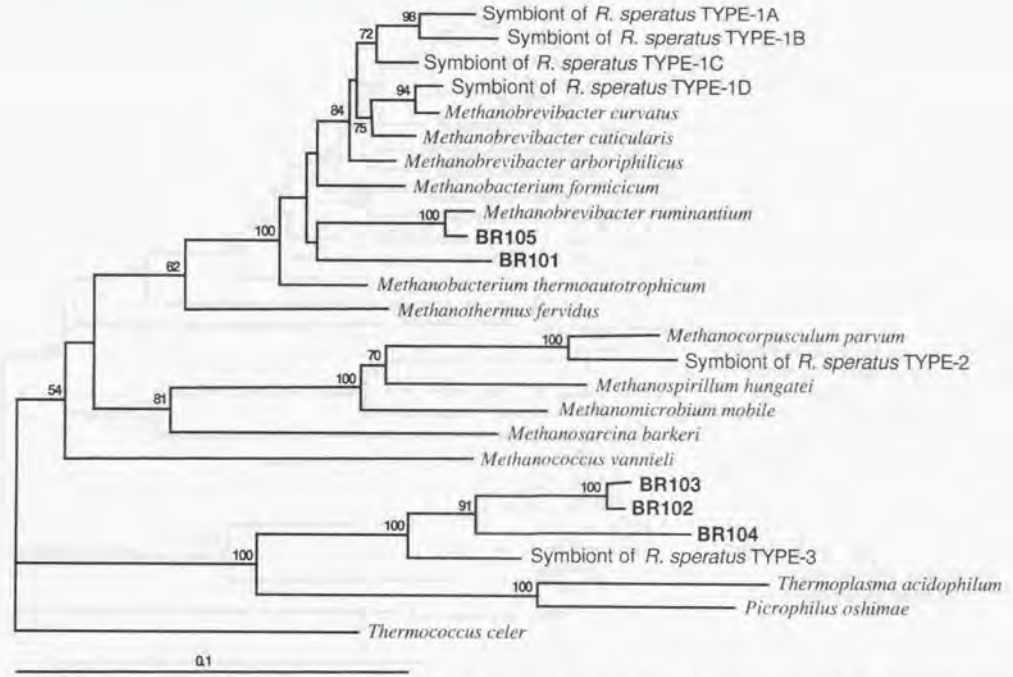


Fig. 3.3. Phylogenetic positions of the clones obtained from the rumen of hay-fed cow within the members of the *Euryarchaeota*. The phylogenetic tree was constructed from 503 unambiguously aligned nucleotide positions in SSU rRNA gene using the neighbor-joining method. The tree topology was tested by 1000 repetitions of bootstrap analysis.

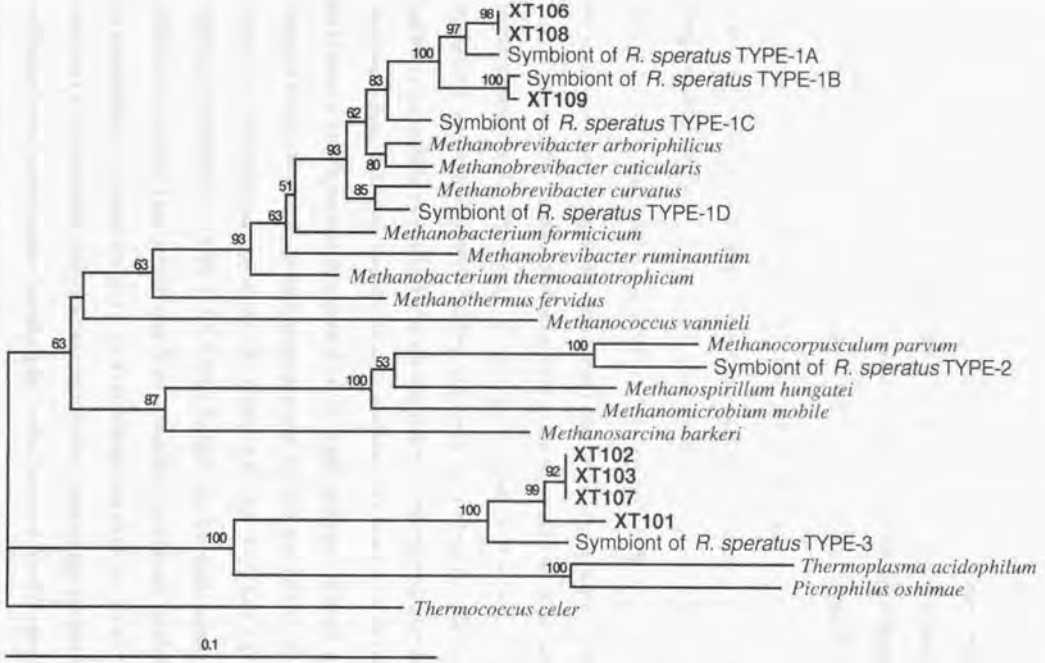


Fig. 3.4. Phylogenetic positions of the clones obtained from the extracted DNA from humas near by termite nests. within the members of the *Euryarchaeota*. The phylogenetic tree was constructed from 503 unambiguously aligned nucleotide positions in SSU rRNA gene using the neighbor-joining method. The tree topology was tested by 1000 repetitions of bootstrap analysis.

GENERAL DISCUSSION

The terminal H_2-consuming process in termite hindgut ecosystem is little different from other natural habitats. The specific physiological group of H_2 sink organisms that dominates this H_2-consuming stage depends on the habitat. In anoxic marine sediments, where the concentration of sulfate is relatively high, sulfate-reducing bacteria usually dominate terminal electron flow (e.g. $4H_2 + SO_4^{2-} \rightarrow S^{2-} + 4H_2O$), which includes the complete anaerobic oxidation of acetate to CO_2 (e.g. $2CH_3COOH + SO_4^{2-} \rightarrow 2CO_2 + S^{2-} + 4H_2O$). In contrast, in anoxic habitats low in sulfate, methanogens usually dominate, using primarily CO_2 and the methyl group of acetate as electron acceptors (e.g. $4H_2 + 2CO_2 \rightarrow CH_4 + 2H_2O$ and $CH_3COOH \rightarrow CH_4 + CO_2$). Indeed, in gastrointestinal tracts, such as ruminant animals, the concentration of terminal electron acceptors other than CO_2 is usually limited, the fermentation of the lignocellulose is primarily toward methanogenesis (63). However, in termite hindgut ecosystems, CO_2-reducing acetogenesis generally outprocess methanogenesis as the primary H_2 sink. For the host termites, intermediates of lignocellulose decomposition by the resident microbiota can be important carbon and energy sources. In such situation, CO_2-reducing acetogenic bacteria could make a significant contribution to the host termite nutrition. Breznak and Switzer estimated that one third of respiratory requirement of the host was fulfilled by the acetate formed by CO_2-reducing acetogens. It seems odd that CO_2-reducing acetogens outcompete methanogens for H_2 in the termite hindgut, as this rarely occurs in natural habitats. One possible factor contributing to its competitive success was its ability to carry out mixotrophy [i.e. to gain energy by the simultaneous use of $H_2 + CO_2$ and an organic substrate(s)]. This property might enable cells to realize more energy per unit time in situ and thereby outcompete resident methanogens, which generally have a narrow range of utilizable substrates (7). However, the distinct reason of this phenomenon is still unclear.

The methanogenic symbionts of the hindgut of termites house in two different niches, i.e. on the epithelial surface of hindgut and in the cytoplasm of flagellated protozoa (41, 57). The peripheral region near the hindgut surface is microoxic, where it is usually unsuitable for methanogens to colonize (17). However, these methanogens have some oxygen tolerance and dominate this habitat (35), where oxygen sensitive CO_2-reducing acetogens probably can not colonize. On the other hand, endosymbiotic relationships between H_2-consuming methanogens and anaerobic protozoa are seen also in natural habitat (18). The cytoplasm of anaerobic protozoa is thought to be the best position in termite hindgut to acquire the substrate, i.e. H_2 and CO_2, for methanogenesis. Under the competitive situation for H_2, methanogens may acquired these physical and physiological advantages to competitive acetogens.

In this study, some of the colonies of the lower termite *N. koshunensis* without methane emission and fluorescent methanogens existed. One of the possible reason of the lack of methanogens from the hindgut is the failure of methanogen transmission between termite generations at colony foundation. However, the compositions of flagellated protozoa in both colonies with and without methanogens were mostly identical which is indicating the successive transmission of protozoa. In the study of *R. speratus*, no endosymbiont was hybridized with *Methanobrevibacter*-specific probes. The possibility that the molecular phylogenies of endosymbiotic methanogens vary from those of free-living ones as the results of coevolution in the distinct mutualism to their host protozoa was indicated. In this case, the endosymbiotic methanogens could be stably transmitted to the next generation of termites along with the protozoa. It is inconsistent with the observations in *N. koshunensis*. In this issue, more significant factors which positively eliminate the methanogens from the hindgut ecosystem may involve, e.g. difference of physicochemical condition in the hindgut between the termites of the colonies with and without methane emission.

In any case, to use of H_2 and CO_2 in the hindgut for acetate synthesis but not for

methane production is thought to serve as an additional energy source for the host termite. If it is true, the termites of the colonies without methane emission could obtain more utilizable energy source per unit food intake than those of the colonies with methane emission. This potential advantage may effect the growth rate of individual termites or the productivity of the queen, which may result in fast colony growth.

ACKNOWLEDGMENT

I express my thanks to Prof. T. Matsumoto, and also thank members of Department of Biology at The University of Tokyo for their encouragement and valuable comments throughout the course of this study.

I am deeply indebted to Assoc. Prof. A. Yamagishi for valuable advice, discussions and warm encouragement during the course. Thanks are also due to Prof. T. Oshima and members of the Oshima laboratory at Tokyo University of Pharmacy and Life Science for providing a comfortable environment for study and my daily life.

I also thank Prof. I. Yamaoka and Dr. K. Yara for providing an opportunity to start this work.

Finally, I would like to express my deep appreciation to my parents who allowed me to study further in graduate school.

REFERENCES

1. **Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe.** 1979. Methanogens: Reevaluation of a unique biological group. Microbiol. Rev. 43:260-296.

2. **Bignell, D. E.** 1984. Direct potentiometric determination of redox potentials of the gut contents in the termites *Zootermopsis nevadensis* and *Cubitermes severus* and in three other arthropods. J. Insect Physiol. 30:169-174.

3. **Bignell, D. E., and J. M. Anderson.** 1980. Determination of pH and oxygen status in the guts of lower and higher termites. J. Insect. Physiol. 26:183-188.

4. **Brauman, A., M. D. Kane, M. Labat, and J. A. Breznak.** 1992. Genesis of acetate and methane by gut bacteria of nutritionally diverse termites. Science. 257:1384-1387.

5. **Breznak, J. A.** 1984. Biochemical aspects of symbiosis between termites and their intestinal microbiota, p. 173-203. In J. M. Anderson, A. D. M. Rayner, and D. W. H. Walton (ed.), Invertibrate Microbial Interactions. Cambride University Press, Cambridge.

6. **Breznak, J. A.** 1975. Symbiotic relationships between termites and their intestinal microbiota, p. 559-580. In D. H. Jennings and D. L. Lee (ed.), Symbiosis: 29th Symposium of the Society for Experimental Biology. Cambridge University Press, Cambridge.

7. **Breznak, J. A., and J. S. Blum.** 1991. Mixotrophy in the termite gut acetogen, *Sporomusa termitida*. Arch. Microbiol. 156:105-110.

8. **Breznak, J. A., and J. M. Switzer.** 1986. Acetate synthesis from H_2 plus CO_2 by termite gut microbes. Appl. Environ. Microbiol. 52:623-630.

9. **Britschgi, T. B., and R. D. Fallon. 1994.** PCR-amplification of mixed 16S rDNA genes from an anaerobic cyanide-degrading consortium isolated from anaerobic

waste treatment reactor. FEMS Microbiol. Ecol. 13:225-232.

10.**Brune, A., D. Emerson, and J. A. Breznak.** 1995. The termite gut microflora as an oxygen sink: Microelectrode determination of oxygen and pH gradients in guts of lower and higher termites. Appl. Environ. Microbiol. 61:2681-2687.

11.**Bryant, M. P., B. C. McBride, and R. S. Wolfe.** 1968. Hydrogen-oxidizing methane bacteria. I. Cultivation and methanogenesis. J. Bacteriol. 95:1118-1123.

12.**Collins, N. M., and T. G. Wood.** 1984. Termites and atmospheric gas production. Science. 224:84-86.

13.**Delong, E. F.** 1992. Archaea in coastal marine environments. Proc. Natl. Acad. Sci. USA. 89:5685-5689.

14.**Delong, E. F., G. S. Wickham, and N. R. Pace.** 1989. Phylogenetic strains: Ribosomal RNA-based probes for the identification of single cells. Science. 243:1360-1363.

15.**Delong, E. F., K. Y. Wu, B. B. Prezelin, and R. V. M. Jovine.** 1994. High abundance of Archaea in Antarctic marine picoplankton. Nature. 371:695-697.

16.**Denhardt, D. T.** 1966. A membrane filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. 23:641.

17.**Ebert, A., and A. Brune.** 1997. Hydrogen concentration profiles at the oxic-anoxic interface: a microsensor study of the hindgut of the wood-feeding lower termite *Reticulitermes flavipes* (Kollar). Appl. Environ. Microbiol. 63:4039-4046.

18.**Embley, T. M., and B. J. Finlay.** 1994. The use of small subunit rRNA sequences to unravel the relationships between anaerobic ciliates and their methanogen endosymbionts. Microbiology. 140:225-235.

19.**Esch, G. W., A. O. Bush, and J. M. Aho.** 1990. Parasite communities: Patterns and processes. Chapman and Hall, London.

20.**Felsenstein, J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 39:783-791.

21.**Ferry, J. G.** 1989. Genus II. *Methanospirillum*, p. 2193-2195. In J. T. Staley, M. P. Bryant, N. Pfenning, and J. G. Holt (ed.), Bergey's Manual of Systematic Bacteriology, vol. 3. Williams & Wilkins, Baltimore.

22.**Fraser, P. J., R. A. Rasmussen, J. W. Creffield, J. R. French, and M. A. K. Khalil.** 1986. Termites and global methane - another assessment. Journal of Atmospheric Chemistry. 4:295-310.

23.**Fuhrman, J. A., K. McCallum, and A. A. Davis.** 1993. Phylogenetic diversity of subsurface marine microbial communities from the Atlantic and Pacific Oceans. Appl. Environ. Microbiol. 59:1294-1302.

24.**Guzen, H. J., C. A. M. Broers, M. Barughare, and C. K. Stumm.** 1991. Methanogenic bacteria as endosymbionts of the ciliate *Nyctotherus ovalis* in the cockroach hindgut. Appl. Environ. Microbiol. 57:1630-1634.

25.**Higashi, M., T. Abe, and T. P. Burns.** 1992. Carbon-nitrogen balance and termite ecology. Proc. R. Soc. Lond. B. 249:303-308.

26.**Holton, T. A., and M. W. Graham.** 1990. A simple and efficient method for direct cloning of PCR product using ddT-tailed vectors. Nucleic Acids Res. 19:1156.

27.**Honigberg, B. M.** 1970. Protozoa associated with termites and their role in digestion, p. 1-36. In K. Krishna and F. M. Weesner (ed.), Biology of Termite, vol. 2. Academic, New York.

28.**Hungate, R. E.** 1939. Experiments on the nutrition of *Zootermopsis*. III. The anaerobic carbohydrate dissimilation by the intestinal protozoa. Ecology. 20:230-244.

29.**Kataoka, N., Y. Tokiwa, and K. Takeda.** 1991. Improved technique for identification and enumeration of methanogenic bacteria colonies on roll tubes by epifluorescence microscopy. Appl. Environ. Microbiol. 57:3671-3673.

30.**Khalil, M. A. K., R. A. Rasmussen, J. R. J. French, and J. A. Holt.** 1990. The influence of termites on atmospheric trace gases: CH_4, CO_2, $CHCl_3$, N_2O, CO, H_2, and light hydrocarbons. J. Geophys. Res. 95:3619-3634.

31.**Kimura, M.** 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16:111-120.

32.**Koidzumi, M.** 1921. Studies on the intestinal protozoa found in the termites of Japan. Parasitology. 13:235-309.

33.**Krishna, D.** 1969. Introduction, p. 1-17. In K. Krishna and F. M. Weesner (ed.), Biology of Termites, vol. 1. Academic, New York.

34.**Krishna, K.** 1970. Taxonomy, phylogeny and distribution of termites, p. 127-152. In K. Krishna and F. M. Weesner (ed.), Biology of Termite, vol. 2. Academic, New York.

35.**Leadbetter, J. R., and J. A. Breznak.** 1996. Physiological ecology of *Methanobrevibacter cuticularis* sp. nov. and *Methanobrevibacter curvatus* sp. nov., isolated from the hindgut of the termite *Reticulitermes flavipes*. Appl. Environ. Microbiol. 62:3620-3631.

36.**Leadbetter, J. R., L. D. Crosby, and J. A. Breznak.** 1998. *Methanobrevibacter filiformis* sp. nov., a filamentous methanogen from termite hindguts. Arch. Microbiol. 169:287-292.

37.**Lee, K. E., and T. G. Wood.** 1971. Termites and Soils. Academic, New York.

38.**Lee, M. J., P. J. Schreurs, A. C. Messer, and S. H. Zinder.** 1987. Association of methanogenic bacteria with flagellated protozoa from a termite hindgut. Curr. Microbiol. 15:337-341.

39.**Maidak, B. L., N. Larsen, M. J. McCaughey, R. Overbeek, G. J. Olsen, K. Fogel, J. Blandy, and C. R. Woese.** 1994. The ribosomal database project. Nucleic Acids Res. 22:3620-3631.

40.**Martius, C., R. Wassmann, U. Thein, A. Bandeira, H. Rennenberg, W. Junk, and W. Seiler.** 1993. Methane emission from wood-feeding termites in Amazonia. Chemosphere. 26:623-632.

41.**Messer, A. C., and M. J. Lee.** 1989. Effect of chemical treatments on methane emission by the hindgut microbiota in the termite *Zootermopsis angusticollis*. Microb. Ecol. 18:275-284.

42.**Miller, T. L.** 1989. Genus II. *Methanobrevibacter*, p. 2178-2183, Bergey's Manual of Systematic Bacteriology, vol. 3. Williams & Wilkins, Baltimore.

43.**Mitter, C., and D. R. Brooks.** 1983. Phylogenetic aspects of coevolution, p. 65-98. In D. J. Futuyma and M. Slatkin (ed.), Coevolution.

44.**O'Brien, R. W., and M. Slaytor.** 1982. Role of microorganisms in the metabolism of termites. Aust. J. Biol. Sci. 35:239-262.

45.**Odelson, D. A., and J. A. Breznak.** 1985. Cellulase and other polymer-hydrolyzing activities of *Trichomitopsis termopsidis*, a symbiotic protozoan from termites. Appl. Environ. Microbiol. 49:622-626.

46.**Odelson, D. A., and J. A. Breznak.** 1985. Nutrition and growth characteristics of *Trichomitopsis termopsidis*, a cellulolytic protozoan from termites. Appl. Environ. Microbiol. 49:614-621.

47.**Odelson, D. A., and J. A. Breznak.** 1983. Volatile fatty acid production by the hindgut microbiota of xylophagous termites. Appl. Environ. Microbiol. 45:1602-1613.

48.**Ogimoto, K., and S. Imai.** 1981. Atlas of rumen microbiology. Japan Scientific Societies Press, Tokyo.

49.**Ohkuma, M., S. Noda, K. Horikoshi, and T. Kudo.** 1995. Phylogeny of symbiotic methanogens in the gut of the termite *Reticulitermes speratus*. FEMS Microbiol. Lett. 134:45-50.

50.**Rasmussen, R. A., and M. A. K. Khalil.** 1983. Global production of methane by termites. Nature. 301:700-702.

51.**Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406-425.

52.**Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a

laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.

53.**Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. 74:5463.

54.**Schink, B.** 1992. Syntrophism among prokaryotes, p. 276-299. *In* A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The Prokaryotes, 2 ed, vol. 1. Springer-Verlag, New York.

55.**Schulz, M. W., M. Slaytor, M. Hogan, and R. W. O'Brien.** 1986. Components of cellulase from the higher termite, *Nasutitermes walkeri*. Insect Biochem. 16:929-932.

56.**Seiler, W., R. Conrad, and D. Scharffe.** 1984. Field studies of methane emission from termite nests into the atmosphere and measurements of methane uptake by tropical soils. Journal of Atmospheric Chemistry. 1:171-186.

57.**Shinzato, N., H. Yoshino, and K. Yara.** 1993. Methane production by microbial symbionts in the lower and higher termites of the Ryukyu Archipelago, p. 161-166. *In* S. Sato, M. Ishida, and H. Ishikawa (ed.), Endocytobiology V. Tubingen University Press, Tubingen, Germany.

58.**Stahl, D. A., R. I. Amann, L. K. Poulsen, L. Raskin, and W. C. Capman.** 1995. Use of fluorescent probes for determinative microscopy of methanogenic archaea, p. 111-121. *In* K. R. Sowers and H. J. Schreier (ed.), Archaea: a laboratory manual Methanogens. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.

59.**Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673-4680.

60.**Trager, W.** 1934. The cultivation of a cellulose-digesting flagellate, *Trichomonas termopsidis*, and of certain other termite protozoa. Biol. Bull. 66:182-190.

61.**Vogels, G. D., W. Hoppe, and C. K. Stumm.** 1980. Association of methanogenic bacteria with rumen ciliates. Appl. Environ. Microbiol. 40:608-612.

62.**Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221-271.

63.**Wolin, M. J.** 1974. Metabolic interactions among intestinal microorganisms, p. 1320-1328. *In* M. H. Floch and D. J. Hentges (ed.), Intestinal Microbiology, vol. 27. American Journal of Clinical Nutrition.

64.**Wolin, M. J., and T. L. Miller.** 1983. Carbohydrate fermentation, p. 147-165. *In* D. J. Hentges (ed.), Human Intestinal Microflora in Health and Disease. Academic, New York.

65.**Yamin, M. A.** 1978. Axenic cultivation of the cellulolytic flagellate *Trichomitopsis termopsidis* (Cleveland) from the termite *Zootermopsis*. J. Protozool. 25:535-538.

66.**Zehnder, A. J. B.** 1989. Genus III. *Methanothrix*, p. 2207-2209. *In* J. T. Staley, M. P. Bryant, N. P. Pfenning, and J. G. Holt (ed.), Bergey's Manual of Systematic Bacteriology, vol. 3. Williams & Wilkins, Baltimore.

67.**Zimmerman, P. R., J. P. Greenberg, S. O. Wandiga, and P. J. Crutzen.** 1982. Termites: A potentially large source of atmospheric methane, carbon dioxide and molecular hydrogen. Science. 218:563-565.

68.**Zimmerman, P. R., and S. O. Greenberg.** 1983. Termites and methane. Nature. 302:354-355.

APPENDIX

SSU rRNA gene sequences of symbiotic methanogens in termites.

1. SSU rRNA gene sequences of symbiotic methanogens in *R. speratus*.

RS101-110: colony RS1 from Tokyo

RS201-210: colony RS2 from Tokyo

RS301-310: colony RS3 from Yamaguchi

RS401-410: colony RS4 from Tokyo

RS501-510: colony RS5 from Kobe

RS601-610: colony RS6 from Okinawa

```
          5        15        25        35        45        55        65        75
          |---------+---------+---------+---------+---------+---------+---------+
RS101  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS102  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS103  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS104  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS105  TTAAAGGAATTGGCGGGGGAGCACCACAACAGGTGGAGCCTGTGGTTTAATTGGATTCAGCGCCGGACAGCTCAC
RS106  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS107  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS108  TTAAAGGAATTGGCGGGGGAGCACCGCAACGGGAGGAGCGTGCGGTTTAATTGGATTCAACACCGGAAAACTCAC
RS109  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS110  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS201  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS202  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS203  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS204  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS205  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS206  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS207  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS208  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS209  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS210  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS301  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS302  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS303  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS304  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS305  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS306  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS307  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS308  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS309  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS310  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS401  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
```

```
RS403  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS404  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS405  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS406  TTAAAGGAATTGGCGGGGGAGCACCGCAACGGGAGGAGCGTGCGGTTTAATTGGATTCAACACCGGAAAACTCAC
RS407  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS408  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS409  TTAAAGGAATTGGCGGGGGAGCACCGCAACGGGAGGAGCGTGCGGTTTAATTGGATTCAACACCGGAAAACTCAC
RS410  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS501  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTAAACGCCGGACATCTCAC
RS502  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS503  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS504  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS505  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS506  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS507  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS508  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS509  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS510  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS601  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS602  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS603  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS604  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS605  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS606  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS607  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS608  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS609  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
RS610  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
E.coli TCAAATGAATTGACGGGGGCCCGC-ACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTAC

           85        95        105       115       125       135       145
           ----------+---------+---------+---------+---------+---------+---------+---

RS101  CAGGGGC-GACAGCA--GTATGATGGTCAGATTGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS102  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS103  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS104  CAGGGGC-GACAGCA--GTATGATGGTCAGATTGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS105  CGGATAA-GACAGCT--GCGTGATGGTCAATCTGAAGGTTTTACCT-GACTAGC-TGAGA--GGAGGTGCATGGC
RS106  CAGGGGC-GACAGCA--GTATGATGGTCAGATTGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS107  CAGGGGC-GACAGCA--GTATGATGGTCAGATTGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS108  CAGGGAA-GACTGTT--ACATGAAAGCCAGGCTAACGACCTTGCTC-GATTTTC-AGAGA--GGTGGTGCATGGC
RS109  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS110  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS201  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS202  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS203  CAGGGGC-GACAGCA--GTATGATGGTCAGATTGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS204  CAGGGGC-GACAGCA--GTATGATGGTCAGATTGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS205  CAGGGGC-GACAGCA--GTATGATGGTCAGATTGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS206  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS207  CAGGGGC-GACAGCA--GTATGATGGTCAGATTGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS208  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS209  CAGGGGC-GACAGCA--GTATGATGGTCAGATTGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS210  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS301  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTAATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS302  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
```

```
RS303  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS304  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS305  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTAATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS306  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS307  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS308  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS309  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS310  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS401  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS403  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS404  CAGGGGC-GACAGC---GTATGATAGCCAGTTTGATGAGCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS405  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS406  CAGGGAA-GACTGTT--ACATGAAAGCCAGGCTAACGACCTTGCTC-GATTTTC-AGAGA--GGTGGTGCATGGC
RS407  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS408  CAGGGGC-GACAGCA--GTATGATGGTCAGATTGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS409  CAGGGAA-GACTGTT--ACATGAAAGCCAGGCTAACGACCTTGCTC-GATTTTC-AGAGA--GGTGGTGCATGGC
RS410  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS501  CAGGGGC-GACAGCA--GTATGATGGTCAGATTGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS502  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS503  CAGGGGC-GACAGCA--GTATGATGGTCAGATTGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS504  CAGGGGC-GACAGCA--GTATGATGGTCAGATTGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS505  CAGGGGC-GACAGCA--GTATGATGGTCAGATTGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS506  CAGGGGC-GACAGCA--GTATGATGGTCAGATTGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS507  CAGGGGC-GACAGCA--GTATGATGGTCAGATTGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS508  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS509  CAGGGGC-GACAGCA--GTATGATGGTCAGATTGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS510  CAGGGGC-GACAGCA--GTATGATGGTCAGATTGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS601  CAGGGGC-GACAGCA--GTATGATGGTCAGATTGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS602  CAGGGGC-GACAGCA--GTATGATGGTCAGATTGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS603  CAGGGGC-GACAGCA--GTATGATGGTCAGATTGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS604  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS605  CAGGGGC-GACAGCA--GTATGATGGTCAGATTGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS606  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS607  CAGGGGC-GACAGCA--GTATGATGGTCAGATTGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS608  CAGGGGC-GACAGCA--GTATGATGGTCAGATTGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS609  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RS610  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
E.coli CTGGTCTTGACATCCACGGAAGTTTTCAGAGATGAGAATGTGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGC

          155       165       175       185       195       205       215       220
          ----+---------+---------+---------+---------+---------+---------+---------+

RS101  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
RS102  CCCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS103  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS104  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
RS105  CGTCGTCAGTTCGTACTGTGAAGCATCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCAACAATTGCCAGCAG
RS106  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
RS107  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
RS108  CGTCGTCAGTTCGTACCGTAAGGCGTTCTCTTAAGTGAGATAACGAACGAGACCCTCACCAATAGTTGCTACTTC
RS109  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS110  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS201  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS202  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS203  CGCCGTCAGCTCGTACCGTGAGGCGTCCCGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
```

```
RS204  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
RS205  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
RS206  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS207  CGCCGTCAGCTCGTACCGTGAGGCGTCCCGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
RS208  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS209  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
RS210  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS301  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCGG
RS302  CGCCGCCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS303  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS304  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS305  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCGG
RS306  CGCCGTCAGCTCGTCCCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS307  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS308  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS309  CGCCGCCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS310  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS401  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS403  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS404  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAACAG
RS405  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS406  CGTCGTCAGTTCGTACCGTAAGGCGTTCTCTTAAGTGAGATAACGAACGAGACCCTCACCAATAGTTGCTACTTT
RS407  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS408  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
RS409  CGTCGTCAGTTCGTACCGTAAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCTCACCAATAGTTGCTACTTT
RS410  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS501  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCTTTAGTTACCAGCAA
RS502  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS503  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCTTTAGTTACCAGCAA
RS504  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCTTTAGTTACCAGCAA
RS505  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCTTTAGTTACCAGCAA
RS506  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCTTTAGTTACCAGCAT
RS507  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCTTTAGTTACCAGCAA
RS508  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS509  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCTTTAGTTACCAGCAA
RS510  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCTTTAGTTACCAGCAA
RS601  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
RS602  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
RS603  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
RS604  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS605  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
RS606  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS607  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
RS608  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
RS609  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RS610  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
E.coli TGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGC-G

          235       245       255       265       275       285       295
          ----------+---------+---------+---------+---------+---------+---------
RS101  GT-C-TTTTTTTAAGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
RS102  GT-CC-TTTTTTTGGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGT
RS103  GT-CCTTTTTTT-GGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGT
RS104  GT-C-TTTTTTTAAGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
```

```
RS105  ---C--ATCTCTG-GATGGCTGGGGACATTGTTGGGACCGCCCCTGCGAAAGGGGAGGAAGGAATGGGCAACGGT
RS106  GT-C-TTTTTTTAAGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
RS107  GT-C-TTTTTTTAAGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
RS108  AT-CCTCCG----GGATGGA--GGCACACTATTGGGACCGCTGGCGCTAAGTCAGAGGAAGGAGAGGTCAACGGT
RS109  GT-CCTTTTTTTG-GATGTTTGGGCACACTAAGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGT
RS110  GT-CCTTTTTTT-GGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGT
RS201  GT-CCTTTTTTTG-GATGTTTGGGCACACTAAGGGACCGCCAGTGATAAATCGGAGGAAGGAGTGGACGACGGT
RS202  GT-CCTTTTTTT-GGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGT
RS203  GT-CTTTTTTTA-AGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
RS204  GT-C-TTTTTTTAAGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
RS205  GT-CTTTTTTT-AAGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
RS206  GT-CCTTTTTTT-GGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGT
RS207  GT-C-TTTTTTTAAGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
RS208  GT-CC-TTTTTTTGGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGT
RS209  GT-C-TTTTTT-AAGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
RS210  GT-CCTTTTTTT-GGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGT
RS301  AT-C----CTCCGGGATGCC-GGGCACACTAAGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
RS302  GT-CCTTTTTTTG-GATGTTTGGGCACACTAAGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGT
RS303  GT-CC-TTTTTTTGGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGT
RS304  GT-CC-TTTTTTTGGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGT
RS305  AT-CCTTC----GGGATGCC-GGGCACACTAAGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
RS306  GT-CCTTTTTTT-GGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGT
RS307  GT-CC-TTTTTTTGGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGT
RS308  GT-CC-TTTTTTTGGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGT
RS309  GT-CCTTTTTT--GGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGT
RS310  GT-CC-TTTTTTTGGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGT
RS401  GT-CCTTTTTT--GGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGT
RS403  GT-CCTTTTTTT-GGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGT
RS404  AT-CCTTTATT--GGATGCT-GGGCACACTAAGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
RS405  GT-CCTTTTTTT-GGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGT
RS406  AT-CCTCCGGGATGGA------GGCACACTATTGGGACCGCTGGCGCTAAGTCAGAGGAAGGAGAGGTCAACGGT
RS407  GT-CCTTTTTTT-GGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGT
RS408  GT-C-TTTTTT-AAGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
RS409  AT-C--CTCCG--GGATGG--AGGCACACTATTGGGACCGCTGGCGCTAAGTCAGAGGAAGGAGAGGTCAACGGT
RS410  GT-CCTTTTTTG-GATGTTTGGGCACACTAAGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGT
RS501  GT-C--TTTTTTAAGATGTTTGGGCACACTAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
RS502  GT-CCTTTTTTT-GGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGT
RS503  GT-C-TTTTTTA-AGATGTTTGGGCACACTAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
RS504  GT-C-TTTTTT-AAGATGTTTGGGCACACTAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
RS505  GT-C-TTTTTT-AAGATGTTTGGGCACACTAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
RS506  GT-CCTTTTTT-TGGATGTTTGGGCACACTAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
RS507  GT-C-TTTTTT-AAGATGTTTGGGCACACTAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
RS508  GT-CCTTTTTTTG-GATGTTTGGGCACACTAAGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGT
RS509  GT-C-TTTTTT-AAGATGTTTGGGCACACTAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
RS510  GT-C-TTTTTT-AAGATGTTTGGGCACACTAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
RS601  GT-C-TTTTTT-AAGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
RS602  GT-C-TTTTTT-AAGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
RS603  GT-C-TTTTTT-AAGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
RS604  GT-CCTTTTTT-TGGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAATCGGAGGAAGGAGTGGACGACGGT
RS605  GT--C-TTTTTTAAGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
RS606  GT-CCTTTTTT-TGGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGT
RS607  GT-C-TTTTTT-AAGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
RS608  GT--C-TTTTTTAAGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGT
RS609  GT-CCTTTTTT-TGGATGTTTGGGCACACTAAGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGT
```

```
RS610  GT-CCTTTTTT-TGGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAATCGGAGGAAGGAGTGGACGACGGT
E.coli GTCC----------GGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTC

         305       315       325       335       345       355       365       370
         ----+---------+---------+---------+---------+---------+---------+---------+

RS101  AGGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGG-GTACAATGGCTGAGACAATGGGTTCCGACATTGAAA
RS102  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGGGTTCTGACATTGAAA
RS103  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGGGTTCTGACATTGAAA
RS104  AGGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGG-CTACAATGGCTGAGACAATGGGTTCCGACATTGAAA
RS105  TGGTCAGCATGCCCCGAATTATCCGGCTACACGCGGG-CTACAATGGCCGGGACAATGGGTCACAACACCGAAA
RS106  AGGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGG-CTACAATGGCTGAGACAATGGGTTCCGACATTGAAA
RS107  AGGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGG-CTACAATGGCTGAGACAATGGGTTCCGACATTGAAA
RS108  AGGTCAGTATGCCCCGAATTTCCTGGGCTACACGCGCG-CTACAAAGGGCGGGACAATGGGTCCCTACACCGAAA
RS109  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGGGTTCTGACATTGAAA
RS110  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGGGTTCTGACATTGAAA
RS201  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGGGCTACAATGGCTAAGACAATGGGTTCTGACATTGAAA
RS202  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGGGTTCTGACATTGAAA
RS203  AGGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGG-CTACAATGGCTGAGACAATGGGTTCCGACATTGAAA
RS204  AGGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGG-CTACAATGGCTGAGACAATGGGTTCCGACATTGAAA
RS205  AGGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGG-CTACAATGGCTGAGACAATGGGTTCCGACATTGAAA
RS206  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGGGTTCTGACATTGAAA
RS207  AGGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGG-CTACAATGGCTGAGACAATGGGTTCCGACATTGAAA
RS208  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGGGTTCTGACATTGAAA
RS209  AGGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGG-CTACAATGGCTGAGACAATGGGTTCCGACATTGAAA
RS210  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGGGTTCTGACATTGAAA
RS301  AGGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGG-CTACAATGGCTAAGACAATGGGTTCCAACACTGAAA
RS302  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGGGTTCTGACATTGAAA
RS303  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGGGTTCTGACATTGAAA
RS304  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGGGTTCTGACATTGAAA
RS305  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGAGTTCCGCCACTGAAA
RS306  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGGGTTCTGACATTGAAA
RS307  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGGGTTCTGACATTGAAA
RS308  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGGGTTCTGACATTGAAA
RS309  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGGGTTCTGACATTGAAA
RS310  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGGGTTCTGACATTGAAA
RS401  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGGGTTCTGACATTGAAA
RS403  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGGGTTCTGACATTGAAA
RS404  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGGGTTCCGACATTGAAA
RS405  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGGGTTCTGACATTGAAA
RS406  AGGTCAGTATGCCCCGAATTTCCTGGGCTACACGCGCG-CTACAAAGGGCGGGACAATGGGTCCCTACACCGAAA
RS407  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGGGTTCTGACATTGAAA
RS408  AGGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGG-CTACAATGGCTGAGACAATGGGTTCCGACATTGAAA
RS409  AGGTCAGTATGCCCCGAATTTCCTGGGCTACACGYGCG-CTACAAAGGGCGGGACAATGGGTCCCTACACCGAAA
RS410  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGGGTTCTGACATTGAAA
RS501  AGGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGG-CTACAATGGCTGAGACAATGGGTTCCGACATTGAAA
RS502  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGGGTTCTGACATTGAAA
RS503  AGGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGG-CTACAATGGCTGAGACAATGGGTTCCGACATTGAAA
RS504  AGGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGG-CTACAATGGCTGAGACAATGGGTTCCGACATTGAAA
RS505  AGGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGG-CTACAATGGCTGAGACAATGGGTTCCGACATTGAAA
RS506  AGGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGG-CTACAATGGCTGAGACAATGGGTTCCGACATTGAAA
RS507  AGGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGG-CTACAATGGCTGAGACAATGGGTTCCGACATTGAAA
RS508  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAACACAATGGGTTCTGACATTGAAA
RS509  AGGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGG-CTACAATGGCTGAGACAATGGGTTCCGACATTGAAA
RS510  AGGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGG-CTACAATGGCTGAGACAATGGGTTCCGACATTGAAA
```

```
RS601  AGGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGG-CTACAATGGCTGAGACAATGGGTTCCGACATTGAAA
RS602  AGGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGG-CTACAATGGCTGAGACAATGGGTTCCGACATTGAAA
RS603  AGGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGG-CTACAATGGCTGAGACAATGGGTTCCGACATTGAAA
RS604  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGGGTTCTGACATTGAAA
RS605  AGGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGG-CTACAATGGCTGAGACAATGGGTTCCGACATTGAAA
RS606  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTGAGACAATGGGTTCCGACATTGAAA
RS607  AGGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGG-CTACAATGGCTGAGACAATGGGTTCCGACATTGAAA
RS608  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTGAGACAATGGGTTCCGACATTGAAA
RS609  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGGGTTCTGACATTGAAA
RS610  AGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGG-CTACAATGGCTAAGACAATGGGTTCTGACATTGAAA
E.coli AAGTCATCATGGCCCTTACGACCAGGGCTACACACGTG-CTACAATGGCGCATACAAAGAGAAGCGACCTCGCGA

                385        395        405        415        425        435        445
                |----------|----------|----------|----------|----------|----------|----
RS101  AGTGGAGG-TAATCCCC-TAAACTTAGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCGTCATAAAGCTGG-AA
RS102  GGTGGAGG-TAATCCCC-TAAACTTGGTC-GTAGTTCGGATTGAGGGCTGTGACTCGCCCTCATGAAGCTGG-AA
RS103  GGTGGAGG-TAATCCCC-TAAACTTGGTC-GTAGTTCGGATTGAGGGCTGTGACTCGCCCTCATGAAGCTGG-AA
RS104  AGTGAAGG-TAATCCCC-TAAACTTAGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGG-AA
RS105  GGTGGCGC-CAATCTCC-TAACCCCGGTC-TTAGTTAGGATTGCGGGTTGCAACTCACCCGCATGAATTTGG-AA
RS106  AGTGGAGG-TAATCCCC-TAAACTTAGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGG-AA
RS107  AGTGGAGG-TAATCCCC-TAAACTTAGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGG-AA
RS108  GGTGGAGG-TAATCCCG--AAACCCGTCC-GTAGTTCGGATTGAGGGTTGTAACTCACCCTCATGAAGCTGG-AT
RS109  GGTGGAGG-TAATCCCC-TAAACTTGGTC-GTAGTTCGGATTGAGGGCTGTGACTCGCCCTCATGAAGCTGG-AA
RS110  GGTGGAGG-TAATCCCC-TAAACTTGGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGG-AA
RS201  AGTGGAGG-TAATCCCC-TAAACTTGGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGG-AA
RS202  GGTGGAGG-TAATCCCC-TAAACTTGGTC-GTAGTTCGGATTGAGGGCTGTGACTCGCCCTCATGAAGCTGG-AA
RS203  AGTGGAGG-TAATCCCC-TAAACTTAGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGG-AA
RS204  AGTGGAGG-TAATCCCC-TAAACTTAGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGG-AA
RS205  AGTGAAGG-TAATCCCCCTAAACTTAGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGG-AA
RS206  GGTGGAGG-TAATCCCCCTAAACTTGGTC-GTAGTTCGGATTGAGGGCTGTGACTCGCCCTCATGAAGCTGG-AA
RS207  AGTGGAGG-TAATCCCC-TAAACTTAGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGG-AA
RS208  GGTGGAGG-TAATCCCC-TAAACTTGGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGG-AA
RS209  AGTGAAGG-TAATCCCC-TAAACTTAGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGG-AA
RS210  GGTGGAGG-TAATCCCC-TAAACTTGGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGG-AA
RS301  AGTGAAGG-TAATCCCC-TAAACTTAGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGG-AA
RS302  GGTGGAGG-TAATCCCC-TAAACTTGGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGG-AA
RS303  GGTGGAGG-TAATCCCC-TAAACTTGGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGG-AA
RS304  GGTGGAGG-TAATCCCC-TAAACTTGGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGG-AA
RS305  AGTGAAGG-TAATCTCC-TAAACTTAGTC-GTAGTTCGGATTGAGGGCTGTAACTTGCCCTCATGAAGCTGG-AA
RS306  GGTGGAGG-TAATCCCC-TAAACTTGGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGG-AA
RS307  GGTGGAGG-TAATCCCC-TAAACTTGGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGG-AA
RS308  GGTGGAGG-TAATCCCC-TAAACTTGGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGG-AA
RS309  GGTGGAGG-TAATCCCC-TAAACTTGGTT-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGG-AA
RS310  GGTGGAGG-TAATCCCC-TAAACTTGGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGG-AA
RS401  GGTGGAGG-TAATCCCC-TAAACTTGGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGG-AA
RS403  GGTGGAGG-TAATCCCC-TAAACTTGGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGG-AA
RS404  AGTGGAGG-TAATCCCCT-AAACTTGGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGG-AA
RS405  GGTGGAGG-TAATCCCC-TAAACTTGGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGG-AA
RS406  GGTGGAGG-TAATCCCG--AAACCCGTCC-GTAGTTCGGATTGAGGGTTGTAACTCACCCTCATGAAGCTGG-AT
RS407  GGTGGAGG-TAATCCCC-TAAACTTGGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGG-AA
RS408  AGTGAAGG-TAATCCCC-TAAACTTAGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGG-AA
RS409  GGTGGAGGGTAATCCCG--AAACCCGTCC-GTAGTTCGGATTGAGGGTTGTAACTCACCCTCATGAAGCTGGGAT
RS410  GGTGGAGG-TAATCCCC-TAAACTTGGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGG-AA
```

```
RS501  AGTGAAGG-TAATCCCC-TAAACTTAGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGG-AA
RS502  GGTGGAGG-TAATCCCC-TAAACTTGGTC-GTAGTTCGGATTGAGGGCTGTGACTCGCCCTCATGAAGCTGG-AA
RS503  AGTGAAGG-TAATCCCC-TAAACTTAGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGG-AA
RS504  AGTGAAGG-TAATCCCC-TAAACTTAGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGG-AA
RS505  AGTGAAGG-TAATCCCC-TAAACTTAGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGG-AA
RS506  AGTGAAGG-TAATCCCC-TAAACTTAGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGG-AA
RS507  AGTGAAGG-TAATCCCC-TAAACTTAGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGG-AA
RS508  GGTGGAGG-TAATCCCC-TAAACTTGGTC-GTAGTTCGGATTGAGGGCTGTGACTCGCCCTCATGAAGCTGG-AA
RS509  AGTGAAGG-TAATCCCC-TAAACTTAGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGG-AA
RS510  AGTGAAGG-TAATCCCC-TAAACTTAGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGG-AA
RS601  AGTGAAGG-TAATCCCC-TAAACTTAGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGG-AA
RS602  AGTGAAGG-TAATCCCC-TAAACTTAGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGG-AA
RS603  AGTGAAGG-TAATCCCC-TAAACTTAGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGG-AA
RS604  AGTGCAGG-TAATCCCCT-AAACTTGGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGG-AA
RS605  AGTGAAGG-TAATCCCC-TAAACTTAGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGG-AA
RS606  AGTGAAGG-TAATCCCC-TAAACTTGGTC-GTAGTTCGGATTGAGGGCTGTGACTCGCCCTCATGAAGCTGG-AA
RS607  AGTGAAGG-TAATCCCC-TAAACTTAGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGG-AA
RS608  AGTGAAGG-TAATCCCC-TAAACTTAGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGG-AA
RS609  GGTGGAGG-TAATCCCC-TAAACTTGGTC-GTAGTTCGGATTGAGGGCTGTGACTCGCCCTCATGAAGCTGG-AA
RS610  AGTGGAGG-TAATCCCC-TAAACTTGGTC-GTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGG-AA
E.coli GAGCAAGCGGA--CCTCATAAAGTGCGTC-GTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGG-AA

          455       465       475       485       495       505       515
          ----+---------+---------+---------+---------+---------+---------+---
RS101  TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS102  TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS103  TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS104  TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS105  TCTGTAGTAATCGCGTTTCACTATAGCGCGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS106  TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS107  TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS108  TCCGTAGTAATCGCGAATCAACAACTCGCGGTGAATATGCCCCTGCTCCTTGCACACACCGCCCGTCA
RS109  TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS110  TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS201  TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS202  TGCGTAGTAATCGTGTGTCATAATCGCACGGTAAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS203  TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS204  TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS205  TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS206  TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS207  TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS208  TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS209  TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS210  TGCGTAGTAATCGTGCGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS301  TGCGTAGTAATCGCGTGTCATAATCGCGCGGTGAATACGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS302  TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS303  TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS304  TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS305  TGCGTAGTAATCGCGTGTCATAATCGCGCGGTGAATACGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS306  TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS307  TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS308  TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS309  TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS310  TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
```

```
RS401   TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS403   TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS404   TGCGTAGTAATCGCGTGTCATAATCGCGCGGTGAATACGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS405   TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS406   TCCGTAGTAATCGCGAATCAACAACTCGCGGTGAATATGCCCCTGCTCCTTGCACACACCGCCCGTCA
RS407   TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS408   TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS409   TCCGTAGTAATCGCGAATCAACAACTCGCGGTGAATATGCCCCTGCTCCTTGCACACACCGCCCGTCA
RS410   TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS501   TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS502   TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS503   TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS504   TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS505   TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS506   TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCTTCA
RS507   TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS508   TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS509   TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS510   TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS601   TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS602   TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS603   TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS604   TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS605   TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGCCCCTGCTCCTTGCACACACCGCCCGTCA
RS606   TGCGTAGTAATCGTATGTCATAATCGCACGGTAAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS607   TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS608   TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS609   TGCGTAGTAATCGTGTGTCATAATCGCACGGTAAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RS610   TGCGTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
E.coli  TCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCA
```

2. SSU rRNA gene sequences of symbiotic methanogens in the Japanese termites.

RK101-105: *Reticulitermes kanmonensis*

CF101-105: *Coptotermes formosanus*

NK101-105: *Neotermes koshunensis*

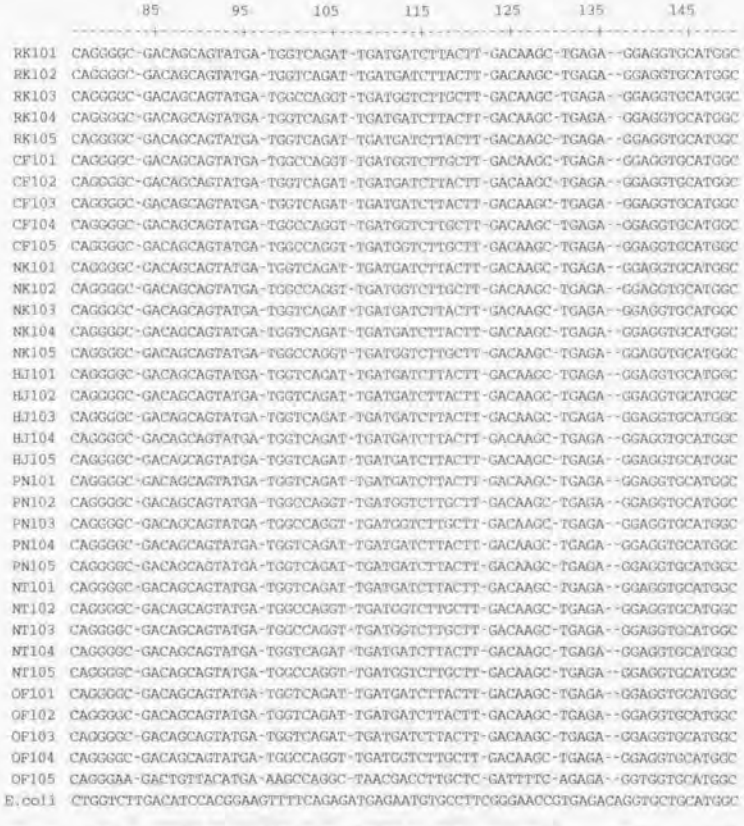
HJ101-105: *Hodotermopsis japonica*

PN101-105: *Pericapritermes nitobei*

NT101-105: *Nasutitermes takasagoensis*

OF101-105: *Odontotermes formosanus*

```
            5         15        25        35        45        55        65        75
            |---------|---------|---------|---------|---------|---------|---------|
  RK101 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  RK102 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  RK103 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  RK104 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  RK105 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  CF101 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  CF102 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  CF103 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  CF104 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  CF105 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  NK101 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  NK102 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  NK103 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  NK104 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  NK105 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  HJ101 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGC-TGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  HJ102 TTAAAGGAATTGGCGGGGGAGCACCGCAACGGGAGGAGCGTGCGGTTTAATTGGATTCAACACCGGAAAACTCAC
  HJ103 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  HJ104 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  HJ105 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  PN101 TTAAAGGAATTGGCCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  PN102 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  PN103 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  PN104 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  PN105 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATCCAACGCCGGACATCTCAC
  NT101 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  NT102 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  NT103 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  NT104 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  NT105 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  OF101 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  OF102 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  OF103 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  OF104 TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
  OF105 TTAAAGGAATTGGCGGGGGAGCACCGCAACGGGAGGAGCGTGCGGTTTAATTGGATTCAACACCGGAAAACTCAC
E.coli  TCAAATGAATTGACGGGGGCCCGC-ACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTAC
```


85 95 105 115 125 135 145
RK101 CAGGGGC-GACAGCAGTATGA-TGGTCAGAT-TGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RK102 CAGGGGC-GACAGCAGTATGA-TGGTCAGAT-TGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RK103 CAGGGGC-GACAGCAGTATGA-TGGCCAGGT-TGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RK104 CAGGGGC-GACAGCAGTATGA-TGGTCAGAT-TGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
RK105 CAGGGGC-GACAGCAGTATGA-TGGTCAGAT-TGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
CF101 CAGGGGC-GACAGCAGTATGA-TGGCCAGGT-TGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
CF102 CAGGGGC-GACAGCAGTATGA-TGGTCAGAT-TGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
CF103 CAGGGGC-GACAGCAGTATGA-TGGTCAGAT-TGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
CF104 CAGGGGC-GACAGCAGTATGA-TGGCCAGGT-TGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
CF105 CAGGGGC-GACAGCAGTATGA-TGGCCAGGT-TGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
NK101 CAGGGGC-GACAGCAGTATGA-TGGTCAGAT-TGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
NK102 CAGGGGC-GACAGCAGTATGA-TGGCCAGGT-TGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
NK103 CAGGGGC-GACAGCAGTATGA-TGGTCAGAT-TGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
NK104 CAGGGGC-GACAGCAGTATGA-TCGTCAGAT-TGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
NK105 CAGGGGC-GACAGCAGTATGA-TGGCCAGGT-TGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
HJ101 CAGGGGC-GACAGCAGTATGA-TGGTCAGAT-TGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
HJ102 CAGGGGC-GACAGCAGTATGA-TGGTCAGAT-TGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
HJ103 CAGGGGC-GACAGCAGTATGA-TGGTCAGAT-TGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
HJ104 CAGGGGC-GACAGCAGTATGA-TGGTCAGAT-TGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
HJ105 CAGGGGC-GACAGCAGTATGA-TGGTCAGAT-TGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
PN101 CAGGGGC-GACAGCAGTATGA-TGGTCAGAT-TGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
PN102 CAGGGGC-GACAGCAGTATGA-TGGCCAGGT-TGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
PN103 CAGGGGC-GACAGCAGTATGA-TGGCCAGGT-TGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
PN104 CAGGGGC-GACAGCAGTATGA-TGGTCAGAT-TGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
PN105 CAGGGGC-GACAGCAGTATGA-TGGTCAGAT-TGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
NT101 CAGGGGC-GACAGCAGTATGA-TGGTCAGAT-TGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
NT102 CAGGGGC-GACAGCAGTATGA-TGGCCAGGT-TGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
NT103 CAGGGGC-GACAGCAGTATGA-TGGCCAGGT-TGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
NT104 CAGGGGC-GACAGCAGTATGA-TGGTCAGAT-TGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
NT105 CAGGGGC-GACAGCAGTATGA-TGGCCAGGT-TGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
OP101 CAGGGGC-GACAGCAGTATGA-TGGTCAGAT-TGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
OP102 CAGGGGC-GACAGCAGTATGA-TGGTCAGAT-TGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
OP103 CAGGGGC-GACAGCAGTATGA-TGGTCAGAT-TGATGATCTTACTT-GACAAGC-TGAGA--GGAGGTGCATGGC
OP104 CAGGGGC-GACAGCAGTATGA-TGGCCAGGT-TGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
OP105 CAGGGAA-GACTGTTACATGA-AAGCCAGGC-TAACGACCTTGCTC-GATTTTC-AGAGA--GGTGGTGCATGGC
E.coli CTGGTCTTGACATCCACGGAAGTTTTCAGAGATGAGAATGTGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGC
155 165 175 185 195 205 215 220
RK101 CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
RK102 CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
RK103 CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
RK104 CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
RK105 CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
CF101 CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
CF102 CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
CF103 CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
CF104 CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
CF105 CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
NK101 CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
NK102 CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
NK103 CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA

```
SK104  CGCCGTCAGCTCGTACCGTGAGGCGTCCCGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
NK105  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
HJ101  CGCCGTCAGCTCGTACCGTGAGGCGTCCCGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
HJ102  CGCCGTCAGCTCGTACCGTGAGGCGTCCCGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
HJ103  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
HJ104  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
HJ105  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
PN101  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
PN102  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
PN103  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
PN104  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
PN105  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
NT101  CGCCGTCAGCTCGTACCGTGAGGCGTCCCGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
NT102  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
NT103  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
NT104  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
NT105  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
OF101  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
OF102  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
OF103  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
OF104  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAT
OF105  CGTCGTCAGTTCGTACCGTAAGGCCTTCTCTTAAGTGAGATAACGAACGAGACCCTCACCAATAGTTGCTACT--
E.coli TGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCG-

          235       245       255       265       275       285       295
-------------------+---------+---------+---------+---------+---------+---------+-----
RK101  GT-CTTTTTTTAAGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTA
RK102  GT-CTTTTTTTAAGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTA
RK103  GTCCTTTTTTT-GGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTA
RK104  GT-C-TTTTTTAAGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTA
RK105  GT-C-TTTTTTAAGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTA
CF101  GTCCTTTTTTT-GGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGTA
CF102  GTC-TTTTTTTAAGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTA
CF103  GTC-TTTTTT-AAGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTA
CF104  GTCCTTTTTTT-GGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTA
CF105  GTCCTTTTTTT-GGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTA
NK101  GT--CTTTTTTAAGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTA
NK102  GTCCTTTTTTT-GGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGTA
NK103  GT--CTTTTTTAAGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTA
NK104  GTCTTTTTTTA-AGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTA
NK105  GTCC-TTTTTTTGGATGTTTGGGCACACTAAGGGGACCGCCAATGATAAATTGGAGGAAGGAGTAGACGACGGTA
HJ101  GTCTTTTTTTA-AGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAGGGAGTGGACGACGGTA
HJ102  GTCTTTTTT-A-AGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTA
HJ103  GT-CTTTTTTTAAGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTA
HJ104  GT-CTTTTTTTAAGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTA
HJ105  GT--CTTTTTTAAGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTA
PN101  GT-CTTTTTTTAAGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTA
PN102  GTCCTTTTTTT-TGGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGTA
PN103  GTCCTTTTTTT-GGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGTA
PN104  GT-CTTTTTTTAAGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTA
PN105  GTC--TTTTTTAAGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTA
NT101  GTCTTTTTTTA-AGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTA
NT102  GTCC-TTTTTTTGGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGTA
NT103  GTCCTTTTTTT-GGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGTA
```

```
NT104  GTCTTTTTT-A-AGATGTTTGGGCACACTAGGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTA
NT105  GTCCTTTTTTT-GGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGTA
OF101  GT--CTTTTTTAAGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTA
OF102  GT--CTTTTTTAAGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTA
OF103  GT--CTTTTTTAAGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTA
OF104  GTCCTTTTTTT-GGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGTA
OP105  -TCATCCTCCG-GGATGGA--GGCACACTATTGGGACCGCTGGCGCTAAGTCAGAGGAAGGAGAGGTCAACGGTA
E.coli GTC-----------CGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCA

          305        315        325        335        345        355        365        370
          ----+---------+---------+---------+---------+---------+---------+---------+
RK101  GGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAAAAG
RK102  GGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAAAAG
RK103  GGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGGCTACAATGGCTAAGACAATGGGTTCTGACATTGAAAGG
RK104  GGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAAAAG
RK105  GGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAAAAG
CF101  GGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGGCTACAATGGCTAAGACAATGGGTTCTGACATTGAAAGG
CF102  GGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAAAAG
CF103  GGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAAAAG
CF104  GGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAAAAG
CF105  GGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAAAAG
NK101  GGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAAAAG
NK102  GGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGGCTACAATGGCTAAGACAATGGGTTCTGACATTGAAAAG
NK103  GGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAAAAG
NK104  GGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAAAAG
NK105  GGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGGCTACAATGGCTAAGACAATGGGTTCTGACATTGAAAGG
HJ101  GGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAAAAG
HJ102  GGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAAAAG
HJ103  GGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAAAAG
HJ104  GGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAAAAG
HJ105  GGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAAAAG
PN101  GGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAAAAG
PN102  GGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGGCTACAATGGCTAAGACAATGGGTTCTGACATTGAAAGG
PN103  GGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGGCTACAATGGCTAAGACAATGGGTTCTGACATTGAAAGG
PN104  GGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAAAAG
PN105  GGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAAAAG
NT101  GGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAAAAG
NT102  GGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGGCTACAATGGCTAAGACAATGGGTTCTGACATTGAAAGG
NT103  GGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGGCTACAATGGCTAAGACAATGGGTTCTGACATTGAAAGG
NT104  GGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAAAAG
NT105  GGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGGCTACAATGGCTAAGACAATGGGTTCTGACATTGAAAGG
OF101  GGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAAAAG
OF102  GGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAAAAG
OP103  GGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAAAAG
OP104  GGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGGCTACAATGGCTAAGACAATGGGTTCTGACATTGAAAGG
OP105  GGTCAGTATGCCCCGAATTTCCTGGGCTACACGCGGGCTACAAAGGGCGGGACAATGGGTCCCTACACCGAAAGG
E.coli AGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGA-G

          385        395        405        415        425        435        445
          ----+---------+---------+---------+---------+---------+---------+---------+
RK101  TGGAGGTAATCCCC-TAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGGAATGCGT
RK102  TGGAGGTAATCCCC-TAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGGAATGCGT
RK103  TGGAGGTAATCCCC-TAAACTTGGTCGTAGTTCGGATTGAGGGCTGTGACTCGCCCTCATGAAGCTGGAATGCGT
RK104  TGAAGGTAATCCCC-TAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGGAATGCGC
```

```
RK105  TGAAGGTAATCCCC-TAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGGAATGCGT
CF101  TGGAGGTAATCCCC-TAAACTTGGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGGAATGCGT
CF102  TGGAGGTAATCCCC-TAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGGAATGCGT
CF103  TGAAGGTAATCCCC-TAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGGAATGCGT
CF104  TGGAGGTAATCCCC-TAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGGAATGCGT
CF105  TGGAGGTAATCCCC-TAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGGAATGCGT
NK101  TGAAGGTAATCCCCCTAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGGAATGCGT
NK102  TGAAGGTAATCCCC-TAAACTTAGTCGTAGTTCGGATTGAGGGCTGTGACTCGCCCTCATGAAGCTGGAATGCGT
NK103  TGAAGGTAATCCCC-TAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGGAATGCGT
NK104  TGGAGGTAATCCCC-TAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGGAATGCGT
NK105  TGGAGGTAATCCCC-TAAACTTGGTCGTAGTTCGGATTGAGGGCTGTGACTCGCCCTCATGAAGCTGGAATGCGT
HJ101  TGGAGGTAATCCCC-TAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGGAATGCGT
HJ102  TGGAGGTAATCCCC-TAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGGAATGCGT
HJ103  TGGAGGTAATCCCC-TAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGGAATGCGT
HJ104  TGGAGGTAATCCCC-TAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGGAATGCGT
HJ105  TGAAGGTAATCCCC-TAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGGAATGCGT
PN101  TGGAGGTAATCCCC-TAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGGAATGCGT
PN102  TGGAGGTAATCCCC-TAAACTTGGTCGTAGTTCGGATTGAGGGCTGTGACTCGCCCTCATGAAGCTGGAATGCGT
PN103  TGGAGGTAATCCCC-TAAACTTGGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGGAATGCGT
PN104  TGGAGGTAATCCCC-TAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGGAATGCGT
PN105  TGAAGGTAATCCCC-TAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGGAATGCGT
NT101  TGGAGGTAATCCCC-TAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGGAATGCGT
NT102  TGGAGGTAATCCCC-TAAACTTGGTCGTAGTTCGGATTGAGGGCTGTGACTCGCCCTCATGAAGCTGGAATGCGT
NT103  TGGAGGTAATCCCC-TAAACTTGGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGGAATGCGT
NT104  TGAAGGTAATCCCC-TAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGGAATGCGT
NT105  TGGAGGTAATCCCC-TAAACTTGGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGGAATGCGT
OF101  TGAAGGTAATCCCC-TAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGGAATGCGT
OF102  TGAAGGTAATCCCC-TAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGGAATGCGT
OF103  TGAAGGTAATCCCC-TAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGGAATGCGT
OF104  TGGAGGTAATCCCC-TAAACTTGGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGGAATGCGT
OF105  TGGAGGTAATCCCG--AAACCCGTCCGTAGTTCGGATTGAGGGTTGTAACTCACCCTCATGAAGCTGGATTCCGT
E.coli AGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCT

          455       465       475       485       495       505
          ----+---------+---------+---------+---------+---------+---------
RK101  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RK102  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RK103  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RK104  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
RK105  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
CF101  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
CF102  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
CF103  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
CF104  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
CF105  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
NK101  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
NK102  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
NK103  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
NK104  AGTAATCGTGTGTCATGATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
NK105  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
HJ101  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCAATCA
HJ102  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
HJ103  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
HJ104  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
```

```
HJ105  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTAA
PN101  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
PN102  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
PN103  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
PN104  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
PN105  AGTAATCGTGTGTCGTAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
NT101  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
NT102  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCCCCTTGCACACACCGCCCGTCA
NT103  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
NT104  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
NT105  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
OF101  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
OF102  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
OF103  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
OF104  AGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
OF105  AGTAATCGCGAATCAACAACTCGCGGTGAATATGCCCCTGCTCCTTGCACACACCGCCCGTCA
E.coli  AGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCA
```

3. SSU rRNA gene sequences of symbiotic methanogens in *M.darwiniensis*.

```
            5         15         25         35         45         55         65         75
            |---------+----------+----------+----------+----------+----------+----------+
MD101  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
MD102  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
MD103  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
MD104  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
MD105  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
E. coli TCAAATGAATTGACGGGGGCCCGC-ACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTAC

                 85         95        105        115        125        135        145
            |---------+----------+----------+----------+----------+----------+----------+
MD101  CAGGGGC-GACAGCA--GAATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
MD102  CAGGGGC-GACAGAG--GAATGATAGCCAGGTTGATGGTTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
MD103  CAGGGGC-GACAGCA--GTATGATGGTCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
MD104  CAGGGGC-GACAGCA--GAATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
MD105  CAGGGGC-GACAGCA--GTATGATGGCCAGGTTGATGGTCTTGCTT-GACAAGC-TGAGA--GGAGGTGCATGGC
E. coli CTGGTCTTGACATCCACGGAAGTTTTCAGAGATGAGAATGTGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGC

            155        165        175        185        195        205        215        220
            |---------+----------+----------+----------+----------+----------+----------+
MD101  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCGG
MD102  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCTCTTAGTTACCAGCAG
MD103  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCGG
MD104  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCGG
MD105  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCGG
E. coli TGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGG

                 235        245        255        265        275        285        295
            |---------+----------+----------+----------+----------+----------+----------+
MD101  ATCCTCCG----GGATGCCGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTAG
MD102  GTCCTTTTT--TGGATGCTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTAG
MD103  ATCCTTTTTTTTGGATGCCGGGCACACTAAGGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGTAG
MD104  ATCCTCCG----GGATGCCGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTAG
MD105  ATCCTC----CGGGATGCCGGGCACACTAACGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTAG
E. coli -TCCG----------GCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAA

            305        315        325        335        345        355        365        370
            |---------+----------+----------+----------+----------+----------+----------+
MD101  GTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGGCTACAATGGCTAAGACAATGGGTTCCAACACTGAAAAGT
MD102  GTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACGCTGAAAGGC
MD103  GTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAAAAGT
MD104  GTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGGCTACAATGGCTAAGACAATGGGTTCCAACACTGAAAAGT
MD105  GTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGGCTACAATGGCTAAGACAATGGGTTCCAACACTGAAAAGT
E. coli GTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAG

                 385        395        405        415        425        435        445
            |---------+----------+----------+----------+----------+----------+----------+
MD101  GAAGGTAATCCCCTAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGGAATGCGTAG
MD102  GAGGGTAATCCTCTAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGGAATGCGTAG
MD103  GGAGGTAATCCTCTAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGGAATGCGTAG
MD104  GAAGGTAATCCCCTAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGGAATGCGTAG
MD105  GAAGGTAATCCCCTAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGGAATGCGTAG
```

```
E. coli  CAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAG

            455        465        475        485        495        505
         ----+---------+---------+---------+---------+---------+------
  MD101  TAATCGCGTGTCATAATCGCGCGGTGAATACGTCCCTGCTCCTTGCACACACCGCCCGTCA
  MD102  TAATCGCGTGTCATAATCGCGCGGTGAATACGTCCCTGCTCCTTGCACACACCGCCCGTCA
  MD103  TAATCGCGTGTCATAATCGTGCGGTGAATACGTCCCTGCTCCTTGCACACACCGCCCGTCA
  MD104  TAATCGCGTGTCATAATCGCGCGGTGAATACGTCCCTGCTCCTTGCACACACCGCCCGTCA
  MD105  TAATCGCGTGTCATAATCGCGCGGTGAATACGTCCCTGCTCCTTGCACACACCGCCCGTCA
E. coli  TAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCA
```

4. SSU rRNA gene sequences of symbiotic methanogens in the cockroach *S. esakii*.

```
           5         15         25         35         45         55         65         75
           |----+----------+----------+----------+----------+----------+----------+----------+
SE101      TTAGAGGAATTGGCGGGGGGGCACCGCAACGGGAGGAGCGTGCGGTTTAATTGGATTCAACACCGGAAAACTCAC
SE102      TTGAAGGGATTGGCGGGGG-GCACCACAACGCGTGGAGC-TGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
SE103      TTTAGGGAATTGGCGGGGGAGCACCACAACGCGTGGAGC-TGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
SE104      TTAAGGGAATTGGCGGGGGAGCACCGCAACGGGAGGAGCGTGCGGTTTAATTGGATTCAACACCGGAAAACTCAC
SE105     GAATTCGATTTGGCGGGGGAGCACCGCAACGGGAGGAGCGTGCGGTTTAATTGGATTCAACACCGGAAAACTCAC
E.coli     TCAAATGAATTGACGGGGG-CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTAC

                  85         95        105        115        125        135        145
           |----+----------+----------+----------+----------+----------+----------+----------+
SE101      CAGGAGC-GACG----GTTACATGAAAGTCAGGCTGATG-ACCTTACTCGATTTTCCGAGAGGTGGTGCATGGC
SE102      CAGGGGC-GACA----GCAGTATGATGGCCAGGCTGATG-GCCTTGCTTGACAAGCTGAGAGGAGGTGCATGGC
SE103      CAGAGGC-GACA----GCAGTATGATGGCCAAGCTGATG-ACTTTGCTTGACAAGCTGAGAGGAGGTGCATGGC
SE104      CAGGCGA-GACT----GTTACATGAAAGCCAGGCTAATG-ACTTTGCTAGATTTTCAGAGAGGTGGTGCATGGC
SE105      CAGCGGA-GACT----GTTACATGAAAGCCAGGTTAATG-ACTTTGCTAGATTTTCAGAGAGGTGGTGCATGGC
E.coli     CTGGTCTTGACATCCACGGAAGTTTTCAGAGATGAGAATGTGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGC

           155        165        175        185        195        205        215        220
           |----+----------+----------+----------+----------+----------+----------+-----|
SE101      CGTCGTCAGTTCGTACCGTAAGGCGTTCTCTTAAGTGAGATAACGAACGAGACCCTCACCAATAGTTGCTACCGT
SE102      CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCGG
SE103      CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGCCAACGAGCGAGACCCACATTCTTAGTTACCAGCGG
SE104      CGTCGTCAGTTCGTACCGTAAGGCGTTCTCTTAAGTGAGATAACGAACGAGACCCTCGTCAATAGTTGCTACTTC
SE105     CGTCGTCAGTTCGTACCGTAAGGCGTTCTCTTAAGTGAGATAACGAACGAGACCCTCGTCAATAGTTGCTACTTT
E.coli     TGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGG

                  235        245        255        265        275        285        295
           |----+----------+----------+----------+----------+----------+----------+-----|
SE101      ATCTTCCGAGGTACGG-CACACTATTGGGACCGCTGGCGCTAAGTCAGAGGAAGGAGAGGTCAACGGTAGGTCA
SE102      ATCCTTTTGGATGCCGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTAGGTCC
SE103      ATCTTC-GGGATGCCGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTAGGTCC
SE104      ATCCTCCGGGGTGGAGG-CACACTATTGAGACCGCTGGCGCTAAGTCAGAGGAAGGAGAGGTCAACGGTAGGTCA
SE105     ATCCTCCGGGATGGAGG-CACACTATTGAGACCGCTGGCGCTAAGTCAGAGGAAGGAGAGGTCAACGGTAGGTCA
E.coli     TCCGGCCGGGA-------ACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCA

           305        315        325        335        345        355        365        370
           |----+----------+----------+----------+----------+----------+----------+-----|
SE101      GTATGCCCCGAATCTCCTGGGCTACACGCGCGCTACAAAGGGCGGGACAATGGGTTCCTACACCGAAAGGTGACG
SE102      GTATGCCCCGAATCCCCTGGGCAACACGCGGGCTACAATGGCTAAGACAATGGGTTCCGACATTGAAAAGTGGAG
SE103      GTATGCCCCGAATCCTCTGGGCAACACGCGGGCTACAATGGCTAAGACAATGGGTTCCGACGCTGAAAAGCGGAG
SE104     GTATGCCCCGAATCTCCTGGGCTACACGCGCGCTACAAAGGGCGGGACAATGGGCTCCGACACCGAAAGGTGAAG
SE105     GTATGCCCCGAATCTCCTGGGCTACACGCGCGCTACAAAGGGCGGGACAATGGGCTCCGACACCGAAAGGTGAAG
E.coli     TCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAG

                  385        395        405        415        425        435        445
           |----+----------+----------+----------+----------+----------+----------+-----|
SE101      GTAATC-CCGAAACCCGTCCGTAGTTCGGATTGAGGGTTGTAACTCACCCTCATGAAGCTGGATTCCGTAGTAAT
SE102      GTAATCCTCTAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGGAATGCGTAGTAAT
SE103      GTAATCCTCTAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGGAATGCGTAGTAAT
SE104      GTAATC-TCGAAACCCGTCCGTAGTTCGGATTGAGGGTTGTAACTCACCCTCATGAAGCTGGATTCCGTAGTAAT
SE105     GTAATC-TCGAAACCCGTCCGTAGTTCGGATTGAGGGTTGTAACTCACCCTCATGAAGCTGGATTCCGTAGTAAT
```

```
E.coli  CGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAAT

          455       465       475       485       495       505
        ----+---------+---------+---------+---------+---------+--
SE101   CGCGGATCAACAATCCGCGGTGAATATGCCCCTGCTCCTTGCACACACCGCCCGGCA
SE102   CGCGTGTCACTATCGCGCGGTGAATACGTCCCTGCTCCTTGCACACACCACCCGTCA
SE103   CGCGTGTCACAATCGCGCGGTGAATACGTCCCTGCTCCTTGCACACACCGCCCGTCA
SE104   CGCGAATCAACAACTCGCGGTGAATATGCCCCTGCTCCTTGCACACACCGCCCGTCA
SE105   CGCGAATCAACAACTCGCGGTGAATATGCCCCTGCTCCTTGCACACACCGCCCGTCA
E.coli  CGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCA
```

5. SSU rRNA gene sequences of symbiotic methanogens in the rumen of cow.

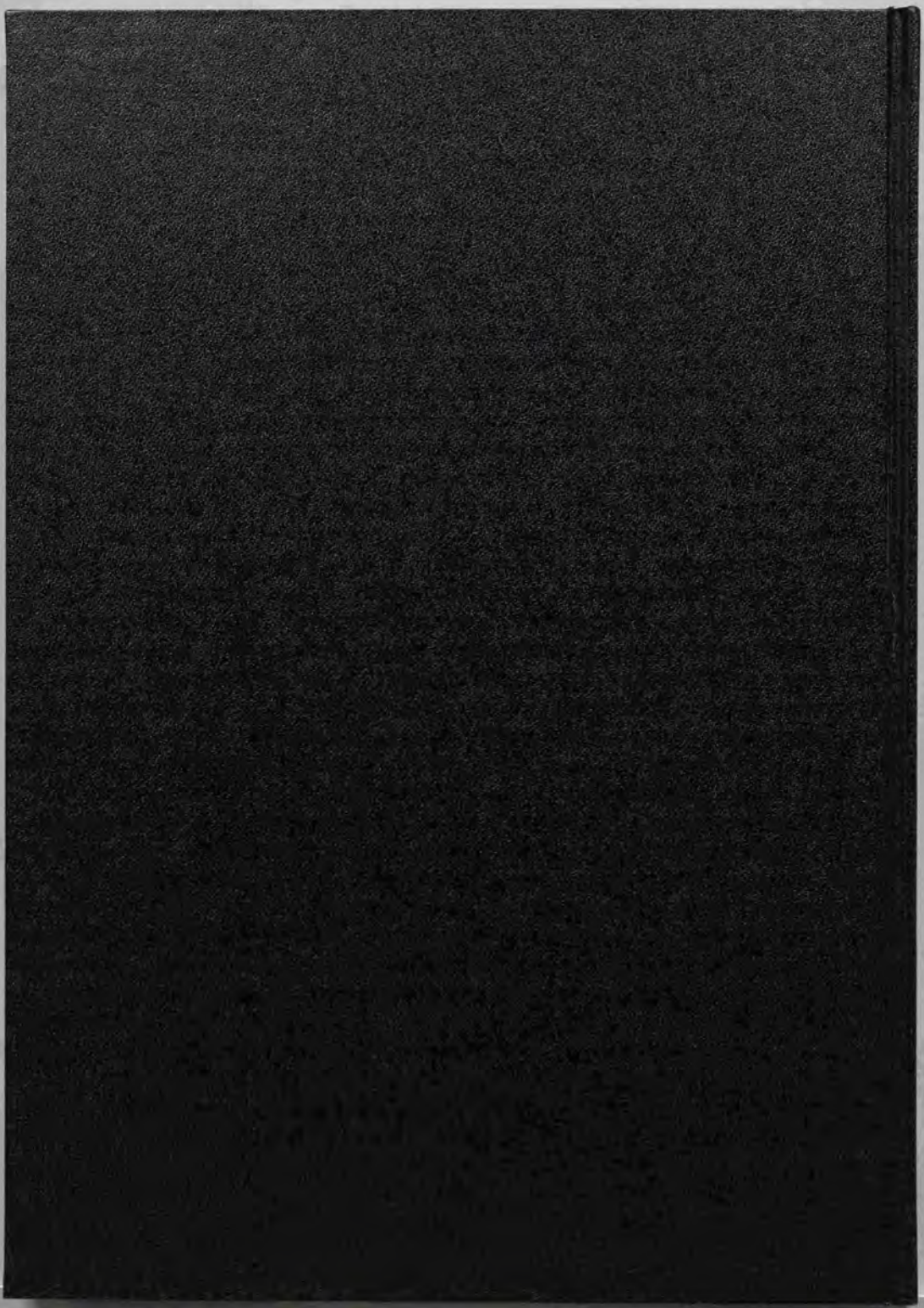
```
           5         15         25         35         45         55         65         75
           |----------|----------|----------|----------|----------|----------|----------|
BR101  TTAAAGGGATTGGCGGGGGGGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
BR102  TTGAAGGGGT-GGCGGGGGAGCACCGCAACGGGAGGAGCGTGCGGTTTAATTGGATTCAACACCGGAAAACTCAC
BR103  TTAAAGGGATCGGCGGGGGAGCACCGCAACGGGAGGAGCGTGCGGTTTAATTGGATTCAACACCGGAAAACTCAC
BR104  TTAAAGGAGTTGGCGGGGGAGCACCGCAACGGGAGGAGCGTGCGGTTTAATTGGATTCAACACCGGAAAACTCAC
BR105  TTGAAGGAGTTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
E.coli TCAAATGAATTGACGGGGGCCCGC-ACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTAC

           85         95        105        115        125        135        145
           |----------|----------|----------|----------|----------|----------|----------|
BR101  CAGAGGC-GACAGCTGT--ATGATAGCCAGGTTGATGACTTTGCTT---GACTAGC-TGAGAGGAGGTGCATGGC
BR102  CAAGGGC-GACTATCAC--ATGAAAGCCAGGCTAACGACCTTGCTT---GATTCTT-AGAGAGGTGGTGCATGGC
BR103  CAAGGGC-GACTATCAC--ATGAAAGCCAGGCTAACGACCTTGCTT---GATTCTT-AGAGAGGTGGTGCATGGC
BR104  CAGGGGA-GACCATCAC--ATGAAGGCCAGGCTGATGACTTTGCCT---GATTCTT-GGAGAAGTGGTGCATGGC
BR105  CAGGAGC-GACAGCTGT--ATGATTACCAGGCTGATGACCTTGTTT---GACTAGC-TGAGAGGAGGTGCATGGC
E.coli CTGGTCTTGACATCCACGGAAGTTTTCAGAGATGAGAATGTGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGC

           155        165        175        185        195        205        215        220
           |----------|----------|----------|----------|----------|----------|----------|
BR101  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCTT
BR102  CGTCGTCAGTTCGTACCGTAAGGCGTTCTCTTAAGTGAGATAACGAACGAGACCCTCATCTATAATTGCTACTTC
BR103  CGTCGTCAGTTCGTACCGTAAGGCGTTCTCTTAAGTGAGATAACGAACGAGACCCTCATCTATAATTGCTACTTC
BR104  CATCGTCAGTTCGTACTGTAAAGCGTTCTCTTAAGTGAGATAACGAACAAGACCCTCACTTGCAATTGCTACTCC
BR105  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCATCAG
E.coli TGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGG

           235        245        255        265        275        285        295
           |----------|----------|----------|----------|----------|----------|----------|
BR101  ATCCTTTTTTGGATGATGGGCACACTAGGGGGACCGCCTATGATAAATAGGAGGAAGGAGTGGACGACGGTAGGT
BR102  ACCCTCCG--GGGTGGAGG-CACATTATCGAGACCGCTGGCGCTAAGTCAGAGGAAGGAGAGGTCAACGGTAGGT
BR103  ACCCTCCG--GGGTGGAGG-CACATTATCGAGACCGCTGGCGCTAAGTCAGAGGAAGGAGAGGTCAACGGTAGGT
BR104  GATCTCCG--GATCGGAGG-CACATTGCCGGGACCGCTGGCGCTAAGCCAGAGGAAGGGGAGGTCAACGGTAGGT
BR105  ATTCTCCG--GAATGCTGGGCACACTAAGGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACGGTAGGT
E.coli ----TCCG------GCCGGGAACTCAAAGGAGACTGGCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGT

           305        315        325        335        345        355        365        370
           |----------|----------|----------|----------|----------|----------|----------|
BR101  CCGTATGCCCCGAATCCTCTGGGCAACACGCGGGCTACAATGGCTGGGACAATGGGTTCCGACACCGAAAGGTGG
BR102  CAGTATGCCCCGAATCTCTTGGGCTACACGCGCGCTACAAAGGGCGGGACAATGGGCTACGACGCCGAAAGGCGA
BR103  CAGTATGCCCCGAATCTCTTGGGCTACACGCGCGCTACAAAGGGCGGGACAATGGGCTACGACGCCGAAAGGCGA
BR104  CAGTATGCCCTGAATCTCCTGGGCTACACGCGCGCTACAAAGGGCGGGACAATGGGCTCCGACGCCGAGAGGCGA
BR105  CCGTATGCCCCGAATCCCCTGGGCTACACGCGGGCTACAATGGCTGGGACAATGGGTTCCGACGCCGAAAGGCGG
E.coli CATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCA

           385        395        405        415        425        435        445
           |----------|----------|----------|----------|----------|----------|----------|
BR101  AGGTAATCCTCTAAACTTAGTCGTAGTTCGGATTGAGGACTGTAACTCGTTCTCATGAAGCTGGAATGCGTAGTA
BR102  AGTCAATCTCG-AAACCCGTCCGTAGTTCGGATTGAGGCTTGTAACTCAGCCTCATGAAGCTGGATTCCGTAGTA
BR103  AGTCAATCTCG-AAACCCGTCCGTAGTTCGGATTGAGGGTTGTAACTCACCCTCATGAAGCTGGATTCCGTAGTA
BR104  AGGTAATCTCG-AAACCCGTCCGTAGTTCGGACTGTGGGTTGTAACTCACCCACACGAAGCTGGATTCCGTAGTA
BR105  TGGTAATCTTTTAAACCTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGGAATGCGTAGTA
```

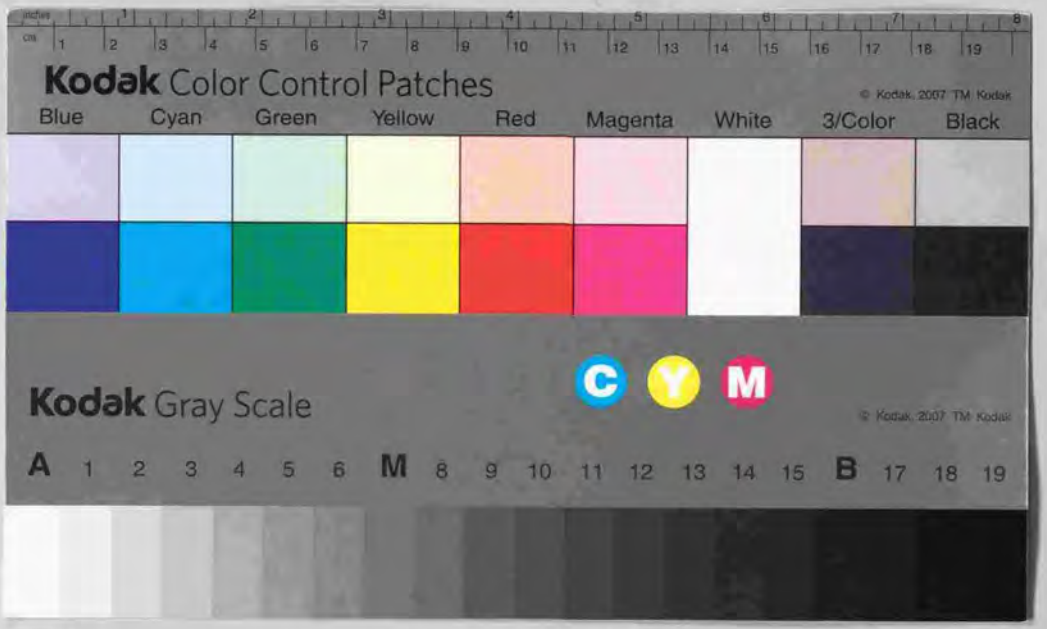
```
E.coli  AGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTA

          455         465         475         485         495         505
        ----+---------+---------+---------+---------+---------+---------+-----
BR101   ATCGCGTATCACTATTGCGCGGTGAATACG-TCCCTGCTCCTTGCACACACCGCCCGTCA
BR102   ATCGCGAATCAAAAACTCGCGGTGAATATGCTCCCTGCTCCTTGCACACACCGCCCGTCA
BR103   ATCGCGAATCAAAAACTCGCGGTGAATATG-CCCCTGCTCCTTGCACACACCGCCCGTCA
BR104   ATCGCGAATCAACAACTCGCGGTGAATATG-CCCCTGCTCCTTGCACACACCGCC-GTCA
BR105   ATCGCGTGTCACAATCGCGCGGTGAATACG-TCCCTGCTCCTTGCACACACCGCCCGTCA
E.coli  ATCGTGGATCAGAATGCCACGGTGAATACGTTCCC-GGGCCTTGTACACACCGCCCGTCA
```

6. SSU rRNA gene sequences of symbiotic methanogens in soil.

```
            5         15         25         35         45         55         65         75
            ----+----------+----------+----------+----------+----------+----------+----------+
XT101  TTAAAGGAATTGGCGGGGGAGCACCGCAACGGGAGGAGCGTGCGGTTTAATTGGATTCAACACCGGAAAACTCAC
XT102  TTAAAGGAATTGGCGGGGGAGCACCGCAACGGGAGGAGCGTGCGGTTTAATTGGATTCAACACCGGAAAACTCAC
XT103  TTAAAGGAATTGGCGGGGGAGCACCGCAACGGGAGGAGCGTGCGGTTTAATTGGATTCAACACCGGAAAACTCAC
XT106  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
XT107  TTAAAGGAATTGGCGGGGGAGCACCGCAACGGGAGGAGCGTGCGGTTTAATTGGATTCAACACCGGAAAACTCAC
XT108  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
XT109  TTAAAGGAATTGGCGGGGGAGCACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCAC
E.coli TCAAATGAATTGACGGGGGCCCGC-ACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTAC

            85         95        105        115        125        135        145
            ----+----------+----------+----------+----------+----------+----------+-----
XT101  CAGGGGA-GACT-----GTTACATGAAAGCCAGGTTAATGACTTTGCTAGA-TTTTCAGAGAGGTGGTGCATGGC
XT102  CAGGGGA-GACT-----GTTACATGAAAGCCAGGCTAATGACTTTGCTAGA-TTTTCAGAGAGGTGGTGCATGGC
XT103  CAGGGGA-GACT-----GTTACATGAAAGCCAGGCTAATGACTTTGCTAGA-TTTTCAGAGAGGTGGTGCATGGC
XT106  CAGGGGC-GACA-----GCAGTATGATGGCCAGGTTGATGGTCTTGCTTGA-CAAGCTGAGAGGAGGTGCATGGC
XT107  CAGGGGA-GACT-----GTTACATGAAAGCCAGGCTAATGACTTTGCTAGA-TTTTCAGAGAGGTGGTGCATGGC
XT108  CAGGGGC-GACA-----GCAGTATGATGGCCAGGTTGATGGTCTTGCTTGA-CAAGCTGAGAGGAGGTGCATGGC
XT109  CAGGGGC-GACA-----GCAGTATGATGGTCAGATTGATGATCTTACTTGA-CAAGCTGAGAGGAGGTGCATGGC
E.coli CTGGTCTTGACATCCACGGAAGTTTTCAGAGATGAGAATGTGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGC

            155        165        175        185        195        205        215        220
            ----+----------+----------+----------+----------+----------+----------+---------+
XT101  CGTCGTCAGTTCGTACCGTAAGGCGTTCTCTTAAGTGAGATAACGAACGAGACCCTCGTCAATAGTT----GCTA
XT102  CGTCGTCAGTTCGTACCGTAAGGCGTTCTCTTAAGTGAGATAACGAACGAGACCCTCGTCAATAGTT----GCTA
XT103  CGTCGTCAGTTCGTACCGTAAGGCGTTCTCTTAAGTGAGATAACGAACGAGACCCTCGTCAATAGTT----GCTA
XT106  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTT----ACCA
XT107  CGTCGTCAGTTCGTACCGTAAGGCGTTCTCTTAAGTGAGATAACGAACGAGACCCTCGTCAATAGTT----GCTA
XT108  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTT----ACCA
XT109  CGCCGTCAGCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCAA
E.coli TGTCGTCAGCTCGTGTCGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTT----GCCA

            235        245        255        265        275        285        295
            ----+----------+----------+----------+----------+----------+----------+-----
XT101  CTTCATCCTCC-----GGGGTGGAGGCACACTATTGAGACCGCTGGCGCTAAGTCAGAGGAAGGAGAGGTCAACG
XT102  CTTCATCCTCC-----GGGGTGGAGGCACACTATTGAGACCGCTGGCGCTAAGTCAGAGGAAGGAGAGGTCAACG
XT103  CTTCATCCTCC-----GGGGTGGAGGCACACTATTGAGACCGCTGGCGCTAAGTCAGAGGAAGGAGAGGTCAACG
XT106  GCATGTCCTTTTTTTGGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACG
XT107  CTTCATCCTCC-----GGGGTGGAGGCACACTATTGAGACCGCTGGCGCTAAGTCAGAGGAAGGAGAGGTCAACG
XT108  GCATGTCCTTTTTTTGGATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAATTGGAGGAAGGAGTGGACGACG
XT109  GTCTTTTTTAA-----GATGTTTGGGCACACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACG
E.coli GCGGTCCGGCC-------GG-----GAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACG

            305        315        325        335        345        355        365        370
            ----+----------+----------+----------+----------+----------+----------+---------+
XT101  GTAGGTCAGTATGCCCCGAATCTCCTGGGCTACACGCGCGCTACAAAGGGCGGGACAATGGGCTCCGACACCGAA
XT102  GTAGGTCAGTATGCCCCGAATCTCCTGGGCTACACGCGCGCTACAAAGGGCGGGACAATGGGCTCCGACACCGAA
XT103  GTAGGTCAGTATGCCCCGAATCTCCTGGGCTACACGCGCGCTACAAAGGGCGGGACAATGGGCTCCGACACCGAA
XT106  GTAGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAA
XT107  GTAGGTCAGTATGCCCCGAATCTCCTGGGCTACACGCGCGCTACAAAGGGCGGGACAATGGGCTCCGACACCGAA
XT108  GTAGGTCCGTATGCCCCGAATCCCCTGGGCAACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAA
```

```
XT109  GTAGGTCCGTATGCCCCGAATCCCCTGGGCTACACGCGGGCTACAATGGCTGAGACAATGGGTTCCGACATTGAA
E.coli TCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCG

          385       395       405       415       425       435       445
---------+---------+---------+---------+---------+---------+---------+---------+-----
XT101  AGGTGAAGGTAATCTCG-AAACCCGTCCGTAGTTCGGATTGAGGGTTGTAACTCACCCTCATTAAGCTGGATTCC
XT102  AGGTGAAGGTAATCTCG-AAACCCGTCCGTAGTTCGGATTGAGGGTTGTAACTCACCCTCATGAAGCTGGATTCC
XT103  AGGTGAAGGTAATCTCG-AAACCCGTCCGTAGTTCGGATTGAGGGTTGTAACTCACCCTCATGAAGCTGGATTCC
XT106  AAGTGAAGGTAATCCCCTAAACTTGGTCGTAGTTCGGATTGAGGGCTGTGACTCGCCCTCATGAAGCTGGAATGC
XT107  AGGTGAAGGTAATCTCG-AAACCCGTCCGTAGTTCGGATTGAGGGTTGTAACTCACCCTCATGAAGCTGGATTCC
XT108  AAGTGAAGGTAATCCCCTAAACTTGGTCGTAGTTCGGATTGAGGGCTGTGACTCGCCCTCATGAAGCTGGAATGC
XT109  AAGTGAAGGTAATCCCCTAAACTTAGTCGTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATAAAGCTGGAATGC
E.coli AGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCG

          455       465       475       485       495       505       510
---------+---------+---------+---------+---------+---------+---------+
XT101  GTAGTAATCGCGAATCAACAACTCGCGGTGAATA-GCCCCTGCTCCTTGCACACACCGCCCGTCA
XT102  GTAGTAATCGCGAATCAACAACTCGCGGTGAATATGCCCCTGCTCCTTGCACACACCGCCCGTCA
XT103  GTAGTAATCGCGAATCAACAACTCGCGGTGAATATGCCCCTGCTCCTTGCACACACCGCCCGTCA
XT106  GTAGTAATCGTATGTCATAATCGCACGGTAAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
XT107  GTAGTAATCGCGAATCAACAACTCGCGGTGAATATGCCCCTGCTCCTTGCACACACCGCCCGTCA
XT108  GTAGTAATCGTATGTCATAATCGCACGGTAAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
XT109  GTAGTAATCGTGTGTCATAATCGCACGGTGAATATGTCCCTGCTCCTTGCACACACCGCCCGTCA
E.coli CTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCA
```




Kodak Color Control Patches
© Kodak, 2007 TM Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
Kodak Gray Scale
C
Y
M
© Kodak, 2007 TM Kodak
A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19