Cultivation of Methanogens under Low-Hydrogen Conditions by Using the Coculture Method[†]

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We previously reported the isolation of novel methanogens by using a new cultivation method, referred to as the coculture method. Here, we extended our coculture method to various anaerobic environmental samples. As a result, we successfully cultivated some uncharacterized methanogens in coculture enrichments and eventually isolated a new methanogen, within the order *Methanomicrobiales*.

So far, almost all cases of the cultivation and isolation of H₂-utilizing methanogenic *Archaea* (methanogens) have been performed under high-H₂ concentrations (e.g., around 100 kPa), even though the concentrations in their natural habitat are far lower (10 to 100 Pa) than in laboratory cultures. This difference between in vitro and in situ physicochemical conditions very likely means that fast-growing methanogens that may prefer high concentrations of H₂ will have to be specifically selected; thus, laboratory cultures under such high-H₂ conditions result in the growth of a very limited range of species. To avoid this situation, we have proposed a new cultivation method, which we named the coculture method, for cultivating H₂-utilizing methanogens (14).

Under anaerobic conditions, methanogens often partner with heterotrophic H₂-producing bacteria, which catalyze the oxidation of a variety of substrates (fatty acids, alcohols, and aromatic compounds). The methanogens use the H₂ produced by these heterotrophic bacteria, and in return, the bacteria benefit from the removal of the H₂ that would otherwise inhibit their growth. This lifestyle is commonly referred to as interspecies H₂ transfer, and the heterotrophic H₂-producing bacteria relying on H₂-utilizing methanogens are called syntrophs (11). In our previous studies, cultivation was performed with propionate as an indirect precursor substrate that is converted to H₂ by syntrophs, with the expectation that methanogens would grow as a result of interspecies H₂ transfer. Based

on this strategy, two novel methanogens representing new genera, *Methanocella paludicola* strain SANAE and *Methanolinea tarda* strain NOBI-1, were successfully isolated (7, 13, 14).

In this study, we extended the method to various types of environmental samples to cultivate and isolate uncharacterized methanogens. Moreover, we also extended the H₂-supplying substrates to include ethanol and butyrate in addition to propionate, because these substances are also known to be decomposed by a syntrophic association of substrate-oxidizing H₂-producing bacteria and H₂-utilizing methanogens (15).

Nine anaerobic environmental samples (marine coastal sediment from Kashiwazaki, Niigata, Japan [KO]; freshwater lake sediment from Lake Suwa, Nagano, Japan [SL]; freshwater pond sediment from Shouzuma Pond, Nagano, Japan [SP]; river sediment of the Azusa River, Nagano, Japan [AR]; sediment from a lotus field located in Nagaoka, Niigata, Japan [LF]; rice field soil from Matsumoto, Nagano, Japan [NR]; rice field soil from Nagaoka, Niigata, Japan [SRP]; rice field soil from Tainan, Taiwan [TNR]; and methanogenic granular sludge obtained from a lab-scale upflow anaerobic sludge blanket reactor treating wastewater from the manufacture of palm oil in our laboratory [MP]) were anaerobically incubated with ethanol (10 mM), butyrate (20 mM), or propionate (20 mM) as the sole carbon and energy source. Additionally, we prepared propionate enrichment cultures with the addition of a pure culture of anaerobic syntrophic propionate-oxidizing bacterium *Syntrophobacter fumaroxidans* strain MPOB (DSMZ 10017) cells (inoculum size, 5% [vol/vol]) to obtain stable cultures (8, 18), except for enrichments from the marine sediment and granular sludge samples because the NaCl resistance of *S. fumaroxidans* was unknown (6) and the granular sludge was expected to contain a large amount of indigenous syntrophic bacteria (5, 16). Moreover, as control experiments, the same environmental samples were used in enrichments by the

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canonical cultivation method in the presence of high concentrations of H_2 (ca. 150 kPa in headspace) or formate (40 mM). All cultivations were performed anaerobically at 37°C without shaking. In total, 52 primary enrichment cultures were prepared for this study (see Table S1 in the supplemental material).

When primary enrichment was made using high concentrations of H_2 and formate, the growth of methanogen-like microbes was confirmed within 3 to 5 days of incubation (as examples, photomicrographs of the enrichment cultures from TNR are shown in Fig. S1A to D in the supplemental material). After three consecutive transfers, 16S rRNA gene-based clone analysis was performed using an archaeal universal primer pair, Ar109f/1490R (14). Twenty-nine phylotypes were detected and were closely related to previously isolated methanogens (Fig. 1 and 2A and B; see Table S1 in the supplemental material). Among them, 26 phylotypes were classified into the genus *Methanobacterium*, two phylotypes in the genus *Methanospirillum*, and one in the genus *Methanogenium*. Moreover, 20 phylotypes showed high similarities (>97%) with the 16S rRNA genes of previously isolated methanogens, whereas the remaining nine phylotypes showed 95 to 96% similarities with the 16S rRNA genes of previously characterized methanogens (see Table S1 in the supplemental material). The methanogens possessing these sequences may be taxonomically novel at least at the species level (17), but they were all affiliated with the well-studied genera *Methanobacterium* and *Methanospirillum*.

In the coculture enrichments, substrate degradation concomitant with methane formation was confirmed after 1 week and more than 1 to 3 months of incubation in ethanol enrichment cultures and butyrate and propionate enrichment cultures, respectively. In particular, the growth of microbes in the propionate enrichments without the addition of *S. fumaroxidans* cells, except for the enrichments constructed from the RF and SRP samples, was very slow and unstable; the growth and methane production stopped unexpectedly and often made successive passages to fresh medium difficult. Additionally, two propionate enrichments in the absence of *S. fumaroxidans* inoculated from the KO and NR samples did not show methane production after a year of incubation. On the other hand, all of the propionate enrichments in the presence of *S. fumaroxidans* cells showed stable growth. During the incubation of the coculture enrichments, the H_2 partial pressures in the cultures were kept at <100 Pa in the ethanol enrichments and at <30 Pa in the butyrate and propionate cultures. Methane, H_2 , short-chain fatty acids, and ethanol were measured as described previously (14). Microscopic observation after three to four transfers showed that those enrichments were comprised mainly of F₄₂₀-autofluorescent methanogen-like cells and oval- or rod-shaped bacterial cells, possibly syntrophs (see Fig. S1E to L in the supplemental material). These observations suggested that ethanol, butyrate, and propionate degradation were carried out by syntrophic association between syntrophic substrate-oxidizing H_2 -producing bacteria and H_2 -utilizing methanogens. To identify the methanogens present in those enrichments, archaeal 16S rRNA gene-based clone analyses were performed. A total of 52 phylotypes were obtained (Fig. 1 and 2C to F; see Table S1 in the supplemental material). Of these, 23 phylotypes were classified into the genera *Meth-*

anobacterium (19 phylotypes) and *Methanospirillum* (4 phylotypes), which were very similar to those obtained from the H_2 and formate enrichments. On the other hand, the remaining 29 phylotypes were comprised of the orders *Methanomicrobiales* (20 phylotypes), *Methanocellales* (5 phylotypes), and *Methanosarcinales* (4 phylotypes, all belonging to the genus *Methanosaeta*). Within the order *Methanomicrobiales*, some phylotypes were affiliated with the genera *Methanoculleus* (nine phylotypes), *Methanofollis* (two phylotypes), *Methanocalculus* (one phylotype), and *Methanoplanus* (one phylotype). Additionally, sequences very closely related to the recently isolated methanogens *Methanolinea tarda* (six phylotypes) (7, 14) and “*Candidatus* Methanoregula boonei” (one phylotype) (1) were also obtained. Both *M. tarda* and “*Ca. Methanoregula boonei*” represent a family-level clade, which had long been recognized as an uncultured archaeal lineage called the group E1/E2 (1) (Fig. 1). Regarding the five phylotypes within the order *Methanocellales*, all were obtained from SRP and SP enrichments. Though the order *Methanocellales* had been recognized as the clone cluster rice cluster I, one strain has been isolated very recently (13, 14) and the rice cluster I methanogens are now being unveiled.

Of the 52 phylotypes obtained from the coculture enrichments, 38 phylotypes (73% of the total phylotypes) were >97% similar to the 16S rRNA genes of the previously characterized (cultivated) methanogens. In contrast, 14 phylotypes (27%) had <96% sequence similarity with those of known methanogens. The organisms represented by these phylotypes were considered to be taxonomically novel at the species or even the genus level. Most of these phylotypes were affiliated with the orders *Methanomicrobiales* and *Methanocellales* with 92 to 96% sequence similarity (see Table S1 in the supplemental material). According to the 16S rRNA gene-based clone analysis, taxonomically novel methanogens were found in abundance in one ethanol, two butyrate, and eight propionate enrichments (from the KO, SP, SL, TNR, LF, and SRP samples). Especially, the ethanol and six propionate enrichments (from the SP, TNR, LF, and SRP samples) contained novel methanogens belonging to the group E1/E2 and/or the order *Methanocellales* (formerly known as rice cluster I), both of which contain only a few cultivated representatives so far. Therefore, we attempted to isolate these methanogens from the enrichments. After several attempts were performed over a year, a novel methanogen, designated strain TNR, was successfully isolated from the propionate enrichment culture (TNR) by serial dilution in liquid medium with H_2 (ca. 150 kPa) as the substrate.

Strain TNR was a nonmotile, rod-shaped methanogen, which utilized H_2/CO_2 and formate for growth and methane production (see Fig. S2 in the supplemental material). The doubling time was 1.2 days at 37°C and pH 7. The most closely related methanogen cultivated so far was *Methanolinea tarda* that we have recently isolated (7), but the similarity of the 16S rRNA genes between the two was only 95% (Fig. 1). On the other hand, the isolation of methanogens from the other enrichments was not successful, i.e., when the coculture enrichments were inoculated into the serial dilution cultures with high concentrations of H_2 or formate, nontargeted methanogens, almost all of which had >97% sequence similarities to the 16S rRNA genes of known *Methanobacterium* and *Methanoculleus* species, outgrew in the cultures. The conventional

By using the coculture method, we successfully enriched methanogens that were absent in previous cultivation attempts and were only detected as environmental clones. In addition, we were able to isolate a methanogen belonging to the group E1/E2 of the order *Methanomicrobiales*. Our study clearly demonstrated that the coculture method is an effective way to cultivate hitherto uncharacterized methanogens. Interestingly, the taxonomic compositions of the phylotypes were clearly different depending on the substrates used in the coculture method (Fig. 1 and 2). When conventional cultivation was employed using high concentrations of H₂ and formate, only very limited phylotypes were obtained, namely, *Methanobacterium*- and *Methanospirillum*-related phylotypes in the H₂ cultures and *Methanobacterium*- and *Methanogenium*-related phylotypes in the formate cultures. When using the coculture

method with ethanol or butyrate, *Methanobacterium*-related phylotypes were also dominant, accounting for 64.3% of the total phylotypes, whereas more diverse methanogen phylotypes than those in the H₂ and formate cultures were retrieved. Contrary to these results, propionate (with and without *S. fumaroxidans*) enrichments allowed quite a different pattern of methanogen phylotypes to become established. The most abundant phylotypes obtained from the propionate enrichments belonged to the orders *Methanocellales* and *Methanomicrobiales*, accounting for 72.8 and 84.7% of the clones examined. The addition of *S. fumaroxidans* cells into the propionate enrichments seemed to have no significant effect on the methanogenic community compositions that emerged, but it helped the stability of the whole community and the capability of the propionate degradation. The theoretical ranges of H₂ partial pressure that allow the anaerobic oxidation of ethanol, butyrate, and propionate to occur are 0.5 to 27,000 Pa; 0.5 to 60 Pa; and 0.5 to 28 Pa, respectively. These values were calculated

per number of clones analyzed for each phylotype. The accession numbers are also shown after each phylotype name. The phylotypes indicated by the same accession numbers have the same sequences (e.g., SP-For-A and SRP-Buty-C, AR-PrOm-A and NR-PrOm-A). All of the clonal sequences were greater than 1,000 nucleotides in length, with the exception of *Methanospirillum* sp. TM20-1 (GenBank acc. no. AB062404; 789 bp). Therefore, the initial tree was constructed with sequences greater than 1,000 nucleotides using the neighbor-joining method. Subsequently, the *Methanospirillum* sp. TM20-1 sequence was inserted into the tree by using the parsimony insertion tool of the ARB program. The scale bar indicates the estimated number of base changes per nucleotide sequence position. The symbols at the branch nodes indicate bootstrap values.

based on the review on energy conservation by Thauer et al. (19), in which the concentrations of products and reactants were 0.35 atm P_{CO_2} , 0.65 atm P_{CH_4} , and 20 mM substances at 37°C and pH 7. For the calculation, a temperature correction was made using the van't Hoff equation. Theoretically, the H_2 partial pressures in the various cultures differ depending on the substrates used, becoming lower in the order of substrates: ethanol > butyrate > propionate. Actually, the H_2 partial pressures measured during substrate degradation in the coculture enrichments remained within these theoretical ranges (data not shown). Given the above theoretical values, the apparent H_2 partial pressure that could be generated from a particular substrate would be the crucial factor affecting the change in the compositions of H_2 -utilizing methanogens in the community. In fact, the relative abundance of members of the genera *Methanobacterium* and *Methanospirillum* increased as the given H_2 partial pressure became higher (propionate → butyrate → ethanol → H_2), and conversely, the relative abundance of members of the orders *Methanomicrobiales* (except for the genus *Methanospirillum*) and *Methanocellales* increased as the H_2 partial pressure became lower (Fig. 1 and 2). We assume that *Methanocellales* spp. and *Methanomicrobiales* spp. (except for *Methanospirillum* spp.) have higher affinities for H_2 than *Methanobacterium* spp. and *Methanospirillum* spp. Several previous studies also support this prediction. Lu et al. reported that *Methanocellales* methanogens incorporated ^{13}C when rice roots were incubated in a low- H_2 atmosphere in the presence of $^{13}CO_2$, while *Methanobacteriales* and *Methanosarcinales* methanogens preferentially incorporated ^{13}C in a high- H_2 atmosphere (10). Also, *Methanocellales* phylotypes were detected from methanogenic environments, usually with a low concentration of H_2 , such as rice fields, fens, and peat bogs (e.g., see references 3, 4, and 9). In addition to the *Methanocellales* methanogens, members of the order *Methanomicrobiales* were frequently found in abundance in low- H_2 -concentration methanogenic environments, such as peat bogs, fens, lake sediments, and rice fields (e.g., see references 2, 12, and 20). Detailed substrate affinity information will provide insight into the relevance between the population structures of methanogens and the H_2 concentrations of their habitats.

Nucleotide sequence accession numbers. Sequence data obtained in this study were deposited in the DDBJ/EMBL/GenBank databases under the accession numbers AB236048 to AB236107, AB236113 to AB236121, and AB447467.

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