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Microbial Community Analysis of Anaerobic Ecosystems - Significance and Importance of Anaerobes -

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Introduction

Anaerobic microorganisms reside everywhere and they are involved in global cycles of carbon, nitrogen, sulfur, and other elements. Of strictly anaerobic microbes, methanogen is one of the most important microorganisms for anaerobic degradation of organic materials in natural and human-made ecosystems such as methane fermenting (biogas) reactors. Methanogens, phylogenetically known as Euryarchaeota in the domain Archaea, are microbes that produce methane as their endproduct and conserve energy from methane formation (methanogenesis). The most characteristic feature of methanogens is that they utilize only very limited substrates such as H_2/CO_2, formate, acetate, methyl compounds (methanol, methylamines and methanethiol), and several alcohols. These substrates are provided by other fermentative microbes that are in charge of degradation of higher organic molecules (Fig. 1). Methanogens are, therefore, responsible for the final step of degradation of organic compounds under anaerobic conditions. As a matter of fact, methane can be detected in most of anaerobic environment unless other electron acceptors such as sulfate, sulfur and Fe (III) that allow sulfate, sulfur, and iron reducing-bacteria, respectively, to oxidize the lower molecule substrates are abundant.

Besides methanogens, to date, a number of different strictly or facultatively anaerobic microorganisms are known and they are associating together in environment to degrade organic materials forming a food web as shown in Fig.1. One of the most characteristic features of anaerobic ecosystems is that degradation of organic materials would not occur without associating with methanogens or other equivalent microbes such as sulfate-reducing bacteria that are responsible for the terminal step of mineralization of organic materials. This is also the case with a very simple substrate, for instance, glucose. Glucose would be an energy-rich substrate for "aerobic" microorganisms and would be easily oxidized to H_2O and CO_2 by one species of microbe yielding sufficient energy to grow. By contrast, there needs to be at least four microbes to completely metabolize glucose to form methane i.e. (A) glucose-fermenting, fatty acids (such as butyrate, propionate and acetate), and H_2-producing microbe, (B) fatty acids-oxidizing acetate/H_2-producing microbe, (C) acetate-metabolizing (aceticlastic) methanogen, and (D) H_2/CO_2-metabolizing (hydrogenotrophic) methanogen. If one of these was missing, complete degradation of glucose to methane would not take place. In particular, oxidation of fatty acids could not proceed without hydrogenotrophic methanogens. This is because oxidation of butyrate or propionate to acetate and H_2/CO_2 is an endergonic reaction so that one has to put exogenous energy to make this reaction energetically feasible. However, if the H_2 partial pressure could be kept very low by coupling with H_2-consuming reaction (methane formation from H_2/CO_2), the whole reaction becomes energetically possible. In this point of view, hydrogenotrophic methanogens are crucial for the whole process of anaerobic degradation of organic matters. Such transfer of H_2 from H_2-producing organisms to H_2-consuming organisms is called

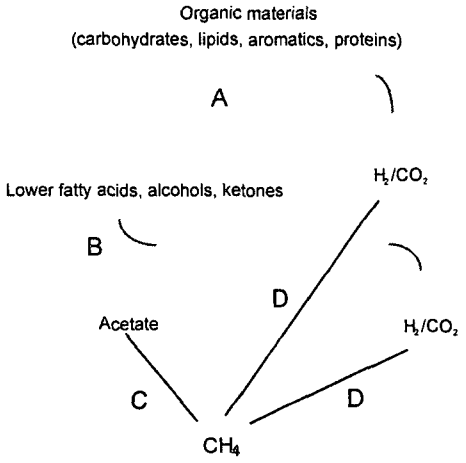

Fig. 1.

interspecies H_2 (electron) transfer and such relation is called syntrophy. We have to keep these in mind to better understand what is occurring in anaerobic microbial communities.

To investigate interspecies H_2 transfer and syntrophic association between organisms, we have been working with a wide variety of anaerobic methanogenic communities. In our earliest studies, we made an enrichment culture which was capable of degrading benzoate to form methane. We took a sludge sample from methanogenic digester and used it as inoculum. After successive transfers to fresh medium containing benzoate as sole carbon and energy source over a year, we obtained a very simplified microbial consortium that was able to metabolize benzoate to form methane. The consortium was found to be comprised of three predominant microorganisms; benzoate degrader (which catalyzes reaction A as shown below), hydrogenotrophic methanogen (reaction B), and acetate-utilizing methanogen (reaction C) . This consortium was the first good exercise for us to learn much about how anaerobic degradation goes and what type of microbial consortium occurs from such a simple aromatic compound. The consortium degraded 5 mM benzoate to stoichiometrically form methane in 3 weeks (reaction D).

$$4C_6H_5COO^- + 28H_2O \text{ ----> } 12CH_3COO^- + 4HCO_3^- + 12H_2 + 12H^+$$
$$DG^{0'} = +359 \text{ (kJ/reaction)} \quad \text{(A)}$$
$$12H_2 + 3HCO_3^- + 3H^+ \text{ ----> } 3CH_4 + 9H_2O$$
$$DG^{0'} = -407 \text{ (kJ/reaction)} \quad \text{(B)}$$
$$12CH_3COO^- + 12H_2O \text{ ----> } 12CH_4 + 12HCO_3^-$$
$$DG^{0'} = -36 \text{ (kJ/reaction)} \quad \text{(C)}$$
$$4C_6H_5COO^- + 31H_2O \text{ ----> } 15CH_4 + 13HCO_3^- + 9H^+$$
$$\Delta G^{0'} = -84 \text{ (kJ/reaction)} \quad \text{(D, overall)}$$

Acetate was transiently accumulated and then methane was formed, suggesting that the benzoate degrader metabolizes benzoate forming acetate together with H_2/CO_2. The consortium was a very stable and all attempts at isolation of the benzoate degrader in pure culture were unsuccessful. By using the enrichment, we examined the effect of H_2 on the benzoate degradation. Exogenous H_2 strongly inhibited the benzoate degradation although methanogenesis occurred from the added H_2. This result strongly indicated that H_2 partial pressure is

the crucial factor affecting the benzoate degradation. This can be explained by the thermodynamic reason that the conversion of benzoate to acetate and H_2/CO_2 is energetically unfavorable without eliminating H_2 to keep the H_2 concentrations sufficiently low (A + B). This is a typical example of interspecies H_2 transfer between the two organisms.

Acetate as another important intermediate in anaerobic degradation of organic materials

Together with H_2, acetate is another key intermediate in anaerobic degradation of organic materials. In the methanogenic benzoate degradation as described above, 80% of methane comes from acetate (see reactions C and D). It is known that in methanogenic natural environment, approx. 70% of methane is derived from acetate. These strongly indicate the importance of acetate-metabolizing (aceticlastic) methanogens. Of over 20 genera of methanogens known to date, only *Methanosaeta* and *Methanosarcina* are the methanogens capable of utilizing acetate. In particular, *Methanosaeta* (previously known as *Methanothrix*) is the primary contributor to acetate degradation in environment and methane reactors. Nevertheless, until early in 1980, there was no detailed information on the genus *Methanosaeta* although the organism was already known early in the last century. The reason why the detailed description on this microorganism appeared far later was that the organism was very difficult to isolate. Surprisingly, it is an extremely slow growing methanogen, having a doubling time of 3-5 days. To make matters worse, the organism does not form colony in agar medium. These traits had hindered researchers from isolating this organism for decades.

Methanosaeta is a filamentous methanogen with a sheathed structure and is distinguished from other methanogens so that we could easily recognize the abundance of this organism in methanogenic communities. Why does *Methanosaeta* grow so slowly but could it predominate in the ecosystems? One thing that can be clearly mentioned is that the energy yield from acetate is theoretically very low. Based on thermodynamic calculations, *Methanosaeta* could obtain only 1 mole of ATP from 1 mole of acetate via methanogenesis. Under anaerobic conditions, acetate is thus an energetically unfavorable substrate compared with other substrates available. Nevertheless, *Methanosaeta* could predominate in anaerobic environment regardless of its slow growth rate and its low energy yield. There are two primary reasons. First, acetate is, as already mentioned, the most abundant intermediate besides H_2 in anaerobic processes. Second, there is almost no competitor for gaining energy from acetate since acetate is the endproduct for most of anaerobic organisms. *Methanosarcina*, another aceticlastic methanogen, could compete with *Methanosaeta* over acetate consumption, but *Methanosarcina* is much more versatile organism capable of utilizing other substrates such as methyl compounds (methanol and methylamines) and H_2, and it preferentially utilizes those substrates.

Biochemistry and energetics of aceticlastic methane formation are of great interest. Currently, the methanogenic pathway from acetate is well-understood, but energy conservation mechanisms still remain to be clarified.

Methanogenic community structure analysis

How methanogens colonize in the anaerobic microbial ecosystems is one of the most intriguing questions not only for researchers who are studying microbial ecology but also engineering people who are working with biogas reactors. Of the methanogenic processes developed so far, upflow anaerobic sludge blanket (UASB) reactor was among the most striking inventions. Since Lettinga et al developed

the reactor for the treatment of high-strength wastewaters from food industry, methane fermentation technology has been becoming very popular. Before this epoch-making reactor appeared, methane fermentation processes had been considered almost over a century to be a very inefficient way and too slow to meet the needs of increased volume in wastewater from urban area and manufacturing processes. UASB process can perform anaerobic degradation of organic matters and subsequent separation in a single vessel. The wastewater is supplied from the bottom of the vessel and the organic matters are digested as the wastewater goes up to the top. During digestion, methane gas bubbles are produced and carry the sludges up resulting in formation of dense sludge flocs (granules) which have high settlability. The reactor can thus select floc forming microbes that are comprised of all members necessary for anaerobic digestion and methanogenesis.

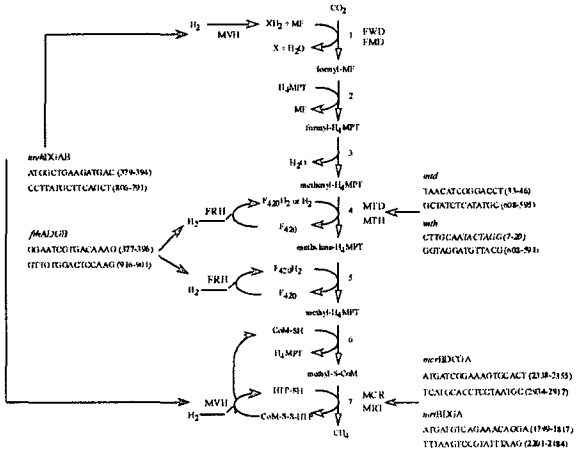

Fig. 2. Methanogenesis from H_2 and CO_2 and primer sets for making probes to detect target genes by Northern analysis. Isoenzymes and functionally equivalent enzymes are shown in bold. Coenzymes: MF, methanofuran; H_4MPT, 5, 6, 7, 8-tetrahydromethanopterin; CoM-SH, coenzyme M; HTP-SH, N-7-mercaptoheptanoyl-L-threonine phosphate; CoM-S-S-HTP, heterodisulfide of CoM-SH and HTP-SH. Enzymes: MVH, methyl viologen-reducing hydrogenase;FRH, F_{420}-reducing hydrogenase; MTD, F_{420}-dependent methylenetetrahydromethanopterin dehydrogenase; MTH, hydrogen-dependent methylenetetrahydromethanopterin dehydrogenase; MCR and MRT, methyl coenzyme M reductase.

Methanogen genomics and expression of methane genes

As mentioned above, H_2 is supplied to hydrogenotrophic methanogens by H_2-producing heterotrophs with very low concentrations. We are particularly focusing on the effect of substrate (H_2) concentrations on the expression of genes responsible for methanogenesis. Previous studies showed that hydrogenotrophic methanogens harbor two isofunctional methyl-coenzyme M reductases and several other enzymes. Of the enzymes, methyl-coenzyme M reductase is the key enzyme that catalyzes the last step of methanogenesis (Fig. 2).

M. thermoautotrophicum genome sequence has allowed us to design various gene probes to detect specific mRNA to determine whether H_2 concentrations affect the expression of those isofunctional genes (Fig. 2). We cultivated *M. thermoautotrophicum* under high H_2 partial pressures and low H_2 partial pressures to do Northern analysis. Cultivation at high H_2 concentrations was simply done in pure culture using serum vials pressurized by

H_2 (1 atm). Cultivation under low H_2 partial pressures (less than 0.001 atm) was possible when the methanogen was cocultured with acetate- or butyrate-oxidizing syntrophs using acetate and butyrate, respectively, as sole carbon and energy source. Acetate or butyrate was oxidized by the syntroph to constantly produce very low concentrations of H_2 that could be immediately converted to methane. Northern analysis and 2-D protein electrophoresis showed that only one of the methyl-coenzyme M reductases was expressed at low H_2 partial pressures whereas both of the genes were expressed at high H_2 partial pressures.

Our experiment is one of the good examples of the usefulness of the complete genome sequence information to investigate genetic and physiological characteristics of methanogens. The genome analysis will shed light not only on genetic traits of the methanogen but also on physiological and ecological characteristics of this environmentally important microorganism.

Published articles related to this topic

Kamagata Y. and E. Mikami: Isolation and characterization of a novel thermophilic *Methanosaeta* strain. Int. J. Syst. Bacteriol., 41, 191-196 (1991).

Kamagata, Y., N. Kitagawa, M. Tasaki, K. Nakamura, and E. Mikami: Degradation of benzoate by an anaerobic consortium and some properties of hydrogenotrophic methanogen and sulfate-reducing bacterium in the consortium. J. Ferment. Bioeng., 73, 213-218 (1992).

Kamagata, Y., H. Kawasaki, H. Oyaizu, K. Nakamura, E. Mikami, G. Endo, Y. Koga, and K. Yamasato: Characterization of three thermophilic strains of *Methanothrix* ("*Methanosaeta*") *thermophila* sp. nov. and rejection of *Methanothrix* ("*Methanosaeta*") *thermoacetophila*. Int. J. Syst. Bacteriol., 42, 463-468 (1992).

Inatomi, K., Y. Kamagata, and K. Nakamura: Membrane ATPase from aceticlastic methanogen *Methanothrix thermophila*. J. Bacteriol., 175, 80-84 (1993).

Tasaki, M., Y. Kamagata, K. Nakamura, K. Okamura, and K. Minami: Acetogenesis from pyruvate by *Desulfotomaculum thermobenzoicum* and differences in pyruvate metabolism among three sulfate-reducing bacteria in the absence of sulfate. FEMS Microbiol. Lett., 106, 259-264 (1993).

Yamada, K., Y. Kamagata, K. Nakamura, Y. Inamori, and I. Nakamura: Selectivity of food bacteria for the growth of *Trimyema compressum*. Arch. Microbiol., 161, 229-233 (1994).

Koyama, O., Y. Kamagata, and K. Nakamura: Degradation of halogenated aromatics by Fenton oxidation and methanogenic sludge. Wat. Res., 28, 895-899 (1994).

Hiraishi, A., Y. Kamagata, and K. Nakamura: Polymerase chain reaction amplification and restriction fragment length polymorphism analysis of 16S rRNA genes from methanogens. J. Ferment. Bioeng., 79, 523-529 (1995).

Yamada, K., Y. Kamagata, and K. Nakamura: The effect of endosymbiotic methanogens on the growth and metabolic profile in the anaerobic free-living ciliate *Trimyema compressum*. FEMS Microbiol. Lett., 149, 129-132 (1997).

Sekiguchi, Y., Y. Kamagata, K. Syutsubo, A. Ohashi, H. Harada, and K. Nakamura: Phylogenetic diversity of mesohilic and thermophilic granular sludges by 16S rRNA gene analysis. Microbiology, 144, 2655-2665 (1998).

Boone, D.R. and Y. Kamagata: Rejection of the species *Methanothrix soehngenii*VP and the genus *Methanothrix*VP as a nomena confusa, and transfer of *Methanothrix thermophila*VP to the genus *Methanosaeta*VP as *Methanosaeta thermophila* comb. nov. Request for Opinion. Int. J. Syst. Bacteriol., 48, 1079-1080 (1998).

Sekiguchi, Y., Y. Kamagata, K. Nakamura, A. Ohashi, and H. Harada: Fluorescent in situ hybridization using 16S rRNA targeted oligonucleotides reveals localization of methanogens and selected uncultured bacteria in mesophilic and thermophilic sludge granules. Appl. Environ. Microbiol., 65, 1280-1288 (1999).

Sekiguchi, Y., Y. Kamagata, K. Nakamura, A. Ohashi, and H. Harada: *Syntrophothermus lipocalidus* gen. nov., sp. nov., a thermophilic syntrophic fatty acid-oxidizing anaerobe which utilizes isobutyrate. Int. J. Syst. Evol. Microbiol., 50, 771-779 (2000).

Hattori, S., Y. Kamagata, S. Hanada, and H. Shoun: *Thermoacetogenium phaeum* gen. nov., sp. nov., a strictly anaerobic, thermophilic, syntrophic acetate-oxidizing bacterium. Int. J. Syst. Evol. Microbiol., 50, 1601-1609 (2000).

Yanagita, K., Y. Kamagata, M. Kawaharasaki, T. Suzuki, Y. Nakamura, and H. Minato: Phylogenetic analysis of methanogens in sheep rumen ecosystem and detection of *Methanomicrobium mobile* by fluorescence in situ hybridization. Biosci. Biotechnol. Biochem., 64, 1737-1742 (2000).

Imachi, H., Y. Sekiguchi, Y. Kamagata, A. Ohashi, and H. Harada: Cultivation and in situ detection of a thermophilic bacterium capable of oxidizing propionate in syntrophic association with hydrogenotrophic methanogens in a thermophilic methanogenic granular sludge. Appl. Environ. Microbiol., 66, 3608-3615 (2000).

Hattori, S., L. Hongwei, H. Shoun, and Y. Kamagata: Involvement of formate as interspecies electron carrier in a syntrophic acetate-oxidizing anaerobic microorganism in coculture with methanogens. J. Biosci. Bioeng., 91, 294-298 (2001).

Sekiguchi, Y., Y. Kamagata and H. Harada: Recent advances in methane fermentation technology. Curr. Opinion. Biotechnol., in press (2001).