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Exploring Novel Hydrogen-Producing Anaerobes in Intestine of Humus Feeders

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1. Introduction.

Recently, several problems about the existing energy-supplying systems, such as the safety of nuclear power methods and exhaustion of fossil fuels have surfaced. Additionally, there has been a lot of environmental problems typified by global warming. Because of these factors, hydrogen have attracted attention as one of the cleaner and sustainable energies.

As the methods of producing hydrogen, the electrolysis of water and the fossil fuel reforming are in practical use, but these are inefficient because of enormous energy consumption.

In contrast to them, the biochemical methods utilizing the metabolizing system of hydrogen-producing microorganisms has been vigorously developed. These methods are more effective and superior than existing methods as mentioned above, because microorganisms can feed industrial wastes as organic substances for hydrogen source. Therefore, the system can not only produce the hydrogen, but also dispose the wastes.

As an organism keeping hydrogen-producing endosymbiotic bacterium, termite (*Reticulitermes speratus*) is well known and have been studied considerably. However, because termites can feed only cellulose, it is presumed that it keeps somewhat limited species of microorganisms. For searching novel microorganisms which have more excellent ability to produce hydrogen, we considered that food habit and growth environment of the host organism were very important. In this point of view, earthworm that is omnivorous and can adapt to an organic-rich soil is very suitable for this purpose. Thus, in this study, to explore the novel hydrogen-producing anaerobes in intense of humus feeders, we tried to detect hydrogenase genes from earth worm (*Eisenia fetida*) and larvae of beetle (*Allomyrina dichotoma*) and analyze them.

2. Results and Discussion.

2.1 Amplification of DNA by PCR

Earthworm, larvae of beetle, and termite were used for the subject of this research. Earthworms were bred in our laboratory, and delivered from Recycle Factory Co. Ltd (Chitose) and Hanamoto Corp. (Asahikawa). Of these, earthworms from Hanamoto Corp. were collected from three different growth environments, i.e. surface and deep layer of breeding ground, and mushroom bed (*Grifola frondosa*), respectively. Larvae of beetle was delivered from Hanamoto Corp.. Termite was purchased from Mushi-megane World Corp (Hiroshima).

PCR amplification was performed on DNAs extracted from the intestine of the organisms using 7 pairs of

Table 1. Primer pairs for PCR amplification

Primers	Sequence (5′-3′)	specificity
Clos[FeFe]1f	GCTGATATGACAATAATGGAAGAA	[FeFe] (*Clostridium* spp)
Clos[FeFe]1r	GCAGCTTCCATAACTCCACCGGTTGCACC	[FeFe] (*Clostridium* spp)
Clos[FeFe]2f	AAATCACCACAACAAATATTTGGTGC	[FeFe] (*Clostridium* spp)
Clos[FeFe]2r	ACATCCACCAGGGCAAGCCATTACTTC	[FeFe] (*Clostridium* spp)
PseuHydA1f	GCTTGTAACAATATTGCAGGTC	[Fe] (*Pseudotrichonympha grassii*)
PseuHydA1r	CAATATCTGTATTCTGCCCAG	[Fe] (*Pseudotrichonympha grassii*)
PseuHydA2f	GCATTTCATGTGGTGCGTGTG	[Fe] (*Pseudotrichonympha grassii*)
PseuHydA2r	CCAACCTTCACCTGCAACGGTGTC	[Fe] (*Pseudotrichonympha grassii*)
HoloHydA1f	TCAACAGTTGCAGGTCAAG	[Fe] (*Holomastigotoides mirabile*)
HoloHydA1r	CCTTCGTTGCGAACTGCTC	[Fe] (*Holomastigotoides mirabile*)
Desu[FeFe]f	TCACCTCGTGCTGCCCCGGCTGG	[FeFe] (*Desulfovibrio.vulgaris*)
Desu[FeFe]r	CAGCCGCCGGGGCAGGCCAT	[FeFe] (*Desulfovibrio.vulgaris*)
Desu[NiFe]f	CCGGTTGCCCGCCGAACCC	[NiFe] (*Desulfovibrio.vulgaris*)
Desu[NiFe]r	CGCAGGCGATGCACGGGTC	[NiFe] (*Desulfovibrio.vulgaris*)

known specific forward and reverse primer for hydrogenase (Table 1). As shown in Table 2, it was found that the primer pairs amplified desired region most effectively was Clos[FeFe]1f-Clos[FeFe], derived from *Clostridium.pasuteurianum* (Fig. 1).

Table 2. The result of PCR amplification

Sample	Clos[FeFe]1f Clos[FeFe]1r	Clos[FeFe]2f Clos[FeFe]2r	PseuhydA1f PseuhydA1r	PseuhydA2f PseuhydA2r	HolohydAf HolohydAr	Desu[FeFe]f Desu[FeFe]r	Desu[NiFe]f Desu[NiFe]r
worm							
研究室	±	−	−	*	−	−	*
花本表層	+	−	−	−	−	−	−
花本深層	+	−	−	−	−	−	−
花本マイタケ	+	−	−	−	−	−	*
リサイクルファクトリー	+	±	−	−	−	−	−
beetle	+	−	−	−	−	−	−
termite	+	−	−	−	−	−	−

+, PCR product of the expected size; −, no PCR product; ±, low yield of PCR product; *, several PCR products or bands with a different size.

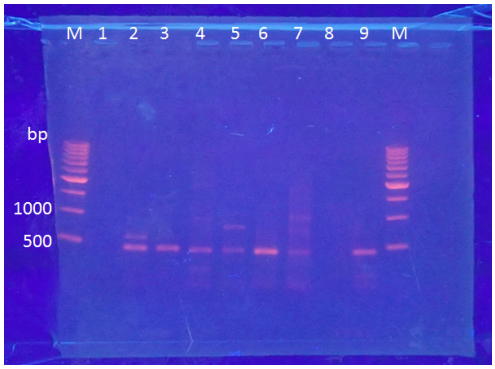

Fig. 1. Amplification of a 460bp of PCR product with primer pairs Clos[FeFe]1f and Clos[FeFe]1r.
1) Earthworm from a) bred in our laboratory, b) Hanamoto: surface layer, c) Hanamoto: deep layer, d) Hanamoto: mushroom bed, e) Recycle Factory, 2) larvae of beetle, 3) termite, 4) blank, 5) *C.pasuteurianum*, M) marker

2.2 Sequential analyses and phylogenetic tree of PCR products

Sequential analyses of genes amplified by PCR with primer pairs Clos[FeFe]1f-Clos[FeFe]1r were performed, and phylogenetic tree was constructed on the basis of the results (Fig. 2).

Homologous analyses of the DNAs were carried out *via* BLAST programs. The percentage of DNA sequence identity was 99% (positive control (9) with hydrogenase of *C. pasteurianum*). The identity of the DNAs from organisms with positive control was 1): 62%, 2):49%, 3): 57%, 4): 66% and 6):53%, respectively. Besides this, all of the sequences disagreed with the sequences of rhe previously reported in GeneBank.

These results revealed the existence of novel DNA sequences of hydrogenase that have been never reported. Thus, the organisms which were tested in this study have possibility to keep the microorganisms owns new hydrogen-producing enzymes.

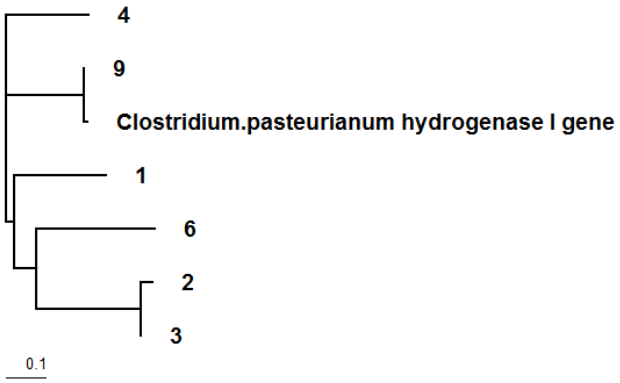

Fig. 2. Phylogenetic tree of amplified [Fe-Fe] hydrogenase genes and *C.pasuteurisnum* hydrogenase gene.

3. Concluding remarks.

We found the novel DNA sequences related with hydrogenase from the microorganism in the intestine of humus feeders. Further studies including the measurement of the performance of hydrogen-producing ability of these microorganisms are going on. And also these results suggested the possibility of the existence of other novel hydrogen-producing microorganisms in the intestines.