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Isolation of Uncultivable Anaerobic Thermophiles of the Family *Clostridiaceae* Requiring Growth-Supporting Factors

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Novel groups of uncultivable anaerobic thermophiles were isolated from compost by enrichment cultivation in medium with a cell-free extract of *Geobacillus toebii*. The cell-free extract of *G. toebii* provided the medium with growth-supporting factors (GSF) needed to cultivate the previously uncultured microorganisms. Twenty-nine GSF-requiring candidates were successfully cultivated, and 16 isolated novel bacterial strains were classified into three different groups of uncultivable bacteria. The similarity among these 16 isolates and a phylogenetic analysis using 16S rRNA gene sequences revealed that these GSF-requiring strains represented novel groups within the family *Clostridiaceae*.

Keywords: Growth-supporting factor (GSF), uncultivable bacteria, cell-free extract, *Geobacillus toebii*, commensal thermophile, compost

About 99% of the microorganisms in nature are still uncultivable using current culture methods because of the complexity of microbial communities and the lack of knowledge concerning their growth [8, 26]. These uncultured microorganisms are notable for their potential as a reservoir of novel enzymes and antibiotic resistance genes [4, 5, 9, 11, 12], and several methods have been developed to physically isolate the microorganisms by sample dilution, filtration, micromanipulation with optical tweezers, density-gradient centrifugation, and cell sorting by flow cytometry [8]. In addition, to cultivate previously uncultivable microorganisms, particular cultivation methods have been developed. These methods involve supplying certain growth-supporting factors (GSF) *via* different direct or indirect techniques, such as the use of a diffusion chamber [10], the addition of culture supernatants [22, 23], the addition of cell-free extract (CFE) of other microorganisms [3, 16, 17, 21], and co-cultivation with helper bacteria [14]. Cultivation with the CFE of other microorganisms has been applied to the cultivation of previously uncultivable microorganisms. For example, *Symbiobacterium toebii*, which was originally isolated by a screening procedure for thermostable enzymes, can grow only with partner microorganisms or with the addition of CFE of a partner microorganism [12, 17]. Understanding the commensal interactions among microorganisms might enable the cultivation of additional previously uncultivable microorganisms.

We previously reported that the growth of commensal thermophiles, which were widely distributed in various environmental samples, was greatly enhanced by adding a CFE of *Geobacillus toebii* to the culture medium [18]. We used denaturing gradient gel electrophoresis (DGGE) to estimate the diversity of the commensal thermophiles that required *G. toebii* CFE for growth, and the DGGE profiles revealed several different 16S rRNA genes among the commensal thermophiles from the soil samples [1]. Bae *et al.* [3] also reported using enrichment with a CFE of *G. toebii* to cultivate novel commensal thermophiles from compost. Here, we report the cultivation and isolation of novel GSF-requiring microorganisms, using a CFE-enriched PETN medium, which contained 30 mM $NaNO_3$ as a source of electron acceptors for denitrification under anaerobic conditions.

Compost samples were collected in August from a traditional composting site near Mt. Aso, Kyushu, Japan. The PETN medium used for cultivation contained 6 g of K_2HPO_4, 2 g of KH_2PO_4, 5 g of bacto-tryptone, 2.55 g of

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$NaNO_3$, and 10 g of yeast extract per liter of deionized water. For the enrichment cultivation, 1.0 g of a compost sample was inoculated into 100 ml of PETN medium in a 100-ml silicon-capped glass bottle, and 200 μg/ml of *G. toebii* cell-free extract was added to provide the growth-supporting factors. The sample was incubated at 60°C for 2 days under anaerobic conditions, which were created by replacing the air in the headspace of the bottle with filter-sterilized 99.9% N_2 gas.

To isolate the GSF-requiring microorganisms, the culture broth was serially diluted after enrichment cultivation and spread on plates of PETN agar containing *G. toebii* CFE. The plates were incubated at 60°C for 2 days in an anaerobic jar (CO_2/H_2, 10:90, BBL GasPak 100 anaerobic system) with BBL GasPak Plus anaerobic system envelopes (Becton Dickinson Microbiology Systems, Cockeysville, MD, U.S.A.). Anaerobic conditions were monitored using GasPak dry anaerobic indicator strips (Becton Dickinson Microbiology Systems). The colonies that grew on the agar plates were isolated, transferred to plates containing PETN agar with or without *G. toebii* CFE, and incubated again as before. The colonies that grew only on the CFE-containing agar plates were selected. This selection step was repeated at least three times to verify the requirement for *G. toebii* CFE.

To prepare the CFE, *G. toebii* cells were harvested at the late exponential phase by centrifugation at 7,000 ×*g* for 10 min at 4°C. The cell pellet was washed twice with 50 mM potassium phosphate buffer, pH 7.2, and broken with a UD-201 ultrasonic disruptor (Tomy, Tokyo, Japan). To remove cell debris, the crude extracts were centrifuged at 9,000 ×*g* for 30 min at 4°C, and then the supernatant was filtered through a 0.22-μm syringe filter (CE membrane; Sartorius AG, Goettingen, Germany).

The chromosomal DNA was extracted from pure cultures of cells for each isolate, according to a previously described method [18]. The 16S rRNA gene from each isolate was amplified by PCR using the eubacterial 16S rRNA universal primers, 27F (forward, 5'-AGA GTT TGA TCC CTG GCT CAG-3') and 1525R (reverse, 5'-AGA AAG GAG GTG ATC CAG CC-3') [15]. The PCR products were purified and used as templates for determining the sequences of the respective 16S rRNA genes, using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit and an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.). The raw 16S rRNA gene sequence data were trimmed and compiled using Seqman software version 4.0 (DNASTAR, Madison, WI, U.S.A.). The 16S rRNA gene sequences of phylogenetically related microorganisms were obtained from the Ribosomal Database Project II (RDP II, http://rdp.cme.msu.edu/) and GenBank database [25]. The 16S rRNA gene sequences were aligned with ClustalX software version 1.83 [24], and partial sequences of 16S rRNAs were edited by GeneDoc software (http://www.nrbsc.org/gfx/genedoc/index.html) [13]. A phylogenetic tree was constructed by the neighbor-joining method, and bootstrap values were calculated from 1,000 trees with the PHYLIP package version 3.67 (http://evolution.genetics.washington.edu/phylip.html) [7]. The 16S rRNA gene sequences of representative new isolates have been deposited in the GenBank database under accession numbers EU178824-EU178839 (CS308, EU178824; CS320, EU178825; CS323, EU178826; CS325, EU178827; CS327, EU178828; CS330, EU178829; CS333, EU178830; CS340, EU178831; CS341, EU178832; CS342, EU178833; CS349, EU178834; CS350, EU178835; CS351, EU178836; CS352, EU178837; CS369, EU178838; and CS533, EU178839).

From the 320 isolates tested, 29 were confirmed as microorganisms requiring GSF for growth. General microbial characterization and 16S rRNA gene sequence analysis were performed to identify their taxonomic positions. The 16S rRNA gene sequence analysis classified the 16 isolated strains into three distinct groups (Fig. 1). Groups I and II were closely related to the previously reported *Clostridiales* bacterium NS5 and NS4 groups [3] with 97.8% and 97.9% similarity, respectively. Group III was related with low similarity (93.7 to 94.9%) to the uncultured bacterium tbr1-1 and *Clostridium* sp. PML group in the whole GenBank database, which includes unidentified strains and uncultured clones. Alternatively, in the analysis with the nomenclatural GenBank database, most of the isolates were 90.0 to 97.1% similar to the family *Clostridiaceae*, *Tepidimicrobium ferriphilum* DSM 16624^T [19], and the family *Peptostreptococcaceae*, *Tissierella praeacuta* ATCC 25539^T [2, 6] (Table 1). The majority of these isolates were most similar to uncultured clones or unidentified isolates in the NCBI database, demonstrating that the strains isolated in this study belong to hard-to-cultivate groups of microorganisms.

In an analysis of the 16S rRNA gene sequence similarity among the isolates (Table 2), the Group I isolates showed 91.0 to 91.7% and 88.2 to 88.8% sequence similarity with the isolates of Groups II and III, respectively. The Group II isolates were 97.8% similar within Group II and 89.9 to 91.7% similar to the Group III isolates. The isolated strains in Group III had >98.7% sequence similarity with each other. Thus, Groups I, II, and III could be classified as three different phylogenetic groups with distances greater than those at the genus level. Additionally, the 16S rRNA gene sequence similarity between strains CS323 and CS333 in Group II was 97.8%, which indicates that these strains may be different species. According to the guidelines for species circumscription [20], a sequence similarity of 97.8% requires DNA-DNA hybridization to determine the exact identification at the species level.

A phylogenetic analysis based on 16S rRNA gene sequences showed that the 16 isolated strains were located in a branch of uncultured microorganisms that is closely

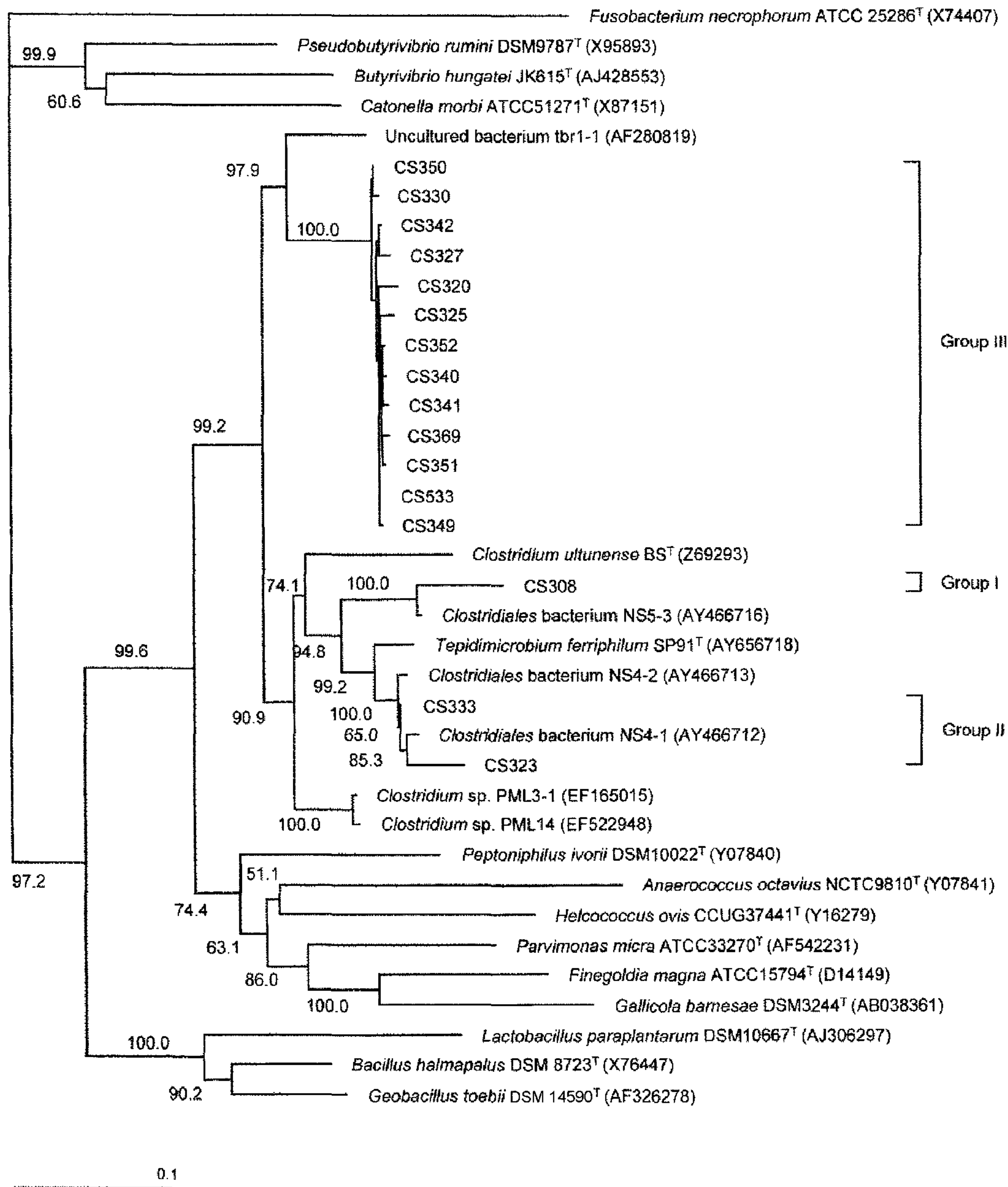

Fig. 1. Phylogenetic tree based on the 16S rRNA gene sequences of isolates and those of related microorganisms.

The reference 16S rRNA gene sequences were obtained from the Ribosomal Database Project II (RDP II, http://rdp.cme.msu.edu/) and the GenBank database. The tree was constructed using the neighbor-joining method, and bootstrap values were calculated from 1,000 replications. Numbers on the tree represent the percentages of bootstrap values. The sequence of *Fusobacterium necrophorum* ATCC 25286T served as the outgroup. The scale bar represents 0.1 substitutions per nucleotide position.

related to the family *Clostridiaceae* (Fig. 1). Bae *et al.* [3] reported that the *Clostridiales* bacterium NS4 and NS5 groups belonged to different species according to DNA-DNA hybridization experiments. The similarity analysis of the 16S rRNA sequences of Groups I and II quite clearly demonstrated that Groups I and II belong to different genera because of their low sequence similarity (about 91%; Table 2). Moreover, the location in the phylogenetic tree also demonstrated that these two groups occurred in different branches, separated by an evolutionary distance of more than 0.04 fixed-point mutations per sequence position (Fig. 1). This result strongly indicates that Groups I and II belong to different genera. Thus, based on the similarity and phylogenetic analyses of their 16S rRNA gene sequences, the isolates were divided into three groups, Group I (1 isolate), Group II (2 isolates), and Group III (13 isolates). All three groups belong to uncultivable bacterial groups in the family *Clostridiaceae*.

Table 1. Characterization of the isolated microorganisms that required cell-free extract for growth.

Group	Strain	Sequence length (nt)	Whole GenBank database		Nomenclatural GenBank database	
			Closest relative in Blast search	Similarity (%)	Closest relative in Blast search	Similarity (%)
I	CS308	1,365	*Clostridiales* bacterium NS5-3	97.8	*Tepidimicrobium ferriphilum* DSM 16624^T	91.7
II	CS323	1,496	*Clostridiales* bacterium NS4-1	97.9	*Tepidimicrobium ferriphilum* DSM 16624^T	94.9
	CS333	1,496	*Clostridiales* bacterium NS4-2	99.3	*Tepidimicrobium ferriphilum* DSM 16624^T	97.1
III	CS320	1,382	Uncultured bacterium tbr1-1	94.2	*Tepidimicrobium ferriphilum* DSM 16624^T	91.1
	CS325	1,377	Uncultured bacterium tbr1-1	94.4	*Tissierella praeacuta* ATCC 25539^T	91.2
	CS327	1,471	Uncultured bacterium tbr1-1	94.6	*Tissierella praeacuta* ATCC 25539^T	91.0
	CS330	1,466	Uncultured bacterium tbr1-1	94.9	*Tepidimicrobium ferriphilum* DSM 16624^T	91.5
	CS340	1,463	Uncultured bacterium tbr1-1;	94.7	*Tepidimicrobium ferriphilum* DSM 16624^T	91.6
	CS341	1,467	*Clostridium* sp. PML14	93.9	*Tissierella praeacuta* ATCC 25539^T	91.0
	CS342	1,464	Uncultured bacterium tbr1-1	94.8	*Tissierella praeacuta* ATCC 25539^T	91.2
	CS349	1,465	Uncultured bacterium tbr1-1	94.7	*Tissierella praeacuta* ATCC 25539^T	91.4
	CS350	1,460	Uncultured bacterium tbr1-1	94.9	*Tepidimicrobium ferriphilum* DSM 16624^T	92.1
	CS351	1,459	Uncultured bacterium tbr1-1	94.9	*Tissierella praeacuta* ATCC 25539^T	91.0
	CS352	1,456	Uncultured bacterium tbr1-1	94.8	*Tissierella praeacuta* ATCC 25539^T	90.0
	CS369	1,460	*Clostridium* sp. PML14	93.7	*Tissierella praeacuta* ATCC 25539^T	91.3
	CS533	1,466	*Clostridium* sp. PML3-1	93.9	*Tissierella praeacuta* ATCC 25539^T	91.2

Previous studies have investigated the distribution of commensal thermophiles in various soil samples [1, 18] and have shown that the cell number of commensal thermophiles increased after enrichment cultivation in medium with CFE [1, 3, 18]. In addition, several different 16S rRNA gene sequences were detected among the commensal thermophiles from various soil samples, based on the DGGE method [1, 3]. Although the DGGE analysis indicated various lineages of microorganisms, such as phylum *Firmicutes*, *Proteobacteria*, and *Bacteroidetes*, in the soil samples, the most predominant microorganisms were those belonging to the genus *Symbiobacterium* [1].

In this study, all isolates were classified into the family *Clostridiaceae*. Considering the experiment above, supplying a cell-free extract of bacteria to the culture medium is a viable method for cultivating uncultivable microorganisms using current cultivation techniques. The enrichment conditions used in this study are suitable for the cultivation of GSF-requiring bacteria belonging to the family *Clostridiaceae*. We suppose that various uncultivable microorganisms, which

Table 2. Sequence similarity among the 16S rRNAs of the isolated strains.

Similarity (%)[a]																	
III													II		I		
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		
	99.6	99.5	99.8	99.6	99.5	99.5	99.5	99.5	99.7	99.5	99.1	99.3	90.2	91.4	88.5	1	CS349
		99.8	99.8	99.6	99.5	99.5	99.4	99.2	99.5	99.3	98.9	99.2	90.2	91.4	88.6	2	CS351
			99.7	99.5	99.5	99.5	99.3	99.1	99.5	99.2	98.9	99.2	90.1	91.3	88.5	3	CS369
				99.7	99.6	99.6	99.6	99.4	99.8	99.5	99.2	99.4	90.3	91.5	88.5	4	CS533
					99.7	99.6	99.3	99.2	99.5	99.4	99.0	99.2	90.1	91.3	88.5	5	CS340
						99.5	99.2	99.1	99.5	99.2	98.9	99.1	90.1	91.3	88.6	6	CS341
							99.2	99.2	99.5	99.3	98.9	99.2	90.2	91.4	88.5	7	CS352
								99.5	99.5	99.3	98.8	99.0	90.2	91.4	88.5	8	CS350
									99.4	99.2	98.7	98.9	90.5	91.7	88.8	9	CS330
										99.5	99.0	99.2	90.1	91.3	88.4	10	CS342
											98.9	99.1	90.1	91.2	88.5	11	CS327
												99.0	89.9	91.0	88.3	12	CS320
													90.3	91.2	88.2	13	CS325
														97.8	91.0	14	CS323
															91.7	15	CS333
																16	CS308

[a]Similarity was calculated using 1,320-nucleotides in a region homologous among the 16S rRNA gene sequences of microorganisms, after eliminating the sequences of the 5' and 3' diverse regions.

cover the various lineages in nature, can be cultivated successfully using a CFE after changing the enrichment conditions, such as temperature, oxygen tension, and supply of various electron donors or acceptors. The bacterial origin of the CFE (*e.g.*, mesophiles or extremophiles) may also be an important consideration.

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