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Effects of Plant Residue Extract and Cobalamin on Growth and Propionate Production of *Propionicimonas paludicola* Isolated from Plant Residue in Irrigated Rice Field Soil

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Since most of the anaerobic bacterial isolates from rice plant residue in irrigated rice field soil grew slowly or weakly in the medium (PY medium) used, growth factors for the isolates were investigated. Plant residue extract (RE) was prepared by autoclaving plant residue collected from the soil, and RE was added to the medium as a possible source of growth factors. With the addition, growth of the slowly-growing, propionate-producing strains of *Propionicimonas paludicola* was considerably improved. Lactate was the dominant product of these strains in the presence of lower concentrations of RE. Moreover, amounts of acetate and propionate produced increased in proportion to the RE concentration added. The factor in RE affecting the growth of these strains appeared to be cobalamin, and addition of cobalamin to the medium remarkably improved their growth. With a sufficient amount of cobalamin added, propionate was the dominant product from the onset of fermentation, while lactate was only a minor product. Concentrations of cobalamin in RE were determined using the microbiological method. Almost exactly the same amount of cobalamin was detected in RE prepared from plant residue samples collected in different years. Cobalamin was also detected in extracts of the rice field soil, although the concentrations were much lower than those in RE. It was suggested that these cobalamin-requiring, propionate-producing bacteria survive in rice field soil by using cobalamin supplied by other microbes, which endogenously produce cobalamin and release it into the environment.

Key words: plant residue in rice field, anaerobic bacteria, propionate, cobalamin or vitamin B_{12}, methanogenic community, *Propionicimonas paludicola*

Flooded rice field soil is a typical anoxic environment, and rice fields have been thought to be one of the major sources of atmospheric methane[7,9,11,13,18,24,34]. In anoxic environments, organic matter is anaerobically decomposed by hydrolytic and fermentative bacteria to mainly lower fatty acids such as formate, acetate, propionate, and butyrate, as well as H_2 and CO_2. Propionate is often detected as the second most abundant fatty acid next to acetate in methanogenic paddy soil[8,20]. Although formate, acetate, and H_2+CO_2 are directly used as methanogenic substrates, propionate and butyrate are further oxidized to acetate and H_2 and then consumed as substrates for methanogens[5,8,25,27,30,39].

Organic fertilizers such as rice straw applied to rice fields are actively decomposed by various anaerobic bacteria living on it at a high population density[14,15]. Thus, a large amount of fermentation products including fatty acids should be supplied through the degradation and stimulate methanogenesis in the plant residue to increase methane emission rates from the fields[33,35,37]. However, little is known about the structure of the anaerobic microbial community and relationships among microbes involved in the degradation of plant residue in flooded rice field soil[1,8,15,17].

In investigations to analyze the composition of the cultur-

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able anaerobic microbial community in soil of a Japanese irrigated rice field, we previously isolated anaerobic bacteria from rice plant residue as well as from living roots of rice plants in the soil. Anaerobic bacterial isolates from these samples were phylogenetically affiliated based on 16S rRNA gene (16S rDNA) sequences, and representative strains were partially characterized to estimate their functions in the microbial community[1,23]. The results obtained indicated that many anaerobic bacterial groups, which are only distantly related to known bacterial species, are living in these fractions at a rather high population density. Out of the isolates from plant residue samples, two strains of the propionate-producing group in the *Actinobacteria* phylum were fully characterized, and we have proposed a new genus and species name, *Propionicimonas paludicola*, for them[2].

During the characterization, it was found that the type strain (Wd^T=JCM 11933^T=DSM 15597^T) of *P. paludicola* grew very slowly in the PYG medium used for cultivation. Furthermore, most other isolates from plant residue samples also grew rather slowly or weakly in the medium. Since all the isolates were enumerated at rather high population densities of 10^{8-9} CFU/g dry wt[1], we were interested in how these slowly-growing bacteria could successfully survive in their habitat.

In this study, we sought natural sources of growth factors for these slow-growing anaerobic bacteria. Plant residue extract (RE) was prepared from plant residue samples collected from the flooded rice field soil and added to the medium. Although most of the isolates were unaffected, RE greatly stimulated growth of the slowly growing strains of *P. paludicola*. The end products of fermentation of the strains were also changed by the addition, and production of propionate was increased in proportion to the RE concentration added. The essential growth factor in RE affecting these strains appeared to be cobalamin (vitamin B_{12}), and cobalamin was actually detected in the RE.

Materials and Methods

Rice field used for sample collection

Plant residue samples for the preparation of RE were collected from a rice straw-treated (RS) plot of an irrigated rice field at the Shonai Branch of the Yamagata Agricultural Experimental Station (Fujishima-machi, Yamagata, Japan) during the rice growing season, as described previously[1]. The RS plot was applied annually with 500 kg rice straw ha^{-1} (cut into pieces of approximately 2 cm) for more than 20 years[17,31]. Stubble of rice plants left in the field with roots after harvest has been also plowed into the soil together with the rice straw. Characteristics of the soil and the cultivation practices of the field were previously described[17,31,32].

The soil used for preparing the soil extract was collected from either the plowed layer of the RS plot or a no organic fertilizer (NO) plot. The NO plot was adjacent to the RS plot and had not been treated with organic fertilizers.

Strains and cultivation methods

Six representative strains (W10, W5, Wd^T, WB4, K5, and KB3) selected from 47 anaerobic bacterial isolates from plant residue samples, that is, rice straw or rice stubble with roots, in irrigated rice field soil were used[1]. The "W" and "K" strains are isolates from straw and stubble samples, respectively. Strain Wd^T (=JCM 11933^T=DSM 15597^T) has been named *P. paludicola* as described above[2], and strain K5 was identified as *P. paludicola* based on the similarity of its 16S rDNA with strain Wd^T (99.5%) and other characteristics. All other strains are only distantly related to each other, and phylogenetic affiliations and some physiological properties of the strains have been shown previously[1].

The strains were usually maintained in PY4S agar slants[1] with a mixed gas (N_2/CO_2=95/5) in the headspace. PY4S agar slants supplemented with 5% (v/v) RE, prepared as described below, were also used for maintenance of some strains. The incubation temperature was 30°C.

Preparation of plant residue extract (RE) and soil extract

RE was prepared using the plant residue (mainly rice straw and rice stubble with roots) collected from the flooded plowed layer of the RS plot in June of three different years (1997, 1998, and 2002). RE was prepared based on the method for preparing soil extract as a supplement to media for cultivation of soil microbes[3]. Plant residue samples collected were washed gently with tap water several times to remove the soil and then cut into small pieces. The samples were autoclaved for 30 min. at 121°C with a fivefold amount of distilled water (wet weight basis). After cooling, the autoclaved samples were filtered, and the filtrate was centrifuged at 10,000×*g* for 30 min. The supernatant obtained was used as RE and stored at −20°C until use. Soil extract was also prepared in the same way using soil samples collected at the same time in 2002 from the flooded plowed layer of both the RS and NO plots. Plant residue such as rice straw and rice roots in soil samples collected was removed before the preparation of soil extract[16,17].

Growth experiments

Strains were cultivated in PY liquid medium[1,10] as a basal medium with or without various supplements such as RE, vitamins, or other possible growth factors.

The composition of the vitamin mixture used (100 ml^{-1}) was 0.1 mg biotin, 0.1 mg cobalamin (as cyanocobalamin), 0.3 mg *p*-aminobenzoic acid, 0.5 mg folic acid, 0.5 mg thiamine hydrochloride, 0.5 mg riboflavin, and 1.5 mg pyridoxine hydrochloride. The trace element solution was prepared according to Widdel and Pfennig[36], and a solution of hemin and vitamin K mixture was prepared by mixing respective stock solutions[10]. These solutions were added to PY liquid medium at 1% (v/v) for cultivation experiments. Effects of cobalamin on growth and fermentation products of strains Wd^T and K5 were examined using cyanocobalamin (Kanto Chemical Co. Inc., Tokyo, Japan).

Glucose was added at 1% (w/v) to PY liquid medium (PYG medium). Growth was monitored by measurement of the optical density at 660 nm with a spectrophotometer (Hitachi U-1000, Katsuta, Japan). All the cultivation tests were carried out in duplicate at 30°C. Fatty acids produced as fermentation products were analyzed using the liquid medium used for the cultivation.

Quantification of cobalamin

Quantification of cobalamin in RE or soil extract was carried out by the microbiological assay method using *Lactobacillus delbrueckii* subsp. *lactis* (="*L. leichmannii*" JCM 1557=ATCC 7830) as a cobalamin-requiring bacterium[4]. The strain was aerobically cultivated in Inoculum Broth USP (Difco Laboratories, Detroit, MI, USA) until the early stationary phase, and the cells were inoculated into the vitamin B_{12} Assay Medium USP (Difco Laboratories) in the presence of different concentrations of extracts for the quantification of cobalamin. The cells were also inoculated into the assay medium containing different concentrations of cobalamin as standard cultures. Cyanocobalamin (Kanto Chemical Co. Inc., Tokyo, Japan) was used as a standard reagent of cobalamin. The growth of the bacterium was monitored by measurement of the optical density at 660 nm with a spectrophotometer (Hitachi U-1000). The cultivation was carried out in duplicate at 37°C.

Analytical methods

Fatty acids in the cultivation liquid medium were analyzed with a gas chromatograph (Hitachi G-5000 or 263-30, Katsuta, Japan) or a high-performance liquid chromatograph (Shimadzu LC-10AD, Kyoto, Japan) as described previously[1]. The glucose concentration in the same medium was measured with a D-Glucose kit (Boehringer Mannheim GmbH, Mannheim, Germany) according to the manufacturer's protocol.

Results

Effects of RE on growth of isolates

When representative anaerobic isolates from plant residue were cultivated in PYG liquid medium, most of the strains grew rather slowly or weakly. Figure 1 shows some typical growth curves of the strains examined. Strain WB4 was a relatively fast grower with a specific growth rate (μ) of 0.343 h^{-1} (Fig. 1D). Growth rates of other strains, however, were much lower (e.g., μ=0.051 h^{-1} for strain W5, μ=0.064 h^{-1} for strain W10, and μ=0.042 h^{-1} for strain KB3) (Fig. 1A, B, F), and the growth of both *P. paludicola* strains (Wd^T and K5) was the slowest (μ=0.026 h^{-1} for strain Wd^T and μ=0.014 h^{-1} for strain K5) (Fig. 1C, E) among the strains examined.

To improve the growth of these isolates, RE was added to the medium at 10% (v/v) as a possible source of growth factors. Although most of the strains were not affected by the addition, the growth of strains Wd^T and K5 was considerably improved (Fig. 1C, E). With 10% (v/v) RE added, the specific growth rates were raised to 0.126 h^{-1} for strain Wd^T

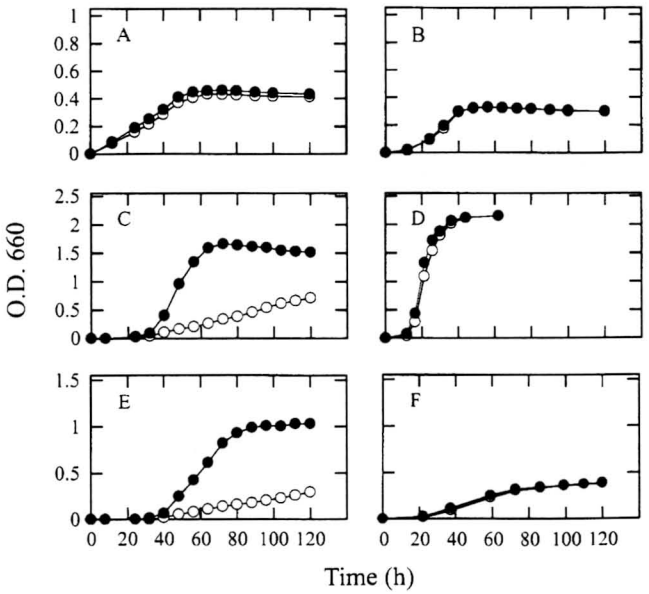

Fig. 1. Growth of six strains (A, W5; B, W10; C, Wd^T; D, WB4; E, K5 and F, KB3) in PYG liquid medium with (●) or without (○) 10% (v/v) plant residue extract (RE). RE prepared in 1997 was used. Values are means of duplicate experiments.

and to 0.075 h^{-1} for strain K5.

Effects of RE on growth and fermentation products of P. paludicola *strains Wd^T and K5*

To confirm the effects of RE on growth and fermentation products of strains Wd^T and K5, both strains were cultivated with different amounts (0–20%, v/v) of RE added. Growth of strains Wd^T (Fig. 2A) and K5 (Fig. 2B) was commonly improved depending on the increase in the RE concentration. Addition of 20% (v/v) RE further improved the growth compared with addition of 10% (v/v) RE, and the specific growth rates of strains Wd^T and K5 reached 0.134 h^{-1} and 0.103 h^{-1}, respectively.

Fermentation products in PYG medium with different amounts of RE were analyzed at the end of cultivation in Fig. 2 for both strains (Fig. 3A, B). In the absence of RE, both strains commonly produced acetate and lactate with propionate as a minor product. Strain Wd^T also produced succinate, while strain K5 did not. Although the lactate produced was increased markedly by addition of 2% (v/v) RE, it was not increased much by further addition in either strain. A concentration of 20% (v/v) RE decreased lactate production compared with 10% (v/v) RE, while amounts of acetate and propionate produced increased in proportion to the concentration of RE added. The succinate production of strain Wd^T decreased slightly in proportion to the increase in the RE concentration.

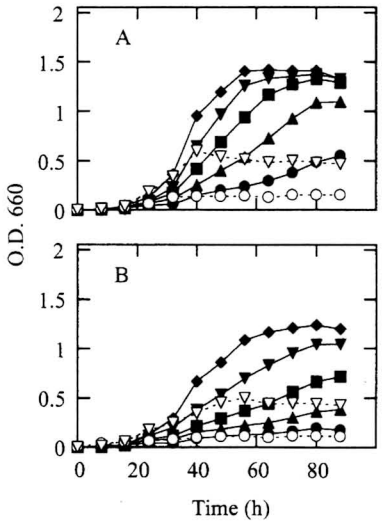

Fig. 2. Effects of concentration of plant residue extract (RE) on growth of strains Wd^T (A) and K5 (B). RE concentration: ○ and ●, 0% (v/v); ▲, 2% (v/v); ■, 5% (v/v); ▽ and ▼, 10% (v/v); ◆, 20% (v/v). Solid line, with 1% (w/v) glucose; broken line, without glucose. RE prepared in 1997 was used. Values are means of duplicate experiments.

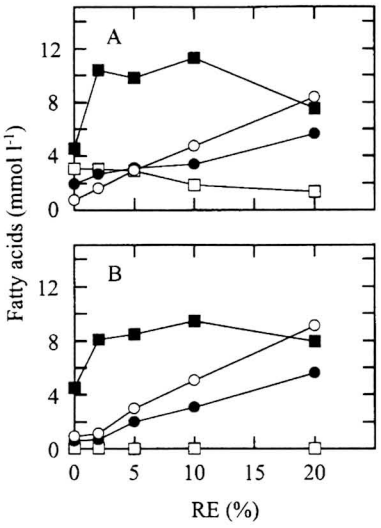

Fig. 3. Effects of concentration of plant residue extract (RE) on fermentation products of strains Wd^T (A) and K5 (B) with 1% (w/v) glucose. Symbols: ●, acetate; ○, propionate; ■, lactate; □, succinate. Values are means of duplicate experiments.

Effects of RE on the growth of both strains without glucose in PY medium were also examined (Fig. 2A, B). The strains showed slight growth without RE, but in both cases growth improved with the addition of 10% (v/v) RE. Without glucose, acetate and propionate were end products, while lactate was not produced regardless of the presence of RE. The addition of RE increased production of acetate by 3.0 mmol l^{-1} and propionate by 4.0 mmol l^{-1} for both strains (data not shown).

Cobalamin requirement of P. paludicola *strains Wd^T and K5*

To define the growth factors in the RE-stimulated growth of strains Wd^T and K5, both strains were cultivated in PYG liquid medium supplemented with various possible growth factors, that is, vitamins, trace elements and hemin, in place of RE. For both strains, almost identical results were obtained.

Trace elements and hemin plus vitamin K did not have any effects on the growth of the strains, while the vitamin mixture caused a remarkable stimulation of growth. The effect of the vitamin mixture on the growth of strains Wd^T and K5 was more distinct than that of 10% (v/v) RE, and both strains became rather fast-growers in the presence of vitamins. When the strains were cultivated in the presence of individual vitamins in the mixture, cobalamin at the same concentration as in the mixture produced almost the

same effect as the vitamin mixture (data not shown).

Figure 4A shows the growth of strain Wd^T in PYG medium with different amounts of cobalamin. Addition of cobalamin to 1 μg l^{-1} considerably raised the growth rate (μ=0.168 h^- with 1 μg l^{-1} of cobalamin), but further addition had little effect (Fig. 4B). End products of fermentation in these cultures were analyzed (Fig. 4C). Addition of cobalamin to 1 μg l^{-1} particularly increased the amount of lactate produced, while on further addition production of lactate decreased and production of acetate and propionate increased. In particular, propionate levels increased considerably in proportion to the cobalamin concentration added and in accordance with the decrease in the amount of lactate produced. Thus, in the presence of 10 μg l^{-1} of cobalamin, propionate was the most dominant end product, and the molar ratio of acetate and propionate produced was about 1:2. Lactate was only a minor product. Almost the same results were obtained for strain K5 (data not shown).

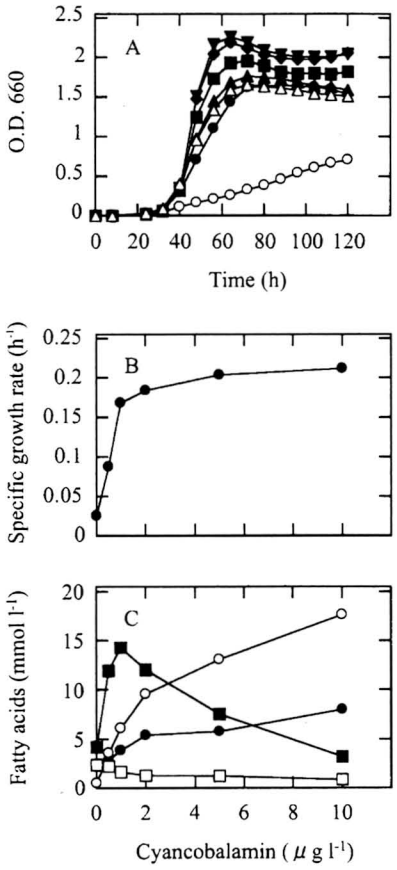

Fig. 4. Effects of concentration of cyanocobalamin on growth of strain Wd^T (A). Symbols: ○, no addition; △, with 10% (v/v) RE. Cyanocobalamin concentration (μg l^{-1}): ●, 0.5; ▲, 1; ■, 2; ◆, 5; ▼, 10. Effects of concentration of cobalamin on the specific growth rate (μ) of strain Wd^T (B). Effects of cobalamin on fermentation products of strain Wd^T (C). Symbols: ●, acetate; ○, propionate; ■, lactate; □, succinate. Values are means of duplicate experiments.

For strain Wd^T, time courses of growth, consumption of glucose, and fatty acid production were investigated using PYG medium with different amounts of cobalamin (0, 1, and 20 μg l^{-1}) (Fig. 5). With 1 μg l^{-1} of cobalamin, lactate was produced dominantly throughout the course of fermentation, while in the presence of 20 μg l^{-1} of cobalamin, lactate production was suppressed and propionate production proceeded most actively from the onset of fermentation.

Detection of cobalamin in RE and soil extract

Concentrations of cobalamin in the RE and the soil extract were determined. It was shown that similar amounts of cobalamin were extracted from the plant residue samples collected in different years, and the concentrations of cobalamin in the RE were about tenfold higher than those in the soil extract (Table 1). Since RE1 in Table 1 was used in the cultivation experiments shown in Figs. 1–3, the addition of 10% (v/v) RE was found to be equivalent to addition of 0.49 μg l^{-1} of cobalamin.

When contents of cobalamin in two undefined components of PY medium (10 g l^{-1} of Trypticase and 5 g l^{-1} of yeast extract) were determined using the same method, it

Table 1. Cobalamin concentration in plant residue extract (RE) and soil extract collected from rice field soil

Extract[a]	Cobalamin concentration	
	μg l^{-1} [b]	μg kg^{-1} [c]
Plant residue extract		
RE1	4.93	24.7
RE2	3.14	15.7
RE3	3.74	18.7
Soil extract		
RS plot	0.43	2.15
NO plot	0.28	1.40

[a] The plant residue samples used for the preparation of RE were collected from the flooded RS plot in June of 1997 (RE1), 1998 (RE2) and 2002 (RE3). Soil samples were collected from the RS plot or the NO plot, which was adjacent to the RS plot and had not been treated with organic fertilizer, at the same sampling time as that of plant residue used for preparation of RE3. The extract was prepared soon after the collection and stored at −20°C until use.

[b] Concentrations in extracts prepared by adding fivefold amounts of water to the samples (wet weight basis).

[c] Amounts of cobalamin in the samples used for extraction (wet weight basis).

The values were roughly estimated on the assumption that all cobalamin present in the sample was released into the water added.

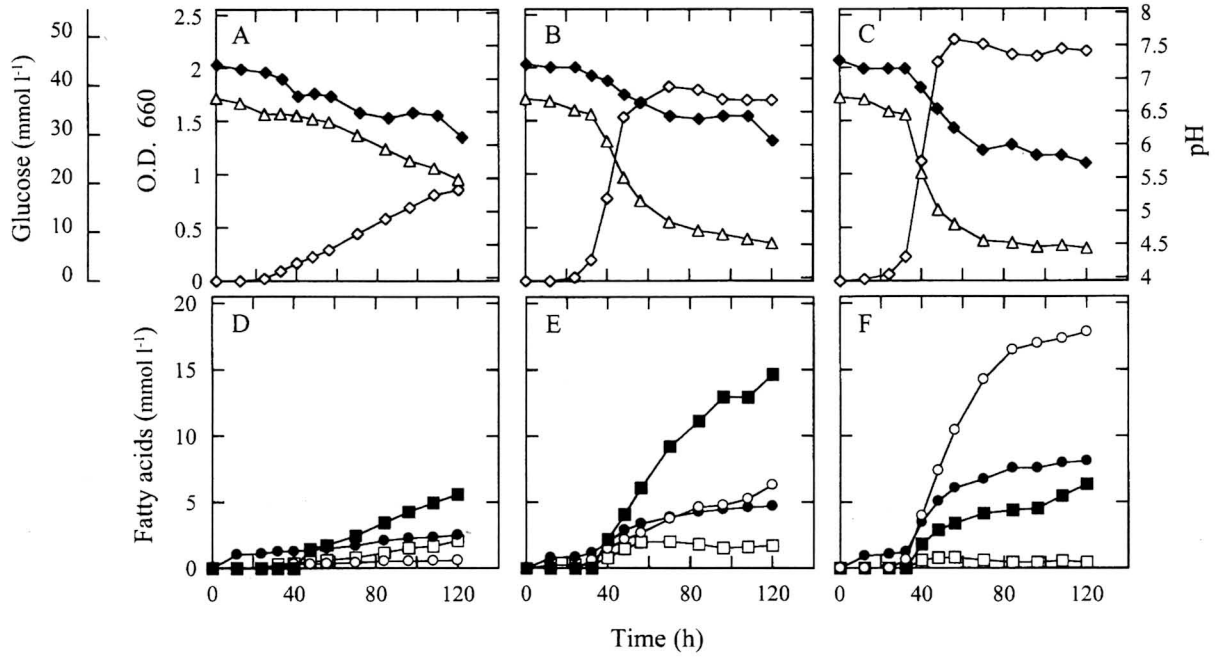

Fig. 5. Time courses of growth and fermentation of strain Wd^T in the presence of different amounts of cyanocobalamin. Cyanocobalamin: (A) and (D), no addition; (B) and (E), 1 μg l^{-1}; (C) and (F), 20 μg l^{-1}. Symbols: ◇, OD; ◆, concentration of glucose; △, pH; ●, acetate; ○, propionate; ■, lactate; □, succinate. Values are means of duplicate experiments.

appeared that these materials might supply about 0.10 μg l^{-1} of cobalamin to the PY medium.

Discussion

The total population densities of culturable anaerobic bacteria on the plant residue samples in the irrigated rice field soil were in the order of 10^9 CFU/g dry wt[1,15], while most of the isolates from the samples grew rather weakly. The strains used for the growth experiment in Fig. 1 were representatives of the dominant groups of the culturable anaerobic bacterial isolates from the plant residue[1], and thus these bacterial groups were considered to successfully thrive on plant residue in the soil.

Defining the optimal growth conditions of microbes newly isolated from natural environments is important to expand the number of culturable species of microbes and to analyze their physiological and functional characteristics. The addition of RE to the medium remarkably improved the growth of the slowest growing, propionate-producing strains, *P. paludicola* Wd^T and K5, and the factor in RE affecting the growth appeared to be cobalamin. The growth of strains Wd^T and K5 in the presence of RE or cobalamin indicated that both strains are strictly dependent on the presence of exogenous cobalamin for their existence. The trace amount of cobalamin derived from the materials in PYG medium without any supplements should support the slow growth of these strains. Furthermore, it was found that strain Wd^T changes the major fermentation products from lactate to propionate depending on the exogenous concentration of cobalamin.

Cobalamin has extensive functions in microbial communities, especially of anaerobic microbes, with many important reactions known to be dependent on it. Most propionate-producing bacterial species produce propionate through the methyl-malonyl CoA pathway. One of the key enzymes in the pathway is methyl-malonyl CoA isomerase, and this enzyme is dependent on cobalamin[22]. Some propionate-producing bacterial species such as *Propionibacterium freudenreichii* are known to produce cobalamin endogenously[38]. The effects of cobalamin on the fermentation pattern of strain Wd^T as shown in Fig. 5 strongly suggested that the strain produced propionate using the methyl-malonyl CoA pathway.

Another major reaction dependent on the cobalamin compound is methyl transfer. Two major groups in anaerobic microbial ecosystems, acetogens and methanogens, possess distinct metabolisms relating to the cobalamin compound. Acetyl-coenzyme A biosynthesis in acetogenic bacteria as well as the acetate catabolism of methanogens involves sim-

ilar corrinoid-dependent methyltransferases[29]. It has been reported that various acetogenic anaerobic bacteria including *Acetobacterium* spp. and *Sporomusa ovata* produce large amounts of corrinoids[12,28], and one of the acetate-utilizing methanogenic archaea, *Methanosarcina barkeri*, produced vitamin B_{12}-compound (Factor III) during cultivation in a methanol-minimum medium. The methanogenic strain excreted 80% of the B_{12} compound produced into the medium without cell lysis[21]. The involvement of corrinoids in anaerobic dehalogenation has also been reported[19,26,29]. Thus, it is likely that the cobalamin compound produced endogenously by these microbial groups serves as an exogenous source of cobalamin for other microbial groups in the community.

In this study, a microbially active cobalamin was actually detected in RE, which was prepared by briefly autoclaving collected plant residue. Cobalamin was also detected in soil extract of the rice field, although the soil extract must be used without dilution (at 100%) to supply almost the same concentration of cobalamin as that in the medium with 10% (v/v) RE. The method used to prepare the extracts was not closely examined in this study, and thus the efficiency of the extraction of cobalamin was unclear. More investigations may be necessary to determine definite concentrations of cobalamin in these samples, however, plant residue may certainly provide a habitat rich in cobalamin for microbes in flooded rice field soil.

A large population of methanogens was detected on plant residue in the RS plot in our previous study[15]. The methanogenic population is one of most probable sources of cobalamin in plant residue under anaerobic decomposition as described above, and thus methanogens, which are supported by other microbes on the substrates to produce methane, might supply an essential growth factor for these propionate-producing bacteria. Furthermore, one of the strains investigated in this study, strain WB4, grew fast even in the absence of RE (Fig. 1) and produced propionate as a major product (data not shown). Although we have not yet examined cobalamin production in the strain, propionate-producing bacterial groups such as strain WB4 in rice field soil are other candidates for producers of cobalamin. The distributions of microbes in plant residue or soil in rice fields might not be homogenous, and thus the sites with high concentrations of cobalamin, which may be located around colonies of cobalamin-producing and -releasing microbes, may be spread heterogeneously in micro-environments.

Propionate is one of the most important intermediates in the carbon flow to methane in anaerobic environments including anoxic rice field soil[8,20]. In anoxic environments like flooded soil, propionate is usually oxidized to acetate and H_2 by syntrophic propionate decomposers and then consumed as substrates for methanogenesis[5]. A propionate-decomposing bacterium, which syntrophically decomposes propionate with methanogens as H_2-utilizing partners, has been isolated from rice field soil[6]. The production and transfer of cobalamin in relation to the methanogenesis and formation and decomposition of propionate seem to be important aspects of methanogenic microbial communities.

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