Characteristics and Propionate Production of *Propionibacterium* Isolated from a Methane fermentation Digester

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Characteristics and propionate production of propionate-producing bacteria in an anaerobic digester were examined. Strains VA-1, MC-1 and KA-2 isolated from sludge were nonmotile, nonspore forming, gram-positive and pleomorphic rods. They fermented lactate to propionate and acetate both with a complex medium and with a mineral medium. The molar ratio of propionate to acetate was 2/1. They utilized glucose, lactate, fumarate, malate, pyruvate and serine for growth, but could not use starch and inulin. From these results the propionate-producing bacteria are considered to be classified into the genus *Propionibacterium* according to Bergey's manual of determinative bacteriology. *Propionibacterium* in methane fermentation sludges may utilize low molecular compounds such as monosaccharides, organic acids and amino acids to produce propionate and acetate. PCR amplification using genus-specific primers supported this identification as *Propionibacterium*. Lactate was converted to propionate and acetate in the dual culture with *Propionibacterium* sp. strain VA-1 and *Desulfovibrio vulgaris* strain F-1 in the presence of sulfate. In the triculture with strain VA-1 and strain F-1 in syntrophic association with *Methanobacterium formicicum* strain HM-1 in the absence of sulfate, lactate was fermented to propionate, acetate and methane. The molar ratio of propionate to acetate in both mixed cultures decreased by about 1/8 as compared with that of the single culture of strain VA-1. The ratio, however, increased 2/1 in the case of the triculture at pH 5.0. Although the population of *Propionibacterium* in the dual culture and the triculture at pH 7.0 was large, the amount of propionate produced was not. Because of the slow growth rate of *Propionibacterium*, the propionate production may increase slowly. Under acidic conditions, propionate produced by *Propionibacterium* is considered to be accumulated in an anaerobic digester as sulfate reducers and methanogens are not able to grow under the conditions.

Key words: methane fermentation, *Propionibacterium*, mixed culture, competitive lactate fermentation

The complete degradation of organic matter into methane and carbon dioxide requires at least three functionally different trophic groups of microorgan-

isms, i) hydrolytic fermentative bacteria, ii) syntrophic acetogenic bacteria, and iii) methanogens¹⁷⁾. Thus, it is important to keep a balance of the various microorganisms which are involved in methane fermentation. Indeed, an accumulation of volatile fatty acids such as propionate and acetate caused a

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decrease of pH in an anaerobic digester and lead to a stop in the methanogenesis¹¹). Propionate was also an inhibitor to bacterial growth^{5,11}). Propionate-producing bacteria which belong to the hydrolytic fermentative bacteria of the first group produce propionate and acetate extracellularly and vitamin B₁₂ intracellularly from saccharides and amino acids as substrates⁷). The microorganisms first described by Orla-Jensen in 1909 were isolated from dairy products, silages and sludges, and were called classical or dairy propionibacteria¹²). The microorganisms have been used in the fermentative industry to produce milk products, herbicides, flavors, preservatives and medicines^{13,18}).

A number of propionate-producing bacteria and sulfate reducers which utilized lactate stayed in methane fermentation sludges¹⁴). The propionate-producing bacteria seem to be competitive with sulfate reducers for lactate as a substrate in an anaerobic digester. Though a sulfate reducer can grow singly in the presence of sulfate, it is able to grow even in a sulfate-free medium when cultured in association with a hydrogen-utilizing methanogen^{1,10}). *Desulfovibrio vulgaris* strain F-1 converted lactate to acetate singly in the presence of sulfate. In the absence of sulfate, strain F-1 converted lactate to acetate when it was grown on lactate in syntrophic association with *Methanobacterium formicicum* strain HM-1¹⁵). The propionate-producing bacteria produced propionate and acetate from lactate. A large amount of propionate produced by the propionate-producing bacteria causes the decrease of pH since it is difficult to degrade propionate in a methane fermentation digester. However, the ecological roles of propionate-producing bacteria in methane fermentation sludges have not been investigated. In this study, characteristics of propionate-producing bacteria isolated from an anaerobic digester acclimatized with a sulfate-free medium were examined. With the propionate-producing bacterium, *D. vulgaris* strain F-1 or *M. formicicum* strain HM-1 isolated from the same sludge, competitive lactate fermentation was studied and the mutual relations of these bacteria in mixed culture were discussed.

Materials and Methods

Source of microorganisms

The propionate-producing bacteria, strains VA-1, MC-1 and KA-2, *Desulfovibrio vulgaris* strain F-1¹⁵) and *Methanobacterium formicicum* strain HM-1⁶), were isolated from bacterial flocs acclimated with a sulfate-free starch medium for a long time in a methanogenic digester¹⁴).

Media and cultivation conditions

The anaerobic procedures used for cell cultivation and media preparation were the same as previously described¹⁴). The basal medium had the following composition (g/l): KH₂PO₄, 0.2; NH₄Cl, 0.25; NaCl, 1.0; MgCl₂·6H₂O, 0.4; KCl, 0.5; CaCl₂·2H₂O, 0.15. The medium was sterilized for 15 min at 121°C. Sodium bicarbonate, sodium sulfide, Se-W solution, trace element solution and vitamin solutions were added to 1 liter of the autoclaved, cooled basal medium from stock solutions. NaHCO₃ and Na₂S were added to obtain final concentrations of 30 mM and 0.5 mM, respectively. The above mentioned solutions were: (1) 1 ml of Se-W solution (mg/l) sterilized by autoclaving; Na₂SeO₃·5H₂O, 3; NaWO₄, 4; NaOH, 500; and (2) 1 ml of trace element solution (g/l) sterilized by autoclaving; ZnCl₂, 0.07; MnCl₂·4H₂O, 0.1; H₃BO₄, 0.062; CoCl₂·6H₂O, 0.2; NiCl₂·6H₂O, 0.02; NaMoO₄·2H₂O, 0.04; CuCl₂·2H₂O, 0.02; FeCl₂·4H₂O, 1.5. (3) 1 ml of vitamin solution (mg/l) sterilized by filtration; biotin, 10; 4-aminobenzoic acid, 40; thiamin, 100; (4) 1 ml of B₁₂ solution (50 mg/l) sterilized by autoclaving; The gas phase was N₂ and the pH of the medium was adjusted to 7.1~7.3. Ten ml of the medium was distributed in 30-ml test tubes with butyl rubber stoppers and screw caps. Strain VA-1 was grown in test tubes with 10 ml of medium at 30°C under an atmosphere of N₂. Strain F-1 was grown in test tubes with 10 ml of medium supplemented with Na₂SO₄ (3 g/l) under an atmosphere of N₂. Strain HM-1 was grown in test tubes with 10 ml of medium under an atmosphere of H₂ and CO₂ (80 : 20, v/v). Strains VA-1 and F-1 were cultured at 30°C in 30-ml test tubes containing 10 ml of medium supplemented with sodium lactate (2.5 g/l)

and Na_2SO_4 (3 g/l) under an atmosphere of N_2 . Strains VA-1, F-1 and HM-1 were tricultured at 30°C in 30-ml test tubes containing 10 ml of medium supplemented with sodium lactate (2.5 g/l) under N_2 . The dual culture of strains VA-1 and F-1 was initiated by inoculating with 0.5 ml of a liquid culture of strain VA-1 (1.28×10^8 cells/ml) and 0.3 ml of that of strain F-1 (1.7×10^7 cells/ml). The triculture of strains VA-1, F-1 and HM-1 was initiated by inoculating with 0.5 ml of a liquid culture of strain VA-1 (1.28×10^8 cells/ml), and 0.2 ml of that of strain F-1 (1.7×10^7 cells/ml) and strain HM-1 (2.28×10^7 cells/ml).

The complex medium used for the taxonomic test of the propionate-producing bacteria had the following composition (g/l): trypticase, 5; yeast extract, 2.5; meat extract, 1; cysteine hydrochloride $\cdot \text{H}_2\text{O}$, 0.25; sodium lactate, 5, salt solution I, 75 ml/l and salt solution II, 75 ml/l. The salt solutions were prepared with (% w/v): (I) K_2HPO_4 , 0.6; (II) KH_2PO_4 , 0.6; $(\text{NH}_4)_2\text{SO}_4$, 1.2; NaCl , 1.2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.12; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.12. The medium was sterilized for 15 min at 121°C. The gas phase was N_2 and the pH of the medium was adjusted to 6.8–7.0. Ten ml of the medium was distributed in 30-ml test tubes with butyl rubber stoppers and screw caps. The bacterial numbers were measured with a bacterial counting plate (Kayagakiirika model EKDS). Whole-cell protein was measured by the method of Lowry *et al*⁹⁾.

Analytical procedures

Propionate and acetate were determined with a gas chromatograph (Shimazu model GC-8A) using a gaskuropack 54 column connected to a flame ionization detector. The oven temperature was 180°C and N_2 was used as the carrier gas. Lactate was methylated with methanol and 50% H_2SO_4 at 60°C and determined with a gas chromatograph (Shimazu model GC-8A) using 10% FFAP on a chromosorb WAW column connected to a flame ionization detector. The oven temperature was 135°C and N_2 was used as the carrier gas. Methane and hydrogen were determined with a gas chromatograph (Shimazu model GC-8A) using a WG-100 column connected to a thermal conductivity detector. The oven temperature was 60°C and Ar was used as the carrier gas.

The bacterial DNA was isolated by a method described previously⁸⁾. The G + C content of the bacterial DNA was measured using a DNA-GC-kit (Yamasa shyou co., ltd.).

Template DNA for PCR

Template DNA from propionate-producing bacteria was prepared as described previously⁸⁾.

PCR amplification

PCR was carried out to amplify a specific region (900 bp) of the 16S rRNA genes from all chromosomal DNA samples with the *Propionibacterium* specific primer pr1 and the eubacteria specific primer pr4 (5'-TGCTTTTCGATACGGGTTGAC-3' and 5'-AGGAGGTGATCCARCCGCA-3', corresponding to the *E. coli* 16S rRNA positions 632–651 and 1522–1540, respectively²⁾). Amplification was performed by using Ampli Taq Gold (Perkin Elmer) according to the manufacturer's instructions. The reaction mixture contained 20 ng of the template DNA, 1.5 mM MgCl_2 and 0.5 μM primers. PCR amplification was carried out for 40 cycles with the following conditions in a DNA thermal cycler (Takara TP3000): precycle denaturation of DNA thermal cycles, 95°C for 15 sec, 56°C for 30 sec and 72°C for 2 min. There was a final extension step at 72°C for 7 min to complete polymerization. The presence and size of amplified products were determined by agarose gel electrophoresis and ethidium bromide staining.

Results and Discussion

Characteristics of the propionate-producing bacteria

The propionate-producing bacteria, strains VA-1, MC-1 and KA-2, fermented lactate to propionate and acetate not only with a complex medium but also with a mineral medium. Growth and fermentation products of strain VA-1 grown on a lactate complex medium are shown in Fig. 1. These strains did not require yeast extract, meat extract and trypticase for growth. It is considered that the propionate-producing bacteria that stayed in the methane fermentation sludges could grow on a lactate defined medi-

Fig. 1. The time course of fermentation of lactate by strain VA-1. Strain VA-1 was grown on a complex medium. Symbols: ■, Lactate; ●, Propionate; ▲, Acetate; ○, Protein.

Fig. 2. A photomicrograph of strain MC-1. Bar represents 10 μm.

um. The three strains were nonmotile, nonspore forming, gram-positive and pleomorphic rods. The cells were 0.8~1 μm in diameter and 1~12 μm in

length. A photomicrograph of strain MC-1 is shown in Fig. 2. The characteristics of these strains coincided well with those of the genus *Propionibacterium* described in Bergey's manual of determinative bacte-

Table 1. Propionate production from saccharides.

strain	VA-1	MC-1	KA-2	<i>P. thoenii</i> ^a	<i>P. acidipropionici</i> ^a
Arabinose	—	+	—	—	+
Cellobiose	+	+	+	—	+
Trehalose	+	—	+	+	+
Mannitol	+	—	+	—	+
Dulcitol	—	—	—	—	—
Fructose	+	+	+	+	+
Galactose	+	+	+	+	+
Glycerol	+	+	+	+	+
Glucose	+	+	+	+	+
Maltose	+	+	+	d+	+
Mannose	+	+	+	+	+
Sucrose	+	+	+	d+	+
Sorbose	—	—	—	—	d+
Starch	—	—	—	+	+
Inulin	—	—	—	—	—

a, Bergey's Manual of Systematic Bacteriology, Vol. 2¹²⁾
d+: reaction positive in 40~90% of strains

Table 2. Propionate production from organic acids and amino acids.

Strain	VA-1	MC-1	KA-2	<i>P. acidipropionici</i> ^a
Organic acids				
Pyruvate	+	+	+	+
Fumarate	+	+	+	—
Malate	+	+	+	—
Lactate	+	+	+	+
Amino acids				
Arg	—	—	—	—
Asp	—	—	—	+
Asn	—	—	—	+
Glu	—	—	—	—
Gln	—	—	—	—
Ser	+	—	—	—
Thr	—	—	—	—

a, *P. acidipropionici* was used as a control strain.

riology¹²⁾. Then, it was concluded that these strains belong to the genus *Propionibacterium* based on PCR amplification of a specific region of the 16S rRNA gene sequence using the *Propionibacterium* specific primer (pr1) and the eubacteria specific primer (pr4) as well as the phenotypic characterization described above. From the 16S rRNA gene sequences of the known *Propionibacterium* species, the products amplified with these primers would be expected to have a size of about 900 bp. All amplified products were 900 bp. Furthermore, the identification of these strains for species was examined. Propionate production from saccharides by these strains is summarized in Table 1. They produced propionate from cellobiose, fructose, glucose, maltose, mannose, sucrose, and glycerol, but did not utilize starch and inulin as substrates for growth. Propionate production from organic acids and amino acids by these strains is summarized in Table 2. *P. acidipropionici* was used as a control

Table 3. Characteristics of propionate-producing bacteria.

strain	VA-1	MC-1	KA-2	<i>P. thoenii</i> ^a	<i>P. acidipropionici</i> ^a
Form	Pleomorphic rods	Pleomorphic rods	Pleomorphic rods	Pleomorphic rods	Pleomorphic rods
Size (μm)	0.8–1 × 1–8	0.8–1 × 1–10	0.8–1 × 1–12	0.5–0.8 × 1–5	0.5–0.8 × 1–5
Color	Cream	Cream	Cream	Red-brown	Cream to orange-yellow
Motility	—	—	—	—	—
Spore formation	—	—	—	—	—
Gram stain	+	+	+	+	+
Aerobic growth	++	+	++	—	++
Catalase activity	—	—	—	+	d+
Indole production	—	—	—	—	—
Esculin degradation	+	+	+	+	d+
Nitrate reduction	—	—	+	—	d+
Gelatin liquefaction	—	—	—	—	—
Milk curd	—	—	—	d—	—
Milk digestion	—	—	—	—	—
Denitrification	—	—	—	nd	nd
Growth of pH	4.4 <	4.9 <	4.3 <	4.7–4.9 <	4.1–4.9 <
GC content (mol%)	72	65.5	73.2	66–67	66–68

a, Bergey's Manual of Systematic Bacteriology, Vol. 2¹²⁾

d+, reaction positive in 40–90% of strains

d–, reaction positive in 10–40% of strains

nd, no data

strain for the experiment. Strains VA-1, MC-1 and KA-2 produced propionate from pyruvate, lactate, fumarate and malate, but not from arginine, aspartic acid, asparagine, glutamic acid, glutamine and threonine except strain VA-1 which produced propionate from serine. The characteristics of these strains are summarized in Table 3. Esculin hydrolysis, nitrate reduction and indole production of strains VA-1 and MC-1 coincided with the characteristics of *P. thoenii*. Strain VA-1, however, differed from *P. thoenii* in color of colony and catalase activity as well as in GC content which was high. In GC content, strain MC-1 was very similar to *P. thoenii*. However, strain MC-1 differed from *P. thoenii* in color of colony, aerobic growth and catalase activity. Strain KA-2 hydrolyzed esculin and reduced nitrate to nitrite, but did not produce indole. These characteristics coincided with those of *P. acidipropionici*. However, the GC content of strain KA-2 was high as compared with that of *P.*

acidipropionici.

The number of propionate-producing bacteria in the methane fermentation sludges was 10^{10} – 10^{11} cells/ml and predominated over other anaerobes¹⁴. Toerien¹⁶ reported that the majority of the anaerobic bacteria isolated from anaerobic digesters produced mainly acetate, propionate and lactate. According to a report by Hayashi and Furusaka^{3,4}, the most numerous anaerobic bacteria was *Propionibacterium* in paddy soils. The strains isolated from paddy soils produced propionate and acetate from low molecular weight compounds such as monosaccharides, amino acids and fatty acids. Therefore, it is considered that *Propionibacterium* play an important role in the consumption of degrading products of organic matters in methane fermentation sludges. Although the propionate-producing bacteria isolated from methane fermentation sludges are considered to be classified into the genus *Propionibacterium*, further experiments will be needed for the identification of the bacteria and the real substrates for fermentation in sludges.

Competitive lactate fermentation by dual culture with Propionibacterium sp. strain VA-1 and D. vulgaris strain F-1 with a sulfate medium

Competitive lactate fermentation by dual culture with strains VA-1 and F-1 was studied with a lactate medium supplemented with sulfate. The bacterial number (7×10^7 cells/10 ml) of strain VA-1 inoculated was about 10 times higher than that (6×10^6 cells/10 ml) of strain F-1. Either strain was able to grow under the conditions when grown singly. Strain VA-1 fermented lactate to propionate and acetate in single culture. The molar ratio of propionate to acetate was 2/1. In the presence of sulfate, strain F-1 converted lactate to only acetate. The time course of fermentation of lactate by dual culture is shown in Fig. 4. Lactate was completely degraded in the 5th day of incubation and a small amount of propionate and a large amount of acetate were produced in the dual culture as compared with single culture of strain VA-1. The molar ratio of propionate to acetate was 1/4. The propionate and acetate produced by strains VA-1 and F-1 were calculated according to the following

Fig. 3. Genus-specific amplification with primer set pr1–pr4. DNA from these bacteria were subjected to PCR and analyzed on a 0.8% TAE agarose gel. Lanes: 1: size marker- λ -Hind III digest; 2: *P. acidipropionici*; 3: strain VA-1; 4: strain MC-1; 5: strain KA-2. *P. acidipropionici* was used as a control strain.

Fig. 4. The time course of fermentation by dual culture with strains VA-1 and F-1 on lactate in the presence of sulfate. Symbols: ■, Lactate; ○, Propionate; ●, Acetate.

equations: For the stoichiometry of lactate degradation by strain VA-1, $3\text{CH}_3\text{CHOHCOO}^- \rightarrow 2\text{CH}_3\text{CH}_2\text{COO}^- + \text{CH}_3\text{COO}^- + \text{HCO}_3^- + \text{H}^+$. For the stoichiometry of lactate degradation by strain F-1, $2\text{CH}_3\text{CHOHCOO}^- + \text{SO}_4^{2-} \rightarrow 2\text{CH}_3\text{COO}^- + 2\text{HCO}_3^- + \text{HS}^- + \text{H}^+$. Strain VA-1 produced 2 mM propio-

nate and 1 mM acetate and strain F-1 produced 7 mM acetate from 10.5 mM lactate. The growth rates (μ) of strains VA-1 and F-1 was 0.016 and 0.027 (h^{-1}), respectively when these strains were grown singly with the same medium. Lactate is considered to be consumed at the ratio of 1 : 2 by strains VA-1 and F-1. Since the growth rate of *Propionibacterium* is slow, the propionate production may increase slowly.

Competitive lactate fermentation by triculture with Propionibacterium sp. strain VA-1 and D. vulgaris strain F-1 in syntrophic association with M. formicicum strain HM-1 with a sulfate-free medium

Competitive lactate fermentation by the triculture of strain VA-1 and strain F-1 in syntrophic association with strain HM-1 was studied with a sulfate-free lactate medium at pH 7.0. The bacterial number (7×10^7 cells/10 ml) of strain VA-1 inoculated was about 10 times higher than that (3.4×10^6 cells/10 ml) of strain F-1 and that (4.5×10^6 cells/10 ml) of strain HM-1. Although strain VA-1 was able to grow singly, strains F-1 and HM-1 were unable to grow singly in the medium. However, when strain F-1 grew in syntrophic association with strain HM-1, lactate was degraded to acetate and methane. Photomicrographs of the triculture are shown in Fig. 5. The time course of

Fig. 5. Photomicrographs of triculture. a) Phase-contrast micrograph; b) Epifluorescence micrograph. 1: strain VA-1; 2: strain F-1; 3: strain HM-1. Bars represent 10 μm .

Fig. 6. The time course of fermentation by triculture with strains VA-1, F-1 and HM-1 on lactate in the absence of sulfate. Symbols: ■, Lactate; ○, Propionate; ●, Acetate; △, Methane; ▲, Hydrogen.

fermentation is shown in Fig. 6. A small amount of hydrogen was produced transiently during the early stage of incubation. It was consumed completely after 5 days of incubation, and then methane was produced. Eight mM acetate, 2.1 mM propionate and 6.5 mM methane were produced from 14 mM lactate. The ratio of propionate to acetate in the triculture was almost the same as that in the dual culture. These results indicate that even if a large number of propionate-producing bacteria stayed in methane fermentation sludges, sulfate reducers could grow on lactate syntrophically with hydrogen-utilizing methanogens under sulfate-free conditions.

Competitive lactate fermentation by the triculture of strain VA-1 and strain F-1 in syntrophic association with strain HM-1 under acidic conditions was studied with a sulfate-free lactate medium adjusted to pH 5.0. The time course of fermentation by the triculture at pH 5.0 is shown in Fig. 7. Propionate and acetate were produced with lactate consumption throughout the incubation period, but methane was not produced. The molar ratio of propionate to acetate was 2/1. This value was the same as that for the single culture of

Fig. 7. The time course of fermentation by triculture with strains VA-1, F-1 and HM-1 on lactate in the absence of sulfate at pH 5.0. Symbols: ■, Lactate; ○, Propionate; ●, Acetate.

strain VA-1. The range of pH for the growth of strains VA-1, F-1 and HM-1 was 4.4~8.3, 5.5~8.5 and 6.0~8.0, respectively. These results show that only strain VA-1 in the triculture at pH 5.0 was able to ferment lactate to propionate and acetate. It is considered that propionate produced by *Propionibacterium* is accumulated in anaerobic digesters under acidic conditions as sulfate reducers and methanogens are not able to grow under the conditions.

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