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Enrichment of amino acid-oxidizing, acetate-reducing bacteria

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In anaerobic condition, amino acids are oxidatively deaminated, and decarboxylated, resulting in the production of volatile fatty acids. In this process, excess electrons are produced and their consumption is necessary for the accomplishment of amino acid degradation. In this study, we anaerobically constructed leucine-degrading enrichment cultures from three different environmental samples (compost, excess sludge, and rice field soil) in order to investigate the diversity of electron-consuming reaction coupled to amino acid oxidation. Constructed enrichment cultures oxidized leucine to isovalerate and their activities were strongly dependent on acetate. Analysis of volatile fatty acids (VFAs) profiles and community structure analysis during batch culture of each enrichment indicated that *Clostridium* cluster I coupled leucine oxidation to acetate reduction in the enrichment from the compost and the rice field soil. In these cases, acetate was reduced to butyrate. On the other hand, *Clostridium* cluster XIVb coupled leucine oxidation to acetate reduction in the enrichment from the excess sludge. In this case, acetate was reduced to propionate. To our surprise, the enrichment from rice field soil oxidized leucine even in the absence of acetate and produced butyrate. The enrichment would couple leucine oxidation to reductive butyrate synthesis from CO_2. The coupling reaction would be achieved based on trophic link between hydrogenotrophic acetogenic bacteria and acetate-reducing bacteria by sequential reduction of CO_2 and acetate. Our study suggests anaerobic degradation of amino acids is achieved yet-to-be described reactions.

[**Key words:** Amino acid degradation; Acetate reduction; Anaerobic bacteria; Enrichment culture; Bacterial community]

Oxidative deamination is the first step of amino acid degradation in anaerobic condition, which is usually followed by decarboxylation of produced 2-keto acids. This step is endergonic in certain amino acids (e.g., branched chain amino acids) and proceeds only if excess electrons liberated are rapidly removed (Table 1, Eq. 1). Removal of excess electrons can be achieved by interspecies hydrogen transfer to hydrogen consumer such as methanogens, acetogenic bacteria, or sulfate-reducing bacteria (1,2). Fermentative degradation is also possible by using amino acids as electron acceptor via so-called Stickland reaction in which the oxidation of an amino acid is coupled to reductive deamination of other amino acids (3–5). In addition, acetate can serve as electron acceptor for amino acid oxidation and is reduced to butyrate via reversed β oxidation (Table 1, Eq. 2) (2,6,7).

Our interest in this study is the diversity of the electron-consuming reaction coupled to amino acid oxidation. Theoretically, electron-consuming reaction involved in amino acid degradation is not limited to those described above, but it has been less surveyed. In current paper, we anaerobically constructed leucine-degrading enrichment culture from three different environmental samples (compost, rice field soil, and anaerobic digestion sludge) in order to find novel electron-consuming reaction involved in anaerobic amino acid degradation. For this purpose, the function of acetate as electron acceptor under strictly anaerobic condition was revisited because acetate reduction to higher volatile fatty acids was exergonic (Table 2, Eqs. 2 and 3). Volatile fatty acids (VFAs) metabolism of each enrichment culture was characterized, and enriched microbes were identified by culture-independent methods. With these approaches, we found that leucine oxidation is coupled to acetate reduction, and that acetate was reduced to propionate. It was also suggested that reductive butyrate synthesis from CO_2 is coupled to oxidative deamination based on metabolic link between acetogenic bacteria and acetate-reducing bacteria.

MATERIALS AND METHODS

Source of microbes Excess sludge of anaerobic digestion process used in this study was generated from laboratory-scale digester that treated raw garbage. The sludge has been stored for two years at room temperature. The rice field soil was obtained from Zama city, Kanagawa, Japan. The compost was constructed from cow feces in Isehara city, Kanagawa, Japan. The latter two samples were collected in November 2011 and stored at 4°C until use.

Clostridium neopropionicum DSM 3847 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany) and routinely maintained as described below. Before the substrate utilization test, *C. neopropionicum* was precultured on 20 mM ethanol for two days. The preculture

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TABLE 1. Gibbs free energy change of reactions discussed in this study.[a]

Reaction			Value of Gibbs free energy change	Equation number
$Leucine + 3H_2O$	→	$2H_2(4[H]) + Isovalerate^- + NH_4^+ + H^+ + HCO_3^-$	$\Delta G^{o\prime} = +4.2$ kJ	1
$2H_2(4[H]) + 2Acetate^- + H^+$	→	$Butyrate^- + 2H_2O$	$\Delta G^{o\prime} = -48.1$ kJ	2
$3H_2(6[H]) + Acetate^- + H^+ + HCO_3^-$	→	$Propionate^- + 3H_2O$	$\Delta G^{o\prime} = -76.1$ kJ	3
$4H_2(8[H]) + H^+ + 2HCO_3^-$	→	$Acetate^- + 4H_2O$	$\Delta G^{o\prime} = -104.6$ kJ	4
$10H_2(20[H]) + 4HCO_3^- + 3H^+$	→	$Butyrate^- + 10H_2O$	$\Delta G^{o\prime} = -257.3$ kJ	5
$5Leucine + 5H_2O$	→	$5Isovalerate^- + Butyrate^- + 5NH_4^+ + 2H^+ + HCO_3^-$	$\Delta G^{o\prime} = -236.3$ kJ	6

[a] The values of Gibbs free energy change were calculated from Thauer et al. (19).

was inoculated to the medium containing alcohols (ethanol and propanol) or BCAAs (valine and leucine) in the presence or absence of acetate at 2.5%.

Media and growth condition All incubation was performed under strictly anaerobic condition. The basal medium used in this study contained (per liter) 0.3 g of KH_2PO_4, 0.6 g of NaCl, 0.1 g of $MgCl_2 \cdot 6H_2O$, 0.08 g of $CaCl_2 \cdot 2H_2O$, 3.5 g of $KHCO_3$, 0.15 g of $Na_2S \cdot 9H_2O$, 0.15 g of Cys-HCl·H_2O, 1 mg of resazurin sodium salt, and 0.2 g of Bacto yeast extract (Becton, Dickinson and Company, Tokyo, Japan). In addition, a vitamin solution (10 ml/L, the composition is the same as one in DSM medium 141), a trace element solution SL-10 (1 ml/L, the composition is the same as one in DSM medium 461), and a Selenate–Tangstate solution (1 ml/L, the composition is the same as one in DSM medium 385) were added to the medium. Other nutrients were added from anaerobic stock solution prepared under N_2 atmosphere. Branched chain amino acids were added as sodium salts. Enrichment cultures were constructed under 35°C. The pH of the autoclaved medium was adjusted to 7.4 with HCl and Na_2CO_3.

The medium used for cultivation of *C. neopropionicum* contained (per liter) 1.0 g of NH_4Cl, 0.6 g of NaCl, 0.1 g of $MgCl_2 \cdot 6H_2O$, 0.08 g of $CaCl_2 \cdot 2H_2O$, 0.3 g of $Na_2S \cdot 9H_2O$, 0.3 g of Cys-HCl·H_2O, 0.5 g of Bacto yeast extract (Becton, Dickinson and Company), 0.5 g of Bacto trypticase (Becton, Dickinson and Company), and 2.0 g of $KHCO_3$. In addition, a vitamin solution (10 ml/L), a trace element solution SL-10 (1 ml/L), and a Selenate–Tangstate solution (1 ml/L) were added to the medium. Cultivation was performed at 30°C. The pH of the autoclaved medium was adjusted to 6.9 with HCl and Na_2CO_3.

In order to remove oxygen from the medium, the medium was boiled for 10 min and cooled to room temperature under N_2/CO_2 (80:20, vol/vol) atmosphere. Ten milliliters or 20 ml of the medium were dispensed to 70 ml volume of glass bottles with the same gas atmosphere, sealed with black butyl rubber septa, and autoclaved at 121°C for 15 min.

Construction of enrichment culture A branched chain amino acid, leucine, was chosen as a substrate for the construction of the enrichment from environmental samples and leucine oxidation was checked by quantification of produced isovalerate. Acetate was added to the enrichment cultures as an electron acceptor. As acetate, propionate, and butyrate are not produced through the anaerobic oxidation of leucine, the utilization of leucine as the substrate can also be detected by acetate reduction to propionate or butyrate by enriched microbes specifically.

For the construction of an enrichment culture from the excess sludge, 10 ml of the sludge was firstly mixed with 10 ml of the basal medium. On the other hand, for the construction from the rice field soil and the compost, 100 g of the samples (wet weight) was firstly mixed with 150 ml of autoclaved deionized water, and 10 ml of the mixture was further mixed with 10 ml of the basal medium. Then, leucine and Difco tryptone (Becton, Dickinson and Company) were supplemented at final concentration of 10 mM and 0.5 g/L, respectively. After incubation for 8 days at 35°C, the culture was serially diluted (10^{-1}–10^{-4}) into the fresh basal medium which contained 20 mM of leucine plus 24 mM of sodium acetate, and further incubated for several days.

Enriched microbial community in 10^{-4} dilution cultures was transferred again to 20 ml of a fresh basal medium at 0.5% volume, and incubated under two nutrient conditions; supplementation with (i) 20 mM leucine only and (ii) 20 mM leucine and 24 mM sodium acetate. Concentration of volatile fatty acids (VFAs) in culture supernatant was monitored during the incubation period by the methods described in Analytical method. Triplicate cultures were always sacrificed for chemical analysis at every sampling point.

Community structure analysis Microbial biomass was collected by centrifugation (5800 ×*g*, 10 min) from the cultures, suspended in Phosphate Buffered Saline (PBS), and stored at −20°C until use. DNA was extracted from the obtained biomass with ISOIL for beads beating (Nippon Gene, Tokyo, Japan) according to manufacturer's instruction.

Polymerase chain reaction (PCR) targeting bacterial 16S rRNA gene was performed by using AmpliTaq gold (Applied Biosystems, Tokyo, Japan). For terminal restriction fragment polymorphism (T-RFLP), a primer set of 6-carboxyfluorescein (FAM)-labeled Ba27f and Ba907r (8) was used. The PCR protocol entailed an initial denaturation for 10 min at 94°C; 25 cycles of denaturation for 30 s at 94°C, annealing for 45 s at 52°C, and elongation for 90 s at 72°C; and a final 5 min elongation at 72°C. Amplified products were purified using Wizard SV gel and PCR clean-up system (Promega, Tokyo, Japan) and digested by a restriction enzyme MspI (New England BioLabs, Tokyo, Japan). Terminal restriction fragments (T-RFs) were separated by electrophoresis with a 3130*xl* genetic analyzer and the T-RF length was determined by comparison with the size standard.

For clone analysis, the primer set, Ba27f (without 6-FAM) and Ba907r were utilized. Amplified products were separated by agarose gel electrophoresis and purified using Wizard SV gel and PCR clean-up system. Purified fragments were inserted into T-easy cloning vector (Promega). Ligation mixture was directly used for the transformation of *ECOS* Competent *E. coli* JM109 (Nippon Gene). Transformants harboring inserted fragment were distinguished by blue-white selection and white colonies were randomly picked up. Inserted fragments were sequenced using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and a 3130*xl* genetic analyzer.

Phylogenetic analysis Reference sequences phylogenetically related to the obtained sequence were searched from GenBank. These sequences were aligned by Clustal X (ver. 1.83) (9). Phylogenetic trees were constructed by the neighbor-joining method (10) with Kimura two parameter model (11) using MEGA 4 software (ver. 4.00) (12). Bootstrap value was obtained from 1000 replications.

Analytical method The concentrations of VFAs in a culture supernatant were quantified by HPLC (Alliance 2695, Waters, Tokyo, Japan) equipped with OApak P and OApak A (Tosoh, Tokyo, Japan), a photodiode array (2996, Waters) and a refractory index detector (2414, Waters). H_2SO_4 (0.75 mM) was used as mobile phase. Hydrogen (H_2) in a gas phase of a culture was detected by Gas Chromatograph (GC-9A, Shimazu, Tokyo, Japan) equipped with a stainless packed column, molecular sieves 5A (GL Science, Tokyo, Japan) by using argon (Ar) as a carrier gas.

Accession number The nucleotide sequence data of 16S rRNA gene obtained in this study have been deposited in the DDBJ nucleotide sequence database under accession numbers AB889809–AB889892.

TABLE 2. Phylogenetic classification of 16S rRNA gene clones detected in enrichment cultures in the presence of acetate.

Phylogenetic group	Number of clone			Expected T-RF (bp)[a]
	Compost	Excess sludge	Rice field soil	
Clostridium cluster I	14		4	74, 518, 520, **522**, 528
Clostridium cluster XIVb	7	13		39, 481, **491**
Clostridium cluster XI			19	**195**
Porphyromonadaceae	8			76
Peptococcaceae	3			216, 247
Aeromonadaceae			7	90
Bacilli		2		150
Others	6		1	
Total	38	15	31	

[a] Expected length (bp) of the T-RF generated by digestion with Msp I. T-RFs of interest were given as bold face.

RESULTS

VFAs production by enrichment cultures Enrichment cultures showing acetate-dependent leucine oxidation were successfully established from the environmental samples by the methods described in Materials and methods. Each enrichment culture showed a different VFAs production pattern in the cultivation experiment (Fig. 1).

In the presence of acetate, all enrichment cultures produced approximately 15–20 mM of isovalerate (Fig. 1). Along with isovalerate production, the enrichment from the compost consumed