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# $^{13}$C-NMR Studies on Lactate Metabolism in a Porcine Gut Microbial Ecosystem

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Lactate is seldom detected as a major acid in the hindgut because it is a typical intermediate metabolite and is converted to a short-chain fatty acid (SCFA) by lactate-utilizing bacteria. Lactate becomes a key metabolite in the large intestine when lactogenic and bifidogenic dietary supplements are fed to animals. If lactate-utilizers are eliminated, lactate will be accumulated. Accumulation of lactate in hindgut might be responsible for diarrhoea caused by short-bowel syndrome, dyspepsia, and antibiotic treatments. Thus, the lactate metabolism in the hindgut ecosystem is important. L-[3-$^{13}$C]-lactate fermentation was studied in a pig *in vitro* model using $^{13}$C-NMR. Pig cecal digesta were diluted with four weights of an anaerobic phosphate buffer containing $^{13}$C-labeled lactate. The mixture was incubated for 24 h at 37°C with gentle shaking. A portion of the culture was sampled periodically and analysed for SCFA. [3-$^{13}$C] propionate, [2-$^{13}$C] acetate, [2-$^{13}$C] propionate, [2-$^{13}$C] butyrate, and [4-$^{13}$C] butyrate were produced from [3-$^{13}$C]-Lactate in decreasing order. The importance of an acrylate pathway in propionate formation was evidenced by the predominant [3-$^{13}$C] propionate formation. An equal production of [2-$^{13}$C] butyrate and [4-$^{13}$C] butyrate, as well as a negligible production of [3-$^{13}$C] butyrate, indicated that extra non-labeled acetate is required for butyrate synthesis. $^{13}$C-labelled butyrate, together with the detection of [2-$^{13}$C] valerate, suggested the important role for *Megasphaera elsdenii* in lactate utilization in the system. *Key words*: $^{13}$C-NMR, lactate metabolism, large intestine, pig.

## INTRODUCTION

The short-chain fatty acid (SCFA) is a major bacterial metabolite in the large intestine. The role of the SCFA is primary in the interaction between the hosts and their hindgut bacteria because SCFA is absorbed and used by the host as an energy source for the epithelial cells and other peripheral tissues (1). Acetate is a ketogenic acid that is used for the synthesis of fat, while propionate is a glucogenic acid that is used for gluconeogenesis in the liver (2, 3). Butyrate is a specific growth stimulator for epithelial cells (4) that acts as a preventive factor against colonic cancer by the induction of apoptosis and differentiation in tumor cells (5, 6). SCFA is produced from carbohydrate fermentation, but some parts of it are produced from the fermentation of organic acids, such as lactate, succinate, and free amino acids. The lactic acid metabolism to SCFA seems to have a practical importance because lactate is a major end product of lactic acid bacteria, which are frequently used as probiotic bacteria (7) or are the targets of prebiotic substances (8, 9). The metabolism of lactate to SCFA may be helpful in curing ulcerative colitis (10), short bowel syndrome (11), or dyspepsia (12) that cause an accumulation of lactic acid in the large bowel. Lactate can be metabolized to acetate, propionate, butyrate, and valerate. If butyrate is preferentially produced from lactate, this metabolic pathway may further improve the function of the large intestine. The colonic (or cecal) lactate metabolism, however, has not been studied although the metabolic pathways of several anaerobic bacteria are known (13). It is difficult to determine how important each metabolic pathway is, especially when in a complex mixture of intestinal bacteria. Nuclear magnetic resonance (NMR) spectroscopy has been used in an earlier study for semi-quantitative observation of metabolic conversions in a pure culture of bacteria (14). In this report, we will describe the butyrate formation from lactic acid by pig cecal bacteria, a potential model for human hindgut flora, using $^{13}$C-NMR spectroscopy.

## MATERIALS AND METHODS

### *Source of the cecal bacteria*

Cecal bacteria were prepared as the buffer-diluted digesta. Cecal digesta were taken from two cecally fistulated sows that received a diet composed mainly of cracked maize, soya bean meal, meat-bone meal, alfalfa meal, and mineral and vitamin premix (15). The pigs were fed equal portions of the diet at 09:00 and 21:00 h. Water was accessible to the pigs at any time. The pigs were kept on the same feeding condition for at least 2 years. Bacterial composition was once determined for these two animals prior to

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the present experiment. After amplified ribosomal DNA restriction analyses (ARDRA) on bacterial 16S rRNA gene (the region between 534 and 908 *E. coli* nucleotide position), we have detected sequences those resembling to genera *Mitsuokella* (1.3% in total *E. coli* clones), *Megasphaera* (6.4%), *Lactobacillus* (3.8%), *Streptococcus* (3.8%), *Treponema* (1.3%), *Bacteroides* (14.1%), *Prevotella* (7.7%), *Fusobacteria* (5.1%), *Termitobacter* (5.1%), *Clostridium* (37.2%), *Eubacterium* (12.8%), *Butyrivibrio* (1.3%) (Kikuchi and Ushida, unpublished). After morning feeding, about 50 g of cecal digesta was sampled from each pig and mixed.

*Incubation*

Digesta were immediately diluted with four weights of an anaerobic phosphate buffer (pH 6.5, 0.1 M). The buffer was boiled and cooled under an $O_2$-free $N_2$ stream before use. The buffer contained 20% (v/v) deuterium oxide. Diluted digesta were squeezed through a 100-$\mu$m nylon mesh, and portions (50 ml) were dispensed into two 100-ml serum bottles. The manipulations were performed under a $N_2$ atmosphere. L-[3-$^{13}$C] sodium lactate (99% enriched) was obtained from ISOTEC (Miamisburg, OH, USA) and used as the substrate for *in vitro* fermentation. Labeled lactate was introduced into vacant incubation vessels to obtain 20 mM as the final concentration after the inoculation of diluted digesta. Bottles were filled with $O_2$-free $N_2$ and closed with butyl rubber septa (Bellco, Vineland, NJ, USA). Fermentation was performed at 37°C with gentle shaking (50 rpm) in a MW-150 stationary temperature shaker (Miyamoto-riken, Osaka). A portion (2 ml) of the culture was sampled using syringes from a bottle at 0, 6, 12, 18, and 24 h of incubation. A sampled culture was dispensed into two microfuge tubes placed in a crushed ice bath and centrifuged immediately at 20 000 $\times$ *g* at 4°C for 10 min. Supernatants were transferred to the new tubes and frozen at $-20$°C until NMR and HPLC analyses.

*NMR spectroscopy*

A part of supernatants were transferred to NMR tubes (5 mm o.d.) and introduced into Unity Inova 500 NMR spectrometer (Varian, Palo Alto, CA, USA).

The $^2$H resonance of deuterium oxide (20%) was used to lock the field and for shimming. $^{13}$C-NMR spectra were recorded at 125 MHz. Carbon spectra were recorded with 28 891 Hz of spectra width, 45° pulse, 0.3 s relaxation delay, 1.13 s acquisition time, and 3 000–40 000 scans depending on the concentration of the products. Data were analysed with a Sun Ultra 1 computer (Sun Microsystems, Inc., Mountain View, CA, USA) with Vnmr X version 5.3 software (Varian, Palo Alto, CA, USA) on a Solaris 2.5.1 operating system (Sun Microsystems Inc., Palo Alto, CA, USA). The signal of the labeled acid consisted of one main peak with three to seven minor satellite peaks. The former peak originated from the $^{13}$C-labeled compound without any replacement of deuterium. The latter peaks inevitably appeared due to the splittings by the $^{13}$C–$^2$H coupling of methyl or methylene group in the $^{13}$C-labeled compound. The amount of the labeled SCFA was estimated from the sum of the peak areas of both main and satellite peaks. The relative signal intensities for labeled SCFA were calculated as percentage of the signal integral for the specific SCFA in the sum of all the peak integrals.

*HPLC analyses*

Another part of supernatants was analysed for organic acids by the ion-exclusion HPLC according to Hoshi et al. (16). Supernatants were mixed with 10 mM sodium hydroxide containing 5 mM crotonic acid as the internal standard. Fat-soluble substances in the supernatant were removed by extraction with chloroform. The aqueous phase was filtered through a cellulose acetate filter with a 0.20-$\mu$m pore size (Advantec, Tokyo) before injection. Organic acids were separated with two serially connected H-type cation exchanger columns (Shim-pack SCR-102H, 8 $\times$ 300 mm; Shimadzu, Kyoto) and detected by a post-column pH-buffered electro-conductivity detection method using an electro-conductivity detector (CDD-6A; Shimadzu, Kyoto) (16).

## RESULTS

*HPLC results (Fig. 1)*

Acetate, propionate and butyrate were detected in all culture supernatants. The concentration of acetate was the highest among the acids, followed by propionate and butyrate. No significant evolution of the concentration of SCFA was detected after a 6 h incubation. The concentration of lactate at 0 h was smaller than expected. This was caused by the metabolism of lactate during preparation of HPLC samples. Tilling in crushed-ice bath followed by centrifugation was not apparently efficient to stop fermentation promptly. However, we did not use chemicals such as sulfuric acid to stop fermentation to avoid the possible undesirable side effects for the NMR spectroscopy.

*$^{13}$C-NMR spectra (Fig. 2)*

The $^{13}$C-NMR spectra of [3-$^{13}$C] lactate in the cecal digesta at 0 h and those of the metabolites of 24 h incubation are shown in Fig. 2a and b. At the beginning of incubation, the signals of [3-$^{13}$C] lactate (20.6 ppm) and [2-$^{13}$C] acetate (23.5 ppm) were detected. The latter signal was negligible. [3-$^{13}$C] propionate (10.4 ppm), [2-$^{13}$C] acetate (23.5 ppm), [2-$^{13}$C] propionate (30.9 ppm), [2-$^{13}$C] butyrate (40 ppm), [4-$^{13}$C] butyrate (13.5 ppm), [3-$^{13}$C] butyrate (19.5 ppm), [2-$^{13}$C] valerate (37.6 ppm), and [1-$^{13}$C] acetate (181.7 ppm) were detected in supernatants sampled at 24 h. There were small satellite peaks around the above-mentioned major peaks of a specifically labeled acid. Those were caused by

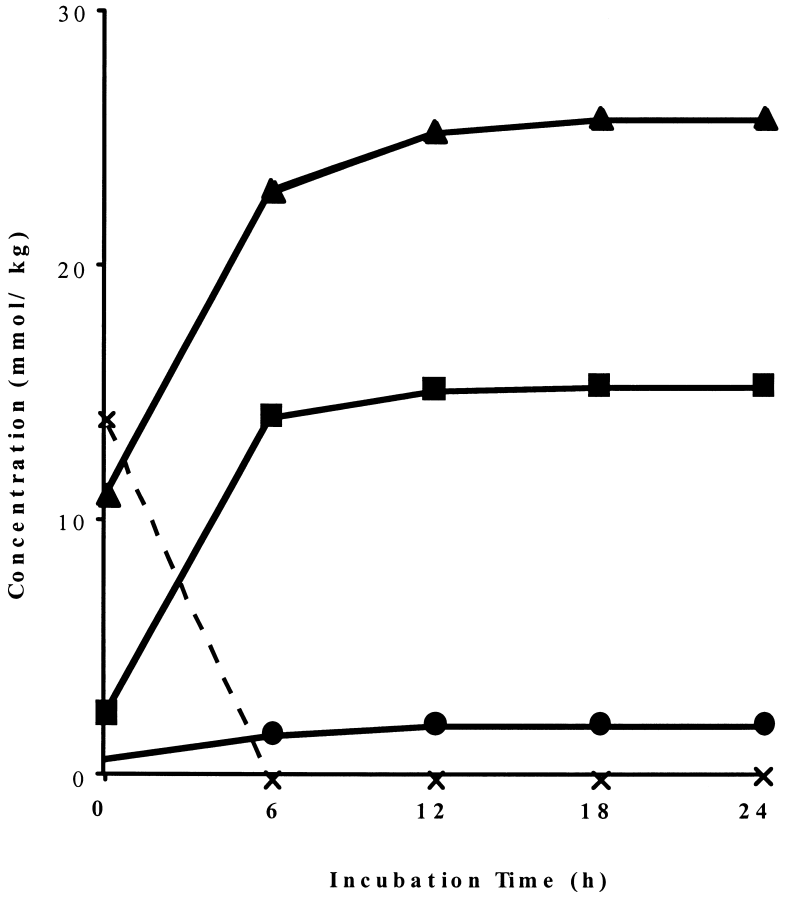


*Fig. 1.* Evolution of SCFA by the fermentation of lactate in pig cecal digesta *in vitro* values were the mean of duplicate incubation. ▲, Acetate; ■, propionate; ●, butyrate; ×, lactate.

the replacement of hydrogen atom(s) with deuterium atom(s) by keto–enol equilibrium.

*Kinetics of the lactate metabolism (Fig. 3)*

The kinetic of the [3-$^{13}$C] lactate-metabolism is shown in Fig. 3. The [3-$^{13}$C] lactate completely disappeared after 6 h of incubation. In place of the [3-$^{13}$C] lactate, [3-$^{13}$C] propionate, [2-$^{13}$C] acetate, [2-$^{13}$C] propionate, [2-$^{13}$C] butyrate, and [4-$^{13}$C] butyrate were produced in decreasing order at 6 h (Fig. 3). The evolution of [3-$^{13}$C] propionate and [2-$^{13}$C] acetate became insignificant after 6 h of incubation. Evolution of [2-$^{13}$C] propionate, [2-$^{13}$C] butyrate, and [4-$^{13}$C] butyrate became insignificant after 12 h incubation. A trace amount of [2-$^{13}$C] valerate was detected at 24 h. The amounts of [3-$^{13}$C] butyrate and [1-$^{13}$C] acetate were always negligible. Relative signal intensities (approximate values) of the metabolites of [3-$^{13}$C] lactate at 24 h incubation were as follows; [3-$^{13}$C] propionate (40%), [2-$^{13}$C] acetate (20%), [2-$^{13}$C] propionate (5%), [2-$^{13}$C] butyrate (3%), [4-$^{13}$C] butyrate (3%) and [2-$^{13}$C] valerate (trace).

## DISCUSSION

There have been studies using $^{13}$C-NMR spectroscopy to detect the specific metabolic pathway in a pure culture of bacteria (14). Applications of the technique are also available toward the complex microbial community, such as that found in the rumen (17), pig large intestine (18), or human feces (19). These three reports demonstrated the potential of $^{13}$C-NMR spectroscopy to estimate the contribution of several possible metabolic pathways leading to the same end product even in a complex mixture of bacteria. The present study also demonstrated the potential of the technique to trace the specific metabolic pathway in a complex ecosystem of hindgut bacteria.

Lactate is a typical intermediate metabolite in the fermentation process in the digestive tract. Therefore, lactate is detected in trace amounts under normal conditions. As shown in Fig. 3 lactate was promptly fermented to acetate, propionate and butyrate in the present study. However, lactate often becomes a major organic acid in the large intestine under certain conditions. A high concentration of lactate is unfavorable because, in the hindgut, it is often associated with disorders of the digestive system, such as ulcerative colitis (10), short bowel syndrome (11), and diarrhea caused by dyspepsia (12). The role of lactic acid in those diseases is still inconclusive. However, there should be an interaction between the mucosa and lactate (20). Maintenance of lactate fermentation may provide a key for the cure to these diseases. When animals receive

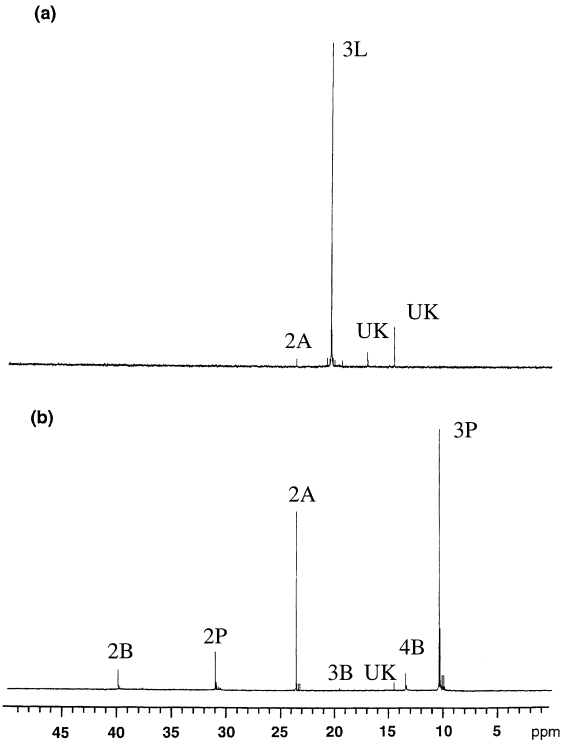


*Fig. 2.* $^{13}$C-NMR spectra of culture supernatant. (a) $^{13}$C-NMR spectrum before incubation. (b) $^{13}$C-NMR spectrum after 24 h of incubation. 3L, 3-$^{13}$C-butyrate; 2A, 2-$^{13}$C-acetate; 2P, 2-$^{13}$C-propionate; 3P, 3-$^{13}$C-propionate; 2B, 2-$^{13}$C-butyrate; 3B, 3-$^{13}$C-butyrate; 4B, 4-$^{13}$C-butyrate; UK, unknown.

bifidogenic prebiotics, such as fructooligosaccharide (8), the resultant increase in the number of bifidobacteria and lactic acid bacteria must enhance lactate production in the hindgut. Therefore, the fermentation (= consumption) of lactate becomes important when bifidogenic or lactogenic materials are employed; otherwise, lactate is accumulated in the hindgut (9, 21).

The present experiment demonstrated the presence of several metabolic pathways of lactate fermentation in the pig caecum. Acetate, propionate, and butyrate were produced as major end products in decreasing order as evidenced by the HPLC. From the NMR spectroscopy (Fig. 3), propionate was the most preferable end product followed by acetate in the present fermentation system. Butyrate formation was less significant than that of propionate and acetate, as evidenced by the lowest relative signal intensity. The contradiction between HPLC and NMR was due to the production of acetate from the endogenous substrate in the digesta.

According to Thauer et al. (22), the thermodynamics of lactate metabolism are as follows:

$$\text{Lactate}^- + 2H_2O \rightarrow \text{Acetate}^- + HCO_3^- + H^+ + 2H_2$$

$$(\Delta G^{\circ\prime} = -4.2 \text{ kJ mol}^{-1})$$

$$\text{Lactate}^- + H_2 \rightarrow \text{Propionate}^- + H_2O$$

$$(\Delta G^{\circ\prime} = -79.9 \text{ kJ mol}^{-1})$$

$$\text{Lactate}^- + \text{Acetate}^- \rightarrow \text{Butyrate}^- + HCO_3^-$$

$$(\Delta G^{\circ\prime} = -52.3 \text{ kJ mol}^{-1})$$

Acetate formation from lactate is the least preferable reaction for the bacteria in terms of Gibb's free energy change. Nevertheless, the relative signal intensity of [2-$^{13}$C] acetate was larger than that of all topoisomers of butyrate (Fig. 3). Acetate formation involves the oxidative decarboxylation of lactate associated with the production of hydrogen. Accordingly, the reaction must be coupled with hydrogen consumption, such as sulfate reduction, methanogenesis, or nitrate reduction, to proceed. The production of acetate may proceed at much faster rate if this coupling occurs because of the large Gibb's free energy changes of these reactions for hydrogen consumption, i.e. $-151.9$, $-135.6$, and $-163.2$ kJ mol$^{-1}$. The most likely case in the pig cecum under the present feeding conditions is the coupling with sulfate reduction (23). In such a case, the reaction would be as follows, and the acetate production would proceed as easily as propionate formation in terms of thermodynamics:

$$\text{Lactate}^- + 1/2\ SO_4^{2-} \rightarrow \text{Acetate}^- + 1/2\ HS^- + HCO_3^- + 1/2\ H^+ \quad (\Delta G^{\circ\prime} = -80.2 \text{ kJ mol}^{-1})$$

This reaction has been demonstrated in a range of Desulfovibrios (24). In our previous study, the level of lactate-utilizing sulfate reducers was at $10^7$ g$^{-1}$ digesta of pig cecum (23). They constituted the major hydrogen disposal system in the porcine hindgut. Acetate formation from lactate, therefore, depends on the number and the activity of the hydrogen disposal systems.

The formation of [3-$^{13}$C] propionate from [3-$^{13}$C] lactate was the most preferred metabolic pathway for lactate fermentation in the presently used pig cecum. In an earlier study done by the group of Prins (25), D-lactate was preferentially converted to propionate, whereas L-lactate was converted to butyrate by mixed rumen bacteria taken from a dairy cow shortly after feeding (within 30 min). However, using the rumen bacteria of the same donor cow sampled at another time relative to feeding, both D- and L-lactate were converted mainly to acetate. These authors stressed the specific role of a lactate-utilizing bacterium, *Megasphaera elsdenii*, in enhanced propionate and butyrate production. In the present study, the level of this bacterium in the sampled digesta was not determined. However, this bacterium was detected by the ARDRA in the same animals as indicated above. *M. elsdenii* is a common member of intestinal microbiota among many animals involving human (26) and detected in high numbers in the pig cecum (27). The contribution of this bacterium is most probable. The relative signal intensity of

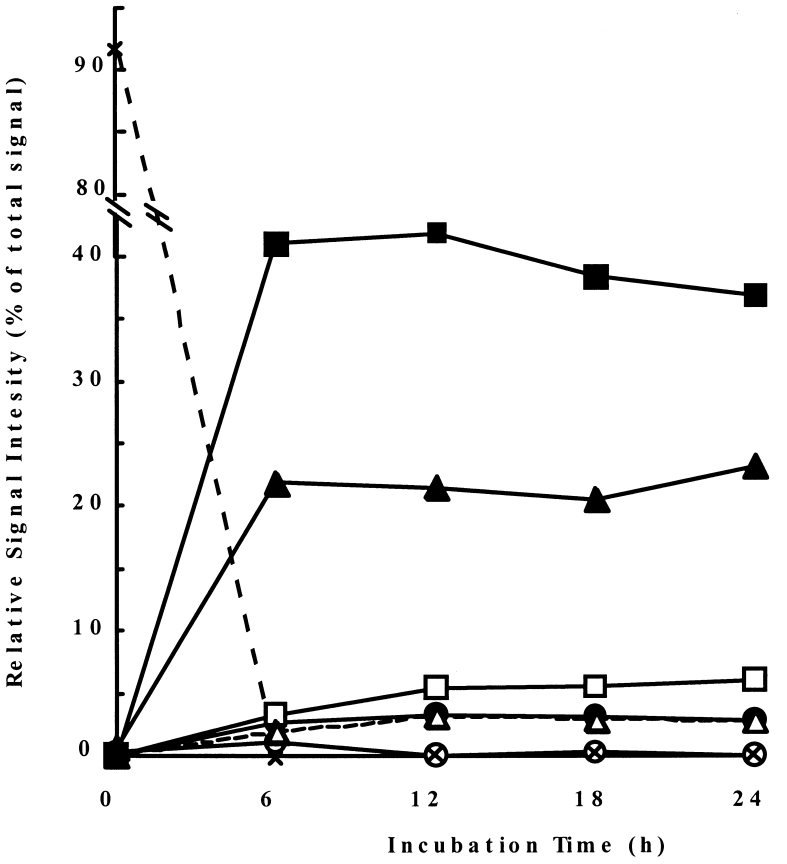


*Fig. 3.* Bacterial conversion of [3-$^{13}$C] lactate into SCFA in the pig cecal digesta *in vitro* culture. ×, 3-$^{13}$C-lactate; ■, 3-$^{13}$C-propionate; ▲, 2-$^{13}$C-acetate; □, 2-$^{13}$C-propionate; ●, 2-$^{13}$C-butyrate; △, 4-$^{13}$C-butyrate and ○, 3-$^{13}$C-butyrate.

[2-$^{13}$C] propionate was about one-eighth of that of [3-$^{13}$C] propionate. The [2-$^{13}$C] propionate should have been formed through a so-called random pathway in which lactate is converted to oxaloacetate that is further metabolized to succinate via malate and fumarate. Succinate was finally decarboxylated to propionate (17). On the other hand, [3-$^{13}$C] propionate should have been formed through the so-called acrylate pathway, in which the position of the labeled carbon is maintained (17). Again, the role of *M. elsdenii* should be stressed because this bacterium is the sole intestinal bacteria possessing the acrylate pathway to ferment lactate to propionate (17). Other lactate-utilizers such as *Veillonella alcalescens*, *Selenomonas ruminantium*, and *Anaerovibrio lypolytica* possess the random pathway (13).

The same relative signal intensities (3%) for [2-$^{13}$C] butyrate and [4-$^{13}$C] butyrate as well as the absence of [3-$^{13}$C] butyrate suggested that lactate was first oxidatively decarboxylated to [2-$^{13}$C] acetyl-CoA via pyruvate. The NMR-spectra suggested that [2-$^{13}$C] butyrate and [4-$^{13}$C] butyrate were both single-labeled. Therefore, unlabeled acetate might be incorporated into $^{13}$C-labeled acetyl-CoA to produce [2-$^{13}$C] or [4-$^{13}$C] acetoacetyl-CoA. The first step of the formation of butyrate from lactate is the dehydrogenation of lactate to pyruvate. This reaction is not reversible because the reaction has a large positive free energy change ($\Delta G^{\circ\prime} = 43.1$ kJ mol$^{-1}$) (22). This must be due to the metabolic hydrogen formation. If metabolic hydrogen is consumed in the formation of butyrate from acetate, the reaction can proceed. The bacteria, therefore, require extracellular acetate to produce butyrate from lactate. This pathway is limited to *M. elsdenii* as well as some strains of *Eubacterium* spp. and *Clostridium* spp. (13, 26). This is because of the rare presence of the constitutive enzyme, NAD-independent lactate dehydrogenase, which catalyzes the oxidation of lactate to pyruvate (28). In the mixture of the cecal bacteria, the limited range of the bacteria is responsible for the butyrate formation from lactate. The present experiment did not provide the information about the conversion of lactate during the first 6 h of incubation, which seems to be important. This may be a limitation of the experiment and should be clarified in the future study. Nevertheless, the results suggested the importance of *M. elsdenii* in lactate conversion in the pig cecum, which is in line with our recent studies (29) in which the potential of this bacterium to remove hyper lactate was demonstrated.

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