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# Valerate production by *Megasphaera elsdenii* isolated from pig feces

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***Megasphaera elsdenii* is able to produce several short-chain fatty acids (SCFAs), such as acetate, propionate, butyrate, and valerate. These SCFAs serve as an energy source for host animals and play an important role in gut health. In this study, *M. elsdenii* was isolated from pig feces that had been collected from two farms located in distinct areas of Japan. These *M. elsdenii* isolates were genotyped, and 7 representative strains were selected. When these 7 strains and *M. elsdenii* JCM 1772$^T$ were cultured with lactate for 24 h, all 7 strains produced valerate as a predominant SCFA. Therefore, the valerate-producing *M. elsdenii* inhabits a wide area of Japan. In contrast, *M. elsdenii* JCM 1772$^T$ produced acetate, propionate, butyrate, and valerate at similar levels. When the Y2 strain, one of the 7 representative strains, was cultured without lactate, low levels of valerate accumulated. In contrast, in a time course of lactate fermentation by the Y2 strain, lactate was rapidly consumed, and acetate and propionate were produced after 6 h of incubation. Thereafter, acetate and propionate were consumed from 6 to 12 h after the start of the incubation, and valerate and butyrate were produced. In most of the previously described *M. elsdenii* strains, valerate was not a predominant SCFA. Therefore, the *M. elsdenii* Y2 strain showed an unique metabolism in which valerate was produced as a primary end product of lactate fermentation.**



[**Key words:** Butyrate; Lactate fermentation; *Megasphaera elsdenii*; Pig; Valerate]

Short-chain fatty acids (SCFAs) are the fermentation products of intestinal microbes (1). Host animals assimilate SCFAs, which provide up to 10% of the daily caloric requirements in pigs and humans (2). In the cecal and mid-colonic digesta of pigs, acetate (C2), propionate (C3), and butyrate (C4) are the most abundant SCFAs, while valerate (C5) and caproate (C6) are limited (3). In the small intestine, lactate is produced by lactic-acid producing bacteria, such as bifidobacteria and lactobacilli; a high level of lactate is present in the pig ileal digesta. Subsequently, the lactate level is markedly decreased, and the SCFA contents simultaneously increase in the colon. This reduction in the level of lactate suggests that part of the lactate is further fermented into SCFAs by lactate-utilizing bacteria (3). *Megasphaera elsdenii* is a major inhabitant of the pig intestine (4,5), and this bacterium converts lactate to various kinds of SCFAs. For example, *M. elsdenii* ATCC 17753 cultured in a broth supplemented with lactate produced acetate and propionate (6,7). An *M. elsdenii* strain isolated from human feces produces butyrate as a main fermented product of lactate (8). Many strains of *M. elsdenii*, such as J6 (9), NIAH 1102 (10), and DSM 20460 (11), utilize lactate to produce acetate, propionate, and butyrate. Two *M. elsdenii* strains, NCIMB 702261 and NCIMB 702410, produce acetate, butyrate, and caproate in a culture medium containing sucrose (12). Three ruminal strains of *M. elsdenii* also produce acetate, propionate, and butyrate from lactate (13). These results suggest an important role for *M. elsdenii* in the lactate metabolism and the production of SCFAs. Some SCFAs perform a physiological function in host animals. For example, butyrate offers multiple benefits, such as reinforcement of the epithelial defense barrier, inhibition of colonic carcinogenesis, and improvement of inflammation (14). Due to its capability to produce butyrate from lactate, *M. elsdenii* is a probiotic candidate for animals. In fact, the combined administration of lactic acid bacteria and *M. elsdenii* to pigs improved the development of the colonic mucosa (15).

Recently, we developed a selective culture medium for *M. elsdenii* called KMI agar medium (16). Three strains of *M. elsdenii* were isolated from pigs raised on a Japanese pig farm. Additionally, we isolated four distinct strains of *M. elsdenii* from another Japanese pig farm. These 7 total strains produced valerate in large amounts after 24 h of incubation in the lactate-containing medium. Thus, it is likely that the valerate-producing *M. elsdenii* widely inhabits the pig intestine. As far as we know, this is the first report of a microorganism that produces valerate as a predominant end product of lactate fermentation.

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## MATERIALS AND METHODS

**Sampling of pig feces** Sows (crossbred Landrace × Large White) and their piglets (crossbred sows × Duroc) were conventionally maintained at two Japanese farms (farms A and B) that are located in distinct areas of Japan. Farm A, Pig Fertilize Matsugaya Co. Ltd., is located in Chiba prefecture at an elevation of 56 m and is on the land with a mixture of andosol and forest soils. Farm B, Ishizeki Swine Farm Ltd., is located in Gunma prefecture at an elevation of 232 m and is on the land with andosol soils. The soil type at the location of each farm was estimated from a web tool provided by the National Agriculture and Food Research Organization (http://soil-inventory.dc.affrc.go.jp). The farms are 168 km apart in a straight line distance. Pigs were treated in accordance with the institutional animal guidelines for the farms. Strains of *M. elsdenii* (M1, M3, and M4) were isolated from the fecal matter collected from 6-month-old pigs raised in farm A as previously described (16). In this study, we also isolated *M. elsdenii* strains from the fecal matter collected from the 6-month-old pigs raised in farm B. Fresh fecal matter was diluted with a 2-fold volume of 30% (v/v) glycerol, which was stored at −80 °C until use.

**Isolation of *M. elsdenii*** Fecal samples from farm B were serially diluted with a 0.9% (w/v) NaCl solution, and the diluted samples were spread onto the KMI agar medium (16). The agar plates were put into an AnaeroPack jar (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan), and incubated at 37 °C for 3 d.

**Colony PCR with *M. elsdenii*-specific primers and *Megasphaera indica*-specific primers** The large, yellow-white colonies that formed on the KMI agar medium were picked and subjected to colony PCR with *M. elsdenii*-specific primers (MeFw and MeRv), as previously described (16). The MeFw and MeRv primer sequences showed low similarities to the 16S rRNA gene sequences of most *Megasphaera* species except for *M. indica*, the closest relative to *M. elsdenii* (17). To distinguish *M. elsdenii* from *M. indica*, we generated reverse primers specific to the 16S rRNA gene sequences of *M. elsdenii* and *M. indica*; MeRv2, 5′-CGTAACGGGTATTGACCGCTACG-3′ (nucleotide positions 472–494 of GenBank accession no. U95027) and MiRv, 5′-TACGACGGGTATTGGCCGTCGTA-3′ (nucleotide positions 470–492 of GenBank accession no. HM990965), respectively. An aliquot of each bacterial colony was added as a template, and PCR was carried out with an initial step at 95 °C for 15 min, followed by 30 cycles of two-step amplification (denaturation at 94 °C for 15 s and annealing/extension at 68 °C for 30 s). A 465-bp DNA fragment is amplified if these primers are annealed with the target genomic DNAs.

**Genotyping of *M. elsdenii* isolates and determination of 16S rRNA gene sequences** The isolates of *M. elsdenii* were genotyped by a PCR assay based on the enterobacterial repetitive intergenic consensus (ERIC-PCR). The representative strains were determined from the electrophoretic pattern of the ERIC-PCR products. The 16S rRNA genes of the representative isolates from farm B were amplified by colony PCR with a universal primer set (27F and 1492R), as previously described (16). The approximately 1.5-kb amplified fragments were purified using a PCR Clean-Up Mini Kit (Favorgen Biotech Corp., Taiwan), and their nucleotide sequences have been deposited in the GenBank database (LC318280-LC318283). The phylogenetic tree was constructed as previously described (18).

**Light microscopy observation** The 7 representative isolates and *M. elsdenii* JCM 1772$^T$ were spread on the EG medium (Nissui Pharmaceutical, Co. Ltd, Tokyo Japan), a recommended medium for *M. elsdenii* (19), and a General Anaerobic Medium (GAM; Nissui Pharmaceutical). Both agar media were supplemented with 0.325% (v/v) DL-lactic acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and designated EG+Lac or GAM+Lac media, respectively. The bacteria that formed on these agar media were stained with 0.125% (w/v) safranin. Cell morphology was observed using light microscopy (Olympus CX41).

**Lactate utilization** The 7 representative isolates and *M. elsdenii* JCM 1772$^T$ were anaerobically pre-cultured at 37 °C in a half-strength GAM broth supplemented with 0.325% (v/v) DL-lactic acid (1/2GAM+Lac) overnight. The GAM broth contained the following components per liter: 10 g of peptic digest of animal tissue, 3 g of papaic digest of soybean meal, 10 g of proteose peptone, 13.5 g of digested serum, 5 g of yeast extract, 2.2 g of beef extract, 1.2 g of liver extract, 3 g of glucose, 2.5 g of $KH_2PO_4$, 3 g of NaCl, 5 g of soluble starch, 0.3 g of L-cysteine HCl, and 0.3 g sodium thioglycollate, with the final pH adjusted to 7.1. Lactate utilization was tested in a 50 mL Erlenmeyer flask containing 20 mL of the 1/2GAM+Lac broth. The volume of the inoculum was 200 μL of the pre-cultured suspension. The flasks were placed in an AnaeroPack jar (Mitsubishi Gas Chemical Company), and the jar was shaken at 80 rpm for 24 h. The production of SCFAs by the *M. elsdenii* strains Y2 and JCM 1772$^T$ was assessed at 6, 9, 12, and 24 h after inoculation. In addition, these two *M. elsdenii* strains (Y2 and JCM 1772$^T$) were cultured in the lactate-free 1/2GAM broth for 24 h. The culture of each replicate was centrifuged at 8000 ×*g* for 10 min, and the SCFA composition in the supernatants was determined using an HPLC Prominence system (Shimadzu Corporation, Kyoto, Japan) equipped with a column (Shim-pack SCR-102H). The mobile phase was 5 mM *p*-toluenesulfonic acid in $H_2O$ and the flow rate was 0.8 mL/min. The eluents were monitored by an electric conductivity detector. The presence of valerate was confirmed by co-chromatography of a standard reagent and fermented products.

## RESULTS

**Isolation of *M. elsdenii* strains from pig feces** In our previous study, the fecal samples from farm A were spread on KMI agar medium, and we picked 7 large, yellow-white colonies. These 7 isolates were genotyped by ERIC-PCR analysis and classified into 3 groups in which the M1, M3 and M4 strains represented (16). In this study, we isolated additional *M. elsdenii* strains from the fecal samples collected from another pig farm (farm B). We picked 20 large, yellow-white colonies that formed on the KMI medium, and these 20 isolates were named Y1 to Y20. All 20 isolates tested positive by PCR with an *M. elsdenii*-specific primer set, MeFw and MeRv (Fig. S1). *M. elsdenii* is the closest relative to *M. indica* (17); thus, it is expected that a partial 16S rRNA gene fragment can be amplified by PCR with the MeFw and MeRv primers in both *M. elsdenii* and *M. indica* (16). To distinguish *M. elsdenii* from *M. indica*, we designed reverse primers that are specific for *M. elsdenii* (MeRv2) and *M. indica* (MiRv). A 465-bp DNA fragment was amplified from all 20 isolates by PCR with the *M. elsdenii*-specific primer pair, MeFw and MeRv2; corresponding fragments were not obtained by PCR with the *M. indica*-specific primer pair, MeFw and MiRv (Fig. S2). These results suggested that all 20 isolates were *M. elsdenii*. Then, these 20 isolates were genotyped by the ERIC-PCR assay and divided into 4 groups (Fig. S3). One strain (Y1) showed a unique ERIC-PCR pattern and was classified as the group 1. Ten of the 20 isolates produced similar, distinctive patterns, and Y2 was chosen as a representative strain for this group (group 2). Seven isolates were classified into group 3, from which Y6 was selected as the representative strain. Finally, group 4 consisted of two isolates, and Y9 was selected as the representative strain. The ERIC-PCR amplified patterns of these 4 representative strains isolated from farm B (Y1, Y2, Y6, and Y9) and 3 representative strains from farm A (M1, M3, and M4) were compared with that of *M. elsdenii* JCM 1772$^T$ (Fig. 1). Interestingly, all 7 representative strains showed distinct ERIC-PCR patterns, indicating that many genotypes of *M. elsdenii* can be found in the pig intestine.

The 16S rRNA gene sequences of the 7 strains shared 98.7–99.2% sequence identity with *M. elsdenii* ATCC 25940$^T$. The phylogenetic analysis showed that all 7 strains were clustered in a clade with *M. elsdenii* ATCC 25940$^T$ (Fig. 2).

When these 7 strains were cultured on the EG+Lac agar medium, they were cocci-shaped and primarily arranged in pairs. Furthermore, when these bacteria were cultured on the GAM+Lac agar medium, the cells of the M3, Y1, Y2, and Y9 strains occurred in chains (Fig. S4). At present, we have not determined the medium component that induced the arrangement of *M. elsdenii* cells in chains.

**Lactate utilization of *M. elsdenii* isolates** Seven strains of *M. elsdenii* and *M. elsdenii* JCM 1772$^T$ were cultured in the 1/2GAM+Lac broth for 24 h, and the amount of SCFAs in the supernatant was determined (Fig. 3). The 1/2GAM+Lac broth contains 0.15% (w/v) glucose as a carbohydrate source. Growth in this broth resulted in nearly complete consumption of input lactate after 24 h of incubation. *M. elsdenii* JCM 1772$^T$ produced acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate as its major SCFAs. This SCFA profile was similar to that of the *M. elsdenii* J6 strain (9). In contrast, all 7 strains (M1, M3, M4, Y1, Y2, Y6, and Y9) mainly produced valerate, followed by isobutyrate, butyrate, and isovalerate (Fig. 3). Although the genotypes of the 7 *M. elsdenii* strains differed from one another (Fig. 2), their SCFA profiles were commonly dominated by valerate. The 3 g/L of lactate and 1.5 g/L of glucose were converted to approximately 1.3–1.7 g/L of valerate by these 7

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