

## Fecal Microflora in a Patient with Short-Bowel Syndrome and Identification of Dominant Lactobacilli

TSUTOMU KANEKO,<sup>1\*</sup> YUKI BANDO,<sup>2,3</sup> HIROKO KURIHARA,<sup>1</sup> KEIKO SATOMI,<sup>1</sup>  
KATSUHITO NONOYAMA,<sup>3</sup> AND NOBUO MATSUURA<sup>3</sup>

Central Research Institute, Meiji Milk Products Co., Higashimurayama, Tokyo 189,<sup>1</sup> and Pediatrics,  
Sagamihara Kyodo Hospital, 2-8-18 Sagamihara,<sup>2</sup> and Pediatrics, Kitasato University  
School of Medicine, 1-15-1 Kitasato, Sagamihara,<sup>3</sup> Kanagawa 228, Japan

Received 8 April 1997/Returned for modification 10 June 1997/Accepted 19 September 1997

**Fecal microflora and lactate concentrations in blood and feces obtained from a patient (a 5 year-old boy) with short-bowel syndrome (SBS) were compared during acidosis to results for the normal condition (no SBS symptoms). The taxonomical position of the lactobacilli found predominantly in the feces sample obtained 2 days before the fifth attack was also studied. The D-lactate level in serum obtained 1 day after the fourth attack was 10-fold higher than that for the normal condition, although there was not a great difference in L-lactate levels. D-Lactate (3.91 mM) and L-lactate (2.86 mM) were also detected in the feces samples collected 2 days before the fifth attack, while no lactate was detected in the feces sample for the normal condition. The counts of total fecal bacteria, especially anaerobic bacteria such as members of the family *Bacteroidaceae*, were found to be low. The counts of lactobacilli and the total population of lactobacilli relative to total fecal bacteria in the feces 2 days before the fifth attack (40.4%) were extremely high. In this case, a majority of the lactobacilli were D-lactate producers as determined by homolactic fermentation. These lactobacilli were identified as *Lactobacillus delbrueckii* subsp. *lactis*. The percentages of bifidobacteria relative to total fecal bacteria in feces samples obtained both 2 days before the fifth attack (50.9%) and for normal condition (61.9%) were also high, although these bacteria were L-lactate producers. In the feces samples for the normal condition, the D-lactate producers decreased to less than 10<sup>9</sup> per g, while the counts of L- or DL-lactate producers were 100-fold higher than the numbers in feces samples obtained 2 days before the fifth attack. These results suggested that an increase in the level of D-lactate producers, such as *L. delbrueckii* subsp. *lactis*, in the colon may be associated with the clinical expression of metabolic acidosis.**

Increased fecal levels of lactate and D-lactic acidosis are seen in patients with short-bowel syndrome (SBS) after jejunoileal bypass surgery, and in some cases the D-lactate levels in blood samples of the patients are also increased (2, 11, 24, 27). Acidosis is believed to result from the absorption of D-lactate produced by the colonic fermentation of carbohydrate that was not absorbed in the remaining small intestine (3). Coronado et al. (5) found that oral antibiotics may induce D-lactic acidosis in patients with SBS by promoting the overgrowth of resistant D-lactate-producing organisms. Colonic D-lactate is mainly formed from pyruvate by bacterial D-lactate dehydrogenase. D-Lactate dehydrogenase is known as a member of the D-isomer-specific 2-hydroxy acid dehydrogenases, distinct from L-lactate dehydrogenase (29). Mammalian tissues contain D-2-hydroxy acid dehydrogenase, which catalyzes the oxidation of D-lactate to pyruvate (4). Therefore, it is suggested that D-lactic acidosis occurs only when the absorption of D-lactate exceeds metabolism or when the D-lactate metabolism is impaired (3). Colonic bacteria producing D- or DL-lactate contribute to metabolic acidosis. Satoh et al. (27) reported that bacteriological analyses of fecal microflora of patients with SBS showed an increase in the number of *Lactobacillus buchneri* or *Lactobacillus fermentum* organisms. These bacteria produce DL-lactate from glucose.

In this study, we analyzed the fecal microflora and lactate concentrations in blood and feces samples from a patient with

SBS 2 days before the fifth attack and compared the results with those for the normal condition (no SBS symptoms). Furthermore, we identified the lactobacilli (producers of D-lactate by homolactic fermentation), which were found predominantly in the feces of this patient.

### MATERIALS AND METHODS

**Clinical case.** A 5-year-old boy with ileus was admitted to Sagamihara Kyodo Hospital on 12 September 1995. After 2 days of hospitalization, he was operated on, and intestinal volvulus was diagnosed. Only 52 cm of small intestine with an intact ileocecal valve remained after resection of a large segment of the small intestine. Subsequent malabsorption was treated with intravenous hyperalimentation and elemental diet for 2 months, although he had watery diarrhea every day. However, metabolic acidosis (first attack) with confusion, speech disturbance, and unusual behavior was suddenly seen in this patient on 10 January 1996. In this case, the levels of L-lactate and pyruvate were normal. After treatment with an infusion of sodium bicarbonate, he recovered within 20 min. Similar attacks were observed six times in 5 months after the first attack. During the episodes, neither hypoglycemia nor ketosis was observed. In this study, fecal and blood samples obtained from this patient 2 days before the fifth attack (19 April 1996) and upon return to a normal condition (3 July 1996) were subjected to lactate and bacteriologic analyses. The fecal samples were placed in a plastic bag for 3 h before bacteriologic analysis. The lactate level in the serum sample obtained 1 day after the fourth attack was also analyzed.

**Fecal pH and bacteriologic analysis.** The fecal pH was determined with a pH meter, and the quantitative bacteriologic analyses were done by the methods described by Mitsuoka et al. (19–21). After 1 g of stool sample was suspended in 9 ml of the anaerobic diluent (20, 21), serial 10-fold dilutions from 10<sup>-1</sup> to 10<sup>-8</sup> were prepared in an anaerobic chamber (N<sub>2</sub>-CO<sub>2</sub>-H<sub>2</sub> [8:1:1]). Aliquots (0.1 ml each) of the serial dilutions from 10<sup>-3</sup> to 10<sup>-8</sup> were spread on four selective agar media (neomycin-Nagler [NN], desoxycholate-hydrogen sulfide-lactose [DHL], neomycin sulfate-brilliant green-taurocholate-blood [NBGT], bifidobacterium [BIF]) and three nonselective agar media (glucose-blood-liver [BL] agars with and without 5% defibrinated horse blood and Trypticase soy [TS] agar with 5% defibrinated horse blood). The selective media used and bacterial groups detected were as follows: NN agar for clostridia such as *Clostridium perfringens*, DHL agar for enterobacteria, NBGT agar for members of the family *Bacteroi-*

\* Corresponding author. Mailing address: Central Research Institute, Meiji Milk Products Co., 1-21-3 Sakae-cho, Higashimurayama, Tokyo 189, Japan. Phone: (0423) 91-2955. Fax: (0423) 95-1829. E-mail: [microbio@ba2.so-net.or.jp](mailto:microbio@ba2.so-net.or.jp).

*daceae*, and BIF agar for bifidobacteria. The NBGT agar medium was prepared with Eggerth Gagnon (EG) agar (19). The BL and DHL agar media were purchased from Eiken Chemical Co. (Tokyo, Japan). The EG and TS agars were purchased from Nissui Pharmacy Co. (Tokyo, Japan) and BBL, Becton Dickinson Microbiology Systems (Cockeysville, Md.), respectively. BIF agar (pH 5.4) was prepared in our laboratory from number 1 medium (14) supplemented with 0.82% (wt/vol) sodium acetate, 0.5% (wt/vol) NaCl, 0.1% (wt/vol) LiCl, and 2.5% (wt/vol) agar. Plates inoculated for the determination of obligate anaerobic bacterial counts were incubated in the anaerobic chamber at 37°C for 48 h. DHL and TS agars were incubated in aerobic conditions at 37°C for 24 and 48 h, respectively. After incubation, preliminary identification of bacterial groups was done by colonial and cellular morphologies, gram reaction, and aerobic and anaerobic growth (19, 21). The results were expressed as log<sub>10</sub> colony counts per gram (wet weight) of feces.

**Isolation of lactobacilli.** Colonies which were found predominantly in 10<sup>-8</sup> and 10<sup>-7</sup> dilution plates (BL agar medium with 5% defibrinated horse blood) of the feces samples obtained 2 days before the fifth attack (36 colonies) and during normal conditions (20 colonies) were picked and isolated by being subcultured twice anaerobically on BL agar medium. The characteristics of the isolates, such as catalase reaction, gram reaction, gas production from glucose, aerobic growth, cell morphology, and acetate and lactate (D-lactate, L-lactate, or DL-lactate) production from glucose, were analyzed. From these results, the counts of lactobacilli (D-lactate, L-lactate, and DL-lactate producers) in the fecal samples were determined.

**Assays of SCFA and lactate.** The short-chain fatty acids (SCFA) in feces were determined with a high pressure liquid chromatography (HPLC) system (model L-6200; Hitachi Co., Tokyo, Japan) in combination with a UV-VIS detector (model L-4200; Hitachi Co.) as described in our previous paper (14). The column (Shodex Ionpak KC-811 plus Ionpak KC-810P guard column; Showa Denko Co., Tokyo, Japan) was run at 63°C. D-Lactate and L-lactate concentrations in feces and serum samples were determined after removal of the protein with Carrez-1 and Carrez-2 solution treatment (14) by an enzymatic analysis method (Boehringer GmbH, Mannheim, Germany). All experiments were repeated twice with the same sample, and the mean results for two experiments were determined.

**Physiological and biochemical characteristics.** Physiological and biochemical tests, such as fermentation of carbohydrates, growth at fixed temperature, and gas production from glucose, were carried out by the methods described by Mitsuoka (22). Ammonia production from arginine and dissimilation of malate were determined by the methods described by Keddie (16).

**Microorganisms and DNA extraction.** The bacteria used were *Lactobacillus fermentum* JCM 1137 (ATCC 11379), *L. fermentum* NRIC 1752<sup>T</sup>, *L. delbrueckii* subsp. *delbrueckii* JCM 1012<sup>T</sup>, *L. delbrueckii* subsp. *lactis* JCM 1148<sup>T</sup>, and *L. delbrueckii* subsp. *lactis* OLL 2654. Each strain was grown anaerobically at 37°C for 15 h in 1,000 ml of medium number 1 (14). To obtain template DNA for PCR, chromosomal DNA of the test strains was extracted and purified as described previously (15). The final DNA concentration in Tris-HCl (1 mM, pH 8.0)-EDTA (1 mM) was 500 ng/μl.

**PCR-ELISA.** Amplification and labelling of the 16S genes coding for rRNA fragments (16S rDNA) of the test strains were performed simultaneously, with a PCR thermal cycler MP (model TP 3000; TAKARA SHUZO Co., Otsu, Japan). Labelling was achieved either by incorporating digoxigenin (DIG)-dUTP (15) and by using the following 5'-DIG-labelled primers to amplify the 16S rDNA (1): forward primer 5'-DIG-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 5'-DIG-GGTTACCTGTACGACTT-3'. PCR was carried out in 100-μl volumes, which consisted of 1.5 μl of DNA isolate (500 μg/ml), 10 μl of 10× PCR buffer (Boehringer), 60.2 μl of ultrapure sterile water, 10 μl of each primer (100 pmol), 0.8 μl of dTTP (80 μM), 1 μl of DIG-11-dUTP (1 μM), 0.5 μl of *Taq* DNA polymerase (2.5 U), and 2 μl each of dATP (200 μM), dCTP (200 μM), and dGTP (200 μM). Cycling conditions were 94°C for 45 s, 50°C for 60 s, and 72°C for 120 s. After 33 cycles, an aliquot (5 μl) of each amplification mixture was run on submarine 0.9% (wt/vol) agarose gels in Tris-acetate buffer (30 min at 100 V), stained with ethidium bromide, and photographed on a UV transilluminator.

The labeled PCR products were analyzed with solution hybridization to a specific capture probe by enzyme-linked immunosorbent assay (ELISA). The capture oligonucleotide, 5'-GCGACAAAATCAATCAGG-3' (30) for the specific detection of *L. fermentum*, was labeled with biotin to allow immobilization of the hybrid to a streptavidin-coated microtiter plate (Boehringer) surface. The 5'-DIG-labelled PCR primers and 5'-biotin-labelled oligonucleotides were synthesized by Nihon Gene Research Labs, Inc. (Sendai, Japan).

Color detection with the PCR product and capture probe was carried out according to the directions in the manual of the PCR-ELISA (DIG detection) kit (Boehringer), with several modifications. In the first step, 10 μl of the DIG-labelled PCR product was denatured with 40 μl of the PCR-ELISA denaturation solution (Boehringer) at 25°C for 10 min. Next, 50 μl of this denatured PCR product and 2 μl of the biotinylated probe (10 pmol/μl) were added to 450 μl of the kit's hybridization buffer and then hybridized at 55°C for 2 h.

**G+C content of DNA.** The G+C content (expressed as moles percent) of DNA was determined by HPLC of its nuclease P<sub>1</sub> hydrolysate. After 50 μl of DNA (500 ng/μl in Tris-HCl [10 mM, pH 8.0] plus EDTA [1 mM]) mixed with 50 μl of 10 mM acetate buffer (pH 5.3) was denatured in boiling water for 5 min, 2.5 μl of nuclease P<sub>1</sub> (400 U/mg; Seikagaku Kogyo Co., Tokyo, Japan) solution (1 mg/ml in 10 mM acetate buffer [pH 5.3]) was added to 50 μl of the denatured

DNA solution and the mixture was incubated at 50°C for 1 h. Next, the reaction mixture was ultrafiltered (0.45-μm-pore-size filter), and the filtrate was analyzed with a HPLC system equipped with a column (LiChrosorb NH<sub>2</sub>; Kanto Chemical Co., Tokyo, Japan). Nucleotides were eluted by 20 mM KH<sub>2</sub>PO<sub>4</sub>-H<sub>3</sub>PO<sub>4</sub> (pH 2.8) at a flow rate of 2 ml/min and a column temperature of 30°C and then determined spectrometrically at 260 nm. A standard mixture of nucleotides (Yamasa Shoyu Co., Choshi, Japan) was used as the reference for calibration of measurements of moles percent.

**Microplate hybridization.** Microplate hybridization assay was essentially carried out by the methods described by Ezaki et al., (7, 8) with several modifications. The DNA was dissolved in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at a concentration of 100 μg/ml and denatured in boiling water for 5 min. The denatured DNA was diluted to a concentration of 10 μg/ml with P buffer (8 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 2.7 mM KCl [pH 8.9]) containing 0.1 M MgCl<sub>2</sub>. Next, a 100-μl portion of this solution was distributed to each well of the microplate (Black Immunoplate II; Nunc, Roskilde, Denmark) and incubated at 30°C for 16 h. After each solution was removed, the wells were washed with 200 μl of P buffer and then dried at 45°C for 3 h.

After 10 μl of purified DNA was mixed with 10 μl of photobiotin solution (1 μg/μl) in an Eppendorf tube, the mixture was cooled in an ice bath and then biotinylated by irradiation with a mercury lamp (SHLS-10024; Toshiba Co., Tokyo, Japan) at a distance of 10 cm for 20 min. Next, the amount of the biotinylated mixture was increased to up to 100 μl with 0.1 M Tris-HCl (pH 9.0), and then 100 μl of 1-butanol was added to the tube. After the mixture was mixed and centrifuged, the upper phase was removed.

The DNA-DNA hybridization was carried out as follows. In the first step, a 200-μl portion of prehybridization solution (2× SSC, 5× Denhardt solution, 50% [vol/vol] formamide, 0.00025% [vol/vol] denatured calf thymus DNA) was added to each microtiter well of a DNA plate and incubated at 37°C for 30 min. Next, the prehybridization solution was removed and replaced with 100 μl of a hybridization solution (0.5 g of dextran sulfate plus 50 μl of biotinylated DNA/10 ml of prehybridization solution). The microplate was then covered, and hybridization was carried out at 42°C for 24 h. After hybridization, the wells were washed twice with 200 μl of 0.2× SSC. Next, 100 μl of PAT solution (50 mg of bovine serum albumin [fraction V] plus 10 mg of Triton X-100/10 ml of P buffer) was distributed to each well and incubated at 25°C for 10 min. A 100-μl portion of enzyme solution (1 μl of streptavidin-β-D-galactosidase complex [PhotoGene; Life Technologies, Inc., Gaithersburg, Md.]/ml of PAT buffer) was distributed to the wells and incubated at 37°C for 30 min. After the wells were washed three times with 200 μl of P buffer, a 200-μl portion of substrate solution (1 mg of 4-methylumbelliferyl-β-D-galactopyranoside/ml of P buffer containing 1 mM MgCl<sub>2</sub>) was distributed to the wells and incubated at 37°C for 30 min. The fluorescence intensity was measured with a microplate reader (model MP-32; Corona Electric Co., Katsuta, Ibaragi, Japan) at wavelengths of 360 and 450 nm for excitation and emission, respectively. The fluorescence intensity of the wells of calf thymus DNA was calculated as 0%, and the intensity of the well for each test organism was calculated as 100%. In this case, the average values for five wells (each) for a calf thymus DNA and a test organism DNA were used for the calculation of DNA-DNA homology value.

## RESULTS

**Fecal microflora.** The distributions of fecal microflora 2 days before the fifth attack (19 April 1996) and for the normal condition (3 July 1996) are listed in Table 1. There was not a great difference in pH values for the two feces samples. The counts of total fecal bacteria and anaerobic bacteria such as the *Bacteroidaceae* were extremely low, while the counts of lactobacilli (9.36 log<sub>10</sub> counts/g) and the total population of lactobacilli in relationship to total fecal bacteria (40.4%) in the feces sample 2 days before the fifth attack were extremely high. The total population of bifidobacteria in relationship to the total fecal bacteria was also high (50.9%), and the total population of lactobacilli plus bifidobacteria was 91.3%. However, the counts of total fecal bacteria in the feces sample for the normal condition increased to 2.7 × 10<sup>10</sup> per g, although the counts for the *Bacteroidaceae* were still low. The total population of lactobacilli in this fecal sample in relationship to total fecal bacteria was lower (14.3%) than that (40.4%) 2 days before the fifth attack.

Some characteristics of isolates obtained from the feces sample 2 days before the fifth attack (36 isolates) and for the normal condition (20 isolates) were analyzed as described in Materials and Methods (15a). It was found that 17 of the 36 isolates from the fecal sample obtained 2 days before the fifth attack were lactobacilli and that these lactobacilli produced

TABLE 1. Microflora in the feces of a patient with SBS

Microorganism	Log <sub>10</sub> CFU/g of feces (%) <sup>a</sup>	
	2 days before fifth attack (19 April 1996)	No SBS symptom (3 July 1996)
Bifidobacteria	9.76 9.46 (50.9)	10.62 10.41 (61.9)
Lactobacilli	9.36 (40.4)	9.78 (14.3)
D-Lactate producers <sup>b</sup>	9.00	8.60
L- or DL-Lactate producers <sup>b</sup>	<7.00	9.47
<i>Bacteroidaceae</i>	7.48	<7.00
Anaerobic cocci	8.48	9.30
<i>Clostridium perfringens</i>	5.39	6.96
Eubacteria and other clostridia	8.00	9.85
Total aerobes	7.45	9.04
<i>Enterobacteriaceae</i>	7.45	9.00

<sup>a</sup> The microorganism population in relationship to total fecal bacteria is indicated in parentheses. The pH values of the fecal samples obtained on 19 April and 3 July were 6.20 and 6.50, respectively.

<sup>b</sup> Isolates (lactobacilli) from BL agar medium with 5% defibrinated horse blood.

exclusively D-lactate from glucose. All of the other isolates (19 isolates) were bifidobacteria. In this case, the number of lactobacilli which produced D-lactate homofermentatively from glucose was 10<sup>9</sup> per g, while the number of lactobacilli which produced L- or DL-lactate was less than 10<sup>7</sup> per g (Table 1).

However, only 3 of 20 isolates from the fecal sample for the normal condition were lactobacilli which produced D-lactate (15a). The others were composed of lactobacilli (10 isolates) which produced D- or L-lactate and bifidobacteria (7 isolates). In this case, D-lactate producers (lactobacilli) decreased to less than 10<sup>9</sup> per g, while the number of L- or DL-lactate producers (lactobacilli) was 100-fold higher than that in the feces sample obtained 2 days before the fifth attack (Table 1).

**Analyses of lactate and SCFA.** The D-lactate level in serum 1 day after the fourth attack was 10-fold higher than that for the normal condition, although there was not a great difference in L-lactate levels between the two blood samples (Table 2). The D- and L-lactate levels in the feces sample 2 days before the fifth attack were 3.91 and 2.86 mM, respectively, although the lactate concentration in serum was low (D-lactate, 0.21 mM; L-lactate, 0.48 mM). We could not detect lactate in the

feces during the normal condition. Furthermore, the SCFA concentration detected from the feces of this patient was lower than that in the human colon described previously (18).

**Identification of lactobacilli.** Bifidobacteria are considered to be independent of D-lactic acidosis because these bacteria are L-lactate producers. Therefore, we studied the taxonomical position of the lactobacilli found predominantly in the feces 2 days before the fifth attack, on the basis of comparative physiological, biochemical, and genomic characteristics, including the respective type strains. The lactobacilli (isolate no. 2, 27, and 64) shared identical phenotypic properties as follows. The lactate isomer was the D type. Positive characteristics included growth at 45°C, gas from malate, arginine hydrolysis, and fermentation of glucose, mannose, fructose, galactose, sucrose, maltose, cellobiose, lactose, melibiose, raffinose, dextrin, starch, inulin, aesculin, salicin, and amygdalin. Negative characteristics included growth at 15°C, gas from glucose, and fermentation of arabinose, xylose, rhamnose, ribose, trehalose, melezitose, glycogen, mannitol, sorbitol, inositol, and α-methyl glucoside. The genomic characteristics are summarized in Table 3. The G+C content of these lactobacilli was approximately 52 mol%, and the hybridization data demonstrated that all three isolates were members of a single genospecies, *L. delbrueckii*. However, many biochemical characteristics of these strains also agreed with those of *L. fermentum*, except the characteristics of the lactate isomer and gas production from glucose. Therefore, we studied the genomic characteristics of these strains by PCR-ELISA and DNA-DNA hybridization assay. In this case, isolate no. 64 was not used for the additional analyses of homology with the various *Lactobacillus* strains. The capture probe for the specific detection of *L. fermentum* showed a positive reaction with the 16S rDNA amplification products of *L. fermentum* NRIC 1752<sup>T</sup> but was negative with those of isolates no. 2 and no. 27 isolated from the fecal sample of this patient (15a). Furthermore, the genetic relatedness by DNA-DNA hybridization assay was 0 to 6% between the isolates and *L. fermentum* strains (NRIC 1752<sup>T</sup> and JCM 1737).

On the other hand, isolates no. 2 and no. 27 showed 83 to 86% DNA-DNA relatedness with *L. delbrueckii* subsp. *delbrueckii* JCM 1012<sup>T</sup> and 92 to 94% DNA-DNA relatedness with *L. delbrueckii* subsp. *lactis* JCM 1148<sup>T</sup>. However, carbohydrate fermentation patterns such as those of maltose, lactose, trehalose, dextrin, and starch, for isolates no. 2 and no. 27, clearly differed from those for *L. delbrueckii* subsp. *delbrueckii* and *L. delbrueckii* subsp. *bulgaricus* (13). Furthermore, *L. delbrueckii* subsp. *bulgaricus* never hydrolyzes arginine (13). From these results, we finally identified the D-lactate producers (lactobacilli) which were found predominantly in the feces 2 days before the fifth attack as *L. delbrueckii* subsp. *lactis*.

TABLE 2. Lactate and SCFA levels in serum and feces

Sample (date of sampling)	Lactate <sup>a</sup>		SCFA (mmol/kg)			
	D-Lactate	L-Lactate	Acetate	Propionate	Butyrate	Total
<b>Serum</b>						
One day after 4th attack (4 April 1996)	2.32	0.82				
Two days before 5th attack (19 April 1996)	0.21	0.48				
No SBS symptom (3 July 1996)	0.17	0.79				
<b>Feces</b>						
Two days before 5th attack (19 April 1996)	3.91	2.86	13.70	9.60	10.90	34.20
No symptom (3 July 1996)	<0.02	<0.02	10.40	7.50	10.30	28.20

<sup>a</sup> Levels are given in millimoles per liter for serum and millimoles per kilogram for feces.



TABLE 3. DNA base composition of lactobacilli isolated from feces and DNA homologies with type strains

Isolate or strain	G+C (mol%)	% homology to probe DNA from:							
		Isolate no. <sup>a</sup>			JCM 1148 <sup>T</sup>	OLL 2654	NRIC 1752 <sup>T</sup>	JCM 1137	JCM 1012 <sup>T</sup>
		2	27	64					
No. 2	52.1	100							
No. 27	52.3	90	100						
No. 64	52.0	100	100	100					
<i>L. delbrueckii</i> subsp. <i>lactis</i> JCM 1148 <sup>T</sup>	50.0	94	92		100				
<i>L. delbrueckii</i> subsp. <i>lactis</i> OLL 2654	49.5	100	96			100			
<i>L. fermentum</i> NRIC 1752 <sup>T</sup>	53.7	0	1		0	1	100		
<i>L. fermentum</i> JCM 1137		3	6		3	3		100	
<i>L. delbrueckii</i> subsp. <i>delbrueckii</i> JCM 1012 <sup>T</sup>		83	86		83	81		6	100

<sup>a</sup> Lactobacilli isolated from the patient with SBS.

## DISCUSSION

In humans, lactobacilli predominate in the stomach and small intestine. In the jejunum and large intestine, strict anaerobes such as the *Bacteroidaceae* and *Eubacteria* are the majority flora and lactobacilli constitute only 0.07 to 1% of the total flora (28). Generally, *L. acidophilus*, *L. fermentum*, and *L. salivarius* are the predominant lactobacilli in the lower intestines and feces of humans, although *L. plantarum* and *L. leichmanii* may sometimes be prevalent (28). However, in this study we found that the counts of lactobacilli and the total population (percent) of these bacteria in relationship to total fecal bacteria in the patient with SBS were much higher than those found in healthy humans (19, 26). In this case, the number of producers (lactobacilli) D-lactate from glucose by homolactic fermentation in the feces 2 days before the fifth attack was approximately  $10^9$  per g, while the number of L- or DL-lactate producers was less than  $10^7$  per g (Table 1). Furthermore, the D-lactate producers (lactobacilli) found predominantly in the feces 2 days before the fifth attack were identified as *L. delbrueckii* subsp. *lactis*. From these results it can be presumed that *L. delbrueckii* subsp. *lactis* was responsible for the D-lactic acidosis.

Finegold et al. (9) and Moore and Holdemann (23) showed that *L. leichmanii* could be isolated from human feces. However, the DNA-DNA homology among the type strains of *L. delbrueckii*, *L. bulgaricus*, *L. lactis*, and *L. leichmanii* is more than 80% (32), and the phenotypical differences are restricted to variations in the fermented carbohydrates (13). For these reasons, *L. leichmanii* is now considered to be *L. delbrueckii* subsp. *lactis* (13). *L. delbrueckii* subsp. *lactis* strains identified in this study may correspond to the former species *L. leichmanii*. Satoh et al. (27) found a marked predominance of lactobacilli in the feces of patients with SBS, and the predominant lactobacilli were identified as *L. buchneri* and *L. fermentum*. However, these lactobacilli are producers of DL-lactate from glucose. Recently, oligonucleotide probes based on the specific genes of variable rRNA regions have been used to characterize the taxonomic relationships among bacteria (1, 6, 17, 25, 31, 33, 34). We also studied the genomic characteristics of the lactobacilli isolated in this study, using PCR-ELISA and DNA-DNA hybridization assay and found that the predominant lactobacilli detected in this patient were clearly different from *L. fermentum*. We are not aware of previous reports showing the predominant presence of *L. delbrueckii* subsp. *lactis* in fecal samples from a patient with SBS. Therefore, this study may be the first case indicating that D-lactate acidosis is attributable to the overgrowth of *L. delbrueckii* subsp. *lactis*.

Under normal conditions, acetate, propionate, and butyrate

are the principal organic acids and L- and D-lactate are either absent or present at very low concentrations in the feces of healthy subjects. This is because the two stereoisomers are converted to SCFA, such as acetate, propionate, and butyrate, in the presence of intestinal bacteria (10, 11) although both L-lactate and D-lactate are produced by intestinal bacteria. The counts of anaerobic bacteria such as the *Bacteroidaceae* in this patient were extremely low (Table 1).

These results are consistent with data in which the SCFA concentrations in the feces of SBS patients were lower than those in healthy humans (3). We also found that SCFA concentrations in the feces of this SBS patient were lower than those previously described for the human colon (18). It is thought that a more aerobic environment which is not favorable to the growth of anaerobic bacteria is formed in the colon because of small intestinal resection. The total population (percent) of bifidobacteria in relationship to total fecal bacteria in each feces sample from this patient was high (Table 1). However, the bifidobacteria are L-lactate producers; hence, D-lactate may be overproduced in the colon and the blood of this patient. In fact, the D-lactate concentration in the serum 1 day after the fourth attack was 10-fold higher than that for the normal condition, although there was not a great difference in L-lactate levels among the samples (Table 2). The concentration of fecal lactate 2 days before the fifth attack was much higher than that for the normal condition, while the concentration of each lactate in serum was low (Table 2). The reason is not clear, but factors such as absorption or impaired metabolism may be necessary for a plasmatic increase of D-lactate, as described by Bustos et al. (3). Furthermore, there is a report indicating that the human organism is able to accumulate and eliminate D-lactate at considerable rates (11).

During the normal condition, the counts of L- or DL-lactate producers in the feces were 100-fold higher than those in the feces 2 days before the fifth attack, while D-lactate producers (lactobacilli) decreased to less than  $10^9$  per g (Table 1). Therefore, an increase in the counts of D-lactate producers (lactobacilli) in the colon may be closely associated with the clinical expression of metabolic acidosis. It is generally accepted that oral administration of antibiotics, treatments with sodium bicarbonate, and restricted intake of simple carbohydrates will correct D-lactic acidosis and improve these symptoms in most patients with SBS (12, 24, 27). In addition to these treatments, it is possible that selective therapy to decrease the counts of D-lactate producers, such as *L. delbrueckii* subsp. *lactis*, in the colon may be more effective for reducing the SBS symptoms of acidosis.

## REFERENCES

1. **Battles, J. K., J. C. Williamson, K. M. Pike, P. L. Gorelick, J. M. Ward, and M. A. Gonda.** 1995. Diagnostic assay for *Helicobacter hepaticus* based on nucleotide sequence of its 16S rRNA gene. *J. Clin. Microbiol.* **33**:1344–1347.
2. **Bongaerts, G., J. Tolboom, T. Naber, J. Bakkerren, R. Severijnen, and H. Willems.** 1995. D-Lactic acidemia and aciduria in pediatric and adult patients with short bowel syndrome. *Clin. Chem.* **41**:107–110.
3. **Bustos, D., S. Pons, J. C. Pernas, H. Gonzalez, M. I. Calderini, K. Ogawa, and J. A. De Paula.** 1994. Fecal lactate and short bowel syndrome. *Dig. Dis. Sci.* **39**:2315–2319.
4. **Cammack, R.** 1969. Assay, purification and properties of mammalian D-2-hydroxy acid dehydrogenase. *Biochem. J.* **115**:55–64.
5. **Coronado, B. E., S. M. Opal, and D. C. Yoburn.** 1995. Antibiotic-induced D-lactic acidosis. *Ann. Intern. Med.* **122**:839–842.
6. **Ehrmann, M., W. Ludwig, and K. H. Schleifer.** 1994. Reverse dot blot hybridization: a useful method for the direct identification of lactic acid bacteria in fermented food. *FEMS Microbiol. Lett.* **117**:143–149.
7. **Ezaki, T., Y. Hashimoto, N. Takeuchi, and H. Yamamoto, S.-L. Liu, H. Miura, K. Matsui, and E. Yabuuchi.** 1988. Simple generic method to identify viridans group streptococci by colorimetric dot blot hybridization and fluorometric hybridization in microdilution wells. *J. Clin. Microbiol.* **26**:1708–1713.
8. **Ezaki, T., Y. Hashimoto, and E. Yabuuchi.** 1989. Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int. J. Syst. Bacteriol.* **39**:224–229.
9. **Finogold, S. M., V. L. Sutter, P. T. Sugihara, H. A. Elder, S. M. Lehmann, and R. L. Phillips.** 1977. Fecal microbial flora in Seventh Day Adventist populations and control subjects. *Am. J. Clin. Nutr.* **30**:1781–1792.
10. **Halperin, M. L., and K. S. Kamel.** 1996. D-Lactic acidosis: turning sugar into acids in the gastrointestinal tract. *Kidney Int.* **49**:1–8.
11. **Hove, H., and P. B. Mortensen.** 1995. Colonic lactate metabolism and D-lactic acidosis. *Dig. Dis. Sci.* **40**:320–330.
12. **Kadokia, S. K.** 1995. D-Lactic acidosis in a patient with jejunioileal bypass. *J. Clin. Gastroenterol.* **20**:154–156.
13. **Kandler, O., and N. Weiss.** 1986. Regular, nonsporing gram-positive rods, p. 1208–1234. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. 2. The Williams & Wilkins Co., Baltimore, Md.
14. **Kaneko, T., H. Mori, M. Iwata, and S. Meguro.** 1994. Growth stimulator for bifidobacteria produced by *Propionibacterium freudenreichii* and several intestinal bacteria. *J. Dairy Sci.* **77**:393–404.
15. **Kaneko, T., and H. Kurihara.** 1997. Digoxigenin-labeled DNA probes for the enumeration of bifidobacteria in fecal samples. *J. Dairy Sci.* **80**:1254–1259.
- 15a. **Kaneko, T.** Unpublished results.
16. **Keddie, R. M.** 1959. The properties and classification of lactobacilli isolated from grass and silage. *J. Appl. Bacteriol.* **22**:403–416.
17. **Kreder, C. A.** 1995. Design and evaluation of *Bacteroides* DNA probes for the specific detection of human fecal pollution. *Appl. Environ. Microbiol.* **61**:1171–1179.
18. **Macfarlane, G. T., and J. H. Cummings.** 1991. Physiology, pathophysiology, and disease, p. 51–92. *In* S. F. Phillips (ed.), *The large intestine*. Raven Press, Ltd., New York, N.Y.
19. **Mitsuoka, T., T. Sega, and S. Yamamoto.** 1965. Eine verbesserte Methodik der qualitativen und quantitativen Analyse der Darmflora von Menschen und Tieren. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. A* **195**:455–469.
20. **Mitsuoka, T., A. Terada, and Y. Morishita.** 1973. Die Darmflora von Mensch und Tier. *Goldschmidt Informiert.* **23**:23–41.
21. **Mitsuoka, T.** 1980. A color atlas of anaerobic bacteria, p. 13–92; 319–327. Sobunsha, Tokyo, Japan (In Japanese.)
22. **Mitsuoka, T.** 1969. Vergleichende Untersuchungen über die Lactobazillen aus den Faeces von Menschen, Schweinen und Hühnern. *Zentralbl. Bakteriol. Abt. 1 Orig.* **210**:32–51.
23. **Moore, W. E. C., and L. V. Holdemann.** 1974. Human fecal flora: the normal flora of 20 Japanese-Hawaiians. *Appl. Microbiol.* **27**:961–979.
24. **Perlmutter, D. H., J. T. Boyle, J. M. Campos, J. M. Egler, and J. B. Watkins.** 1983. D-Lactic acidosis in children: an unusual metabolic complication of small bowel resection. *J. Pediatr.* **102**:234–238.
25. **Saiki, R. K., P. S. Walsh, C. H. Levenson, and H. A. Erlich.** 1989. Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes. *Proc. Natl. Acad. Sci. USA* **86**:6230–6234.
26. **Salminen, S., E. Isolauri, and T. Onnela.** 1995. Gut flora in normal and disordered states. *Chemotherapy* **41**:5–15.
27. **Satoh, T., K. Narisawa, T. Konno, T. Katoh, J. Fujiyama, A. Tomoe, K. Metoki, K. Ilaayasaka, K. Tada, M. Ishibashi, N. Yamane, T. Mitsuoka, and Y. Benno.** 1982. D-Lactic acidosis in two patients with short bowel syndrome: bacteriological analyses of the fecal flora. *Eur. J. Pediatr.* **138**:324–326.
28. **Sharpe, M. E.** 1981. The genus *Lactobacillus*, p. 1653–1679. *In* M. P. Starr (ed.), *The prokaryotes*. Springer-Verlag, Berlin, Germany.
29. **Taguchi, H., and T. Ohta.** 1991. D-Lactate dehydrogenase is a member of the D-isomer-specific 2-hydroxy acid dehydrogenase family. *J. Biol. Chem.* **266**:12588–12594.
30. **Vogel, R. F., G. Bocker, P. Stolz, M. Ehrmann, D. Fanta, W. Ludwig, B. Pot, K. Kersters, K. H. Schleifer, and W. P. Hammes.** 1994. Identification of lactobacilli from sourdough and description of *Lactobacillus pontis* sp. nov. *Int. J. Syst. Bacteriol.* **44**:223–229.
31. **Wang, R.-F., W.-W. Cao, and C. E. Cerniglia.** 1996. PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. *Appl. Environ. Microbiol.* **62**:1242–1247.
32. **Weiss, N., U. Schillinger, and O. Kandler.** 1983. *Lactobacillus lactis*, *Lactobacillus leichimani* and *Lactobacillus bulgaricus*, subjective synonyms of *Lactobacillus delbrueckii* subsp. *lactis* comb. nov. and *Lactobacillus delbrueckii* subsp. *bulgaricus* comb. nov. *Syst. Appl. Microbiol.* **4**:552–557.
33. **Woese, C. R., R. Gutell, R. Gupta, and H. F. Noller.** 1983. Detailed analysis of the higher-order structure of 16S-like ribosomal ribonucleic acids. *Microbiol. Rev.* **47**:621–669.
34. **Woese, C. R.** 1987. Bacterial evolution. *Microbiol. Rev.* **51**:221–271.