J. Gen. Appl. Microbiol., **48**, 143–154 (2002)

Full Paper

Succinate accumulation in pig large intestine during antibiotic-associated diarrhea and the constitution of succinate-producing flora

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(Received December 8, 2001; Accepted May 8, 2002)

Succinate was the major organic acid detected in the hindgut content of pigs suffering from antibiotic-associated diarrhea. Antibiotic-associated diarrhea was induced by an oral dose of polymyxin B sulfate (3,000,000 units/day) or an intramuscular injection of enrofloxacin (0.6 g enrofloxacin/day). In the large intestine of enrofloxacin-treated pigs, Gram-negative facultative anaerobic rods phylogenetically related to *Escherichia coli* and Gram-positive facultative anaerobic non-spore-forming rods phylogenetically related to Lactobacilli were isolated as succinate producers. Succinate-producing Lactobacilli were only isolated as the succinate producer in polymyxin B sulfate-treated pigs. In contrast to antibiotic-associated diarrhea pigs, bacteria belonging to *Bacteroidaceae*, Fusobacteria, and *Enterobacteriaceae* were detected as succinate producers in a non-treated pig. In antibiotic-associated diarrhea conditions, antibiotic-resistant Enterobacteria, *E. coli* in particular, and Lactobacilli may contribute to an abnormal succinate accumulation and may affect water absorption in the hindgut that relates to an expression of antibiotic-associated diarrhea.

Key Words——antibiotic-associated diarrhea; *Enterobacteriaceae*; *Lactobacillaceae*; pig large intestine; succinate accumulation

Introduction

Succinate is usually not detected in the content of the large intestine because it is efficiently decarboxylated to propionate by organic acid-utilizing bacteria such as *Selenomonas ruminantium* (Scheifinger and Wolin, 1973). Succinate accumulation in the hindgut, therefore, indicates an abnormal status of the hindgut fermentation (Rubinstein et al., 1969) and may be involved in antibiotic-associated diarrhea (AAD) (Tsukahara et al., 2000; Tsukahara and Ushida, 2001) because this acid stimulates water secretion from the digestive tract (Shimazaki, 1992). AAD is associated with treatments using broad-spectrum antimicrobials (Hove, 1998) that seriously affect the activity and composition of the hindgut microflora. Low concentrations of fecal short-chain fatty acids (SCFA) are often associated with AAD (Gustafsson et al., 1998; Högenauer et al., 1998; Hove, 1998; Høverstad et al., 1986; Mørtensen and Clausen, 1995). However, these previous papers did not report on the concentration of succinate or lactate. The pathogenic role of *Clostridium difficile* has been discussed in relation to AAD (Aronsson et al., 1981; Högenauer et al., 1998) but without any information on the bacterial flora constitution in AAD.

Therefore, in this study, we intended to analyze the organic acid composition and the bacterial-flora profiles in the hindgut of AAD-induced pigs to find the

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bacteriological explanations for succinate accumulation in the large intestine of pigs.

A part of the study was given at the INRA-RRI Symposium as a poster presentation (Tsukahara and Ushida, 2000).

Materials and Methods

Animals and diet. Six crossbred (Landrace×Large white×Duroc) 25-day-old male piglets weighing 5–7 kg were obtained from a commercial pig farm. The pigs were handled in accordance with the guidelines for studies with laboratory animals of the Kyoto Prefectural University Experimental Animal Committee. They were individually housed in metabolic cages in a temperature-controlled room (25°C) and adapted to the standard diet for weaning piglets (Koromeal GS; Nippon Formula Feed Co., Ltd., Yokohama, Japan) free from intestinal flora modifiers such as antimicrobials, probiotics, and prebiotics. The diet contained 26.0% crude protein, 5.0% ether extracts, 2.4% crude fiber, and 5.0% crude ash. Feed and water were provided ad libitum.

Induction of AAD. After three days of adaptation, we gave two pigs (#s 1 and 2) polymyxin B sulfate (PL; Pfizer, Tokyo, Japan; 3,000,000 units/day orally) and the other two (#s 3 and 4) enrofloxacin (ERFX; Bayer, Tokyo, Japan; 0.6 g ERFX/day injected intramuscularly). The remaining two pigs (#s 5 and 6) were used as the untreated control. Antimicrobials were divided into two equal portions and dosed at 9 : 00 a.m. and 6 : 00 p.m. Feces were collected and induction of AAD was determined from fecal water content higher than 70%. Over this point, the feces spread out when they dropped on the floor. Furthermore, 1 g of feces was immediately analyzed for organic acid by ion-exclusion HPLC as described elsewhere (Ushida and Sakata, 1998). The animals were slaughtered under general anesthesia with ketamine HCl (Ketalar 50; Sankyo, Tokyo, Japan) when succinic acid was detected as one of the major (over 10 mmol/kg wet feces, and over 10 molar % in total organic acid) organic acids in the feces (after five doses in the ERFX pig and after three doses in the PL pig). The control pigs were slaughtered seven days after the adaptation period. After a midline incision, the whole intestine was immediately removed and separated for cecum, gyri centripetales, gyri centrifugales, and rectum. The digesta of each portion were carefully collected and subjected to organic acid (Ushida and Sakata, 1998) and bacterial analyses.

Bacteriology. Digesta were subjected to 10-fold serial dilution with an anaerobic dilution solution (Arakaki et al., 1994) up to 10^{-9} under O_2-free CO_2 for bacterial analyses. Diluted digesta were used for bacterial colony counts on Blood Liver (BL) agar, Eggerth Gagnon (EG) agar, Desoxycholate Hydrogen sulfide Lactose (DHL) agar, Tryptic Soy (TS) agar, modified Lactobacillus Selected (LBS) agar, and modified Columbia Agar Base (COBA) medium. BL, EG, DHL, and TS were obtained from Nissui (Tokyo, Japan) and the others from BBL (Cockeysville, MD, USA). BL, EG, and TS were supplemented with defibrinated horse blood (5% v/v). LBS was supplemented with Oxoid Lab-lemco powder (0.8% w/v: Basingstoke, England), sodium acetate $3H_2O$ (1.5% w/v), and glacial acetic acid (0.37% v/v). COBA was supplemented with defibrinated horse blood (5% v/v), oxolinic acid (0.5 μg/ml; Sigma, St. Louis, MO, USA), and colistin methane sulfonate (0.5 μg/ml; Sigma). COBA medium used here was made selective for Enterococci by the addition of antimicrobials. All the plates were prepared and incubated as indicated in the manufacturer's instructions unless otherwise stated. BL and EG plates were incubated in an anaerobic chamber (Coy Laboratory, Ann Arbor, MI, USA) with a mixture of gas phase composed of $N_2/CO_2/H_2$ (80/10/10). Other plates were incubated under aerobic conditions. The colonies were counted after 24–48 h incubation period at 37°C (Ueno et al., 1982).

Identification of a succinate producer. Colonies developed on BL and EG plates were isolated according to their colony morphotype (Mitsuoka, 1984) and further analyzed for their succinate production in a modified Gifu Anaerobic Medium (GAM) broth (Nissui) in pig #s 1 to 5. Pig #6 was not used for these studies due to a pH lower than the 6.0 that was critical to maintain normal fermentation (Ushida and Sakata, 1998; Table 2). GAM broth was prepared, inoculated, and incubated as indicated by Ueno et al. (1982). After two to three days' incubation at 37°C, organic acids in the culture supernatant were analyzed as described above. The isolates that produced at least 15 molar % succinate in total organic acid were defined here as succinate producers. They were identified according to colony morphotype, cell morphology, Gram staining, and the API bacteria identification system (API 20E for enterobacteria and API 50CHL for lactic acid bacteria,

Nihon bioMerieux, Tokyo, Japan). 16S rDNA of these isolates was partially sequenced for identification of succinate producers. For this purpose, genomic DNA was isolated from the cells grown in the GAM broth, and the 16S rDNA gene was amplified using a forward primer (27f: 5′-AGAGTTTGATCCTGGCTCAG-3′), a reverse primer (1492r: 5′-GGCTACCTTGTTACGACTT-3′), and Ex-*Taq* polymerase (TaKaRa Shuzo, Kyoto, Japan) according to Hiraishi (1995). Amplified products were purified by agarose gel electrophoresis and directly sequenced by a SEQ 4X4 autosequencer (Amershampharmacia Biotech, Tokyo, Japan) and a Thermo Sequenase Cy 5.5 Dye terminator sequencing kit (Amershampharmacia) using EUB 50f (5′-AACACATGCAAGTCAAGTCGCGAAC-3′) as a sequencing primer (single run). The obtained sequences were subjected to a homology search using BLAST software with known sequences in the GenBank.

Microflora analysis of whole cecal digesta. Cloning and sequencing of the amplified 16S rDNA gene from whole cecal contents were done for three pigs (#s 2, 3, and 5). Genomic DNA was extracted according to Blanc et al. (1999). Partial 16S rDNA was amplified using Ex-*Taq* polymerase (TaKaRa Shuzo), primers 517f (5′-CCAGCAGCCGCGGTAAT-3′) and 907r (5′-CCCGTCAATTCATTTGAGTTT-3′), dNTPs, and an Ex-*Taq* buffer. Primers were selected as suggested by Henckel et al. (1999). The PCR conditions other than the thermal cycle were the same as the manufacturer's instructions. Twelve thermal cycles (94°C for 30 s, 54°C for 30 s, and 72°C for 30 s) were adopted according to Wilson and Blitchington (1996) and cloned into pGEM-T as indicated by Blanc et al. (1999). Transformants were subjected to colony PCR under similar conditions, and PCR amplifications were further subjected to amplified ribosomal DNA restriction analysis (ARDRA) using *Hae* III, *Hha* I, and *Sau* 3AI. In the case of pig #3, *Rsa* I was further used to classify one major ARDRA group. Determination of nucleotide sequences of amplifications was made as indicated above with a T7 primer after plasmid extraction with a Quantum Prep Plasmid Mini Prep Kit (Bio-Rad, Tokyo). The data were subjected to a homology search as indicated above.

Results

Water content, pH and organic acid concentration of digesta (Table 1)

Higher water content of digesta was observed in the antimicrobial-treated pigs compared to that in untreated control pigs at least in the distal portions (gyri centrifugales and rectum). The pH of digesta was the lowest at the cecum in all pigs suggesting fermentation activity in this portion.

Succinate was the major acid in the hindgut (cecum, gyri centripetales, gyri centrifugales, rectum) of the antimicrobial-treated pigs. PL-treated pigs also accumulated lactate in the hindgut. Molar percentages of succinate in total organic acid in cecal and colonic digesta were 6 to 10% in the PL-treated pigs and up to 60% in the ERFX-treated pigs. Succinate was not detected or only in small amounts no larger than 1 mmol/kg digesta in the hindgut of the control pigs. Substantial amounts of acetate and propionate were detected in the PL-treated pigs. Accordingly, total organic acid concentration was higher in the hindgut of the PL-treated pigs (70 to 160 mmol/kg digesta) than it was in the ERFX-treated pigs (40 to 80 mmol/kg digesta). One of the control pigs (#6) also showed a high concentration of organic acid in the cecum and gyri centripetales. Pig #6 also accumulated lactic acid in the cecum and gyri centripetales (4.9 to 7.1 mmol/kg digesta).

Bacterial number (Table 2)

Not all cfu numbers on media varied consistently among the segments of the large intestine. The BL plates indicated a higher cfu than those on the EG plates in the antimicrobial-treated pigs. However, similar levels of cfu were detected for both media in the untreated control pigs. Accordingly, the cfu on EG plates was consistently higher in the control than it was in the antimicrobial-treated pigs.

None of the bacteria was detected on DHL plates in the case of the PL-treated pigs, while a substantial number of colonies formed in the case of the ERFX-treated pigs. Significantly different levels of cfu were detected on DHL plates in the two control pigs; one was 10^9 to 10^{10}/g, and the other was 10^5/g.

Succinate producers in AAD (Table 3)

None of the succinate producers were isolated by the EG plates in the PL-treated pigs. In those pigs, the

Table 1. Chemical analyses of large intestinal digesta of pigs administered polymixin B sulfate, enrofloxacin or control.[a]

Large intestine	Measurements	Composition of organic acids (mmol/kg digesta)	Antimicrobials				Control	
			Polymixin B sulfate		Enrofloxacin			
			Pig 1	Pig 2	Pig 3	Pig 4	Pig 5	Pig 6
Cecum	Moisture (%)		85.1	85.3	90.6	87.9	78.6	90.6
	pH		5.1	5.5	6.1	7.1	6.5	5.9
	Organic acids	Total organic acids	165.1	150.6	75.0	80.2	92.8	145.0
		Total SCFA	120.8	100.5	29.2	50.6	88.4	137.5
		Succinate	15.3	39.9	45.8	29.1	0.9	N.D.[b]
		Lactate	29.0	10.0	N.D.	N.D.	3.3	7.1
		Acetate	55.2	57.0	26.7	33.2	46.5	83.1
		Propionate	49.9	39.4	1.7	5.9	27.7	25.8
		n-Butyrate	9.4	1.7	N.D.	7.8	8.8	23.3
Gyri centripetales	Moisture (%)		83.8	82.6	90.2	85.7	72.9	85.3
	pH		5.5	5.7	6.4	7.3	6.7	5.8
	Organic acids	Total organic acid	152.5	164.5	66.9	61.7	73.6	197.5
		Total SCFA	109.1	110.7	22.1	21.1	71.4	191.4
		Succinate	17.0	51.1	43.3	34.7	0.7	0.2
		Lactate	26.5	1.9	N.D.	5.9	1.1	4.9
		Acetate	50.2	54.3	17.9	16.4	37.8	104.9
		Propionate	37.7	33.1	1.4	1.6	15.5	38.6
		n-Butyrate	17.8	20.4	2.8	N.D.	7.6	35.5
Gyri centrifugales	Moisture (%)		74.5	84.5	86.0	81.3	68.0	70.3
	pH		6.0	5.7	6.5	7.4	6.8	6.5
	Organic acids	Total organic acid	78.3	140.2	64.0	42.4	40.0	52.0
		Total SCFA	58.1	85.1	9.6	19.5	38.1	49.0
		Succinate	19.0	53.9	53.8	18.8	0.6	1.0
		Lactate	0.8	1.2	N.D.	4.0	0.7	1.7
		Acetate	33.4	45.8	8.8	13.7	19.3	28.5
		Propionate	22.2	30.1	N.D.	3.0	7.4	8.5
		n-Butyrate	1.3	3.7	N.D.	N.D.	4.2	4.6
Rectum	Moisture (%)		73.0	77.5	82.6	—[c]	66.0	67.7
	pH		6.2	5.7	5.7	—	7.3	6.8
	Organic acids	Total organic acid	71.9	132.1	81.0	—	42.1	38.9
		Total SCFA	50.1	73.9	23.7	—	40.6	37.8
		Succinate	19.6	54.3	56.3	—	0.2	0.1
		Lactate	2.2	3.9	0.7	—	0.6	0.4
		Acetate	21.3	36.8	19.0	—	21.8	17.7
		Propionate	14.4	27.1	0.9	—	7.5	7.3
		n-Butyrate	10.7	10.0	3.0	—	3.7	4.0

[a] Polymixin B sulfate (3,000,000 units/day) orally administered to pigs, and enrofloxacin (0.6 g/day) injected intramuscularly.

[b] N.D.=Not detected.

[c] No content was available in the rectum of pig #4.

majority of isolates (20 of 28) on the EG plates were assigned as Enterococci. Lactobacilli were the only succinate producers isolated by the BL plates from the PL-treated pigs. In pig #s 1 and 2, 29 Lactobacilli were obtained from among 31 isolates of different colony morphotypes from the BL plates. Among the 29 Lactobacilli, six isolates were identified as succinate producers. These six strains of Lactobacilli produced relatively smaller amounts of succinate. They were identified as *L. crispatus*, *L. bucheneri*, *L. acidophilus*, and

Table 2. Colony forming units (log cfu) on selective and non-selective media of intestinal digesta of pigs administered polymixin B sulfate, enrofloxacin or control.[a]

Digestive tracts	Medium[b] (log cfu/g)	Antimicrobials				Control	
		Polymixin B sulfate		Enrofloxacin			
		Pig 1	Pig 2	Pig 3	Pig 4	Pig 5	Pig 6
Jejuno-ileum	BL	8.6	8.7	<7.3	<7.3	<7.3	8.4
	EG	8.1	<7.3	<7.3	<7.3	<7.3	<7.3
	DHL	<2.3	<2.3	6.0	6.6	5.8	<2.3
	TS	8.1	7.6	<7.3	<7.3	<7.3	<7.3
	LBS	8.9	9.0	<7.3	7.4	<7.3	8.1
	COBA	8.3	8.3	7.3	<7.3	7.6	<7.3
Cecum	BL	9.6	9.9	8.3	9.8	10.3	9.4
	EG	9.0	7.5	7.7	7.6	10.1	8.9
	DHL	<2.3	<2.3	7.4	7.6	10.1	4.6
	TS	9.1	8.8	7.4	9.4	10.3	7.8
	LBS	9.6	9.4	8.2	9.8	9.7	10.2
	COBA	9.0	9.2	7.9	9.5	10.3	9.2
Gyri centripetales	BL	10.2	10.0	8.3	9.9	10.1	10.1
	EG	9.0	7.7	7.8	8.0	10.3	10.0
	DHL	<2.3	<2.3	6.6	7.9	9.5	5.1
	TS	9.1	8.8	7.4	9.0	9.9	8.6
	LBS	9.7	9.5	7.9	9.4	9.6	10.0
	COBA	9.4	9.1	8.0	9.7	9.9	10.1
Gyri centrifugales	BL	9.3	10.2	8.2	10.1	10.3	12.6
	EG	8.6	7.6	7.8	8.2	10.4	10.1
	DHL	<2.3	<2.3	7.6	7.9	9.6	5.8
	TS	8.7	8.8	7.9	9.0	10.3	9.5
	LBS	8.5	9.6	8.0	9.5	8.7	9.9
	COBA	8.8	9.1	7.6	9.5	9.5	11.4
Rectum	BL	7.9	9.1	7.8	—[c]	9.6	11.4
	EG	8.6	7.7	7.7	—	9.1	10.5
	DHL	<2.3	<2.3	7.6	—	8.7	5.6
	TS	8.8	9.1	8.2	—	9.6	9.5
	LBS	8.7	9.3	8.2	—	8.9	9.4
	COBA	8.8	9.0	8.2	—	8.9	10.5

[a] See Table 1.

[b] BL=Blood Liver agar; EG=Eggerth Gagnon agar; DHL=Desoxycholate Hydrogen sulfide Lactose agar; TS=Tryptic Soy agar; LBS=modified Lactobacillus Selected agar; COBA=modified Columbia Agar Base medium.

BL and EG were used as anaerobic non-selective media.

TS was used as an aerobic non-selective medium.

DHL was used as selective medium for *Enterobacteriaceae.*

LBS was used as selective medium for *Lactobacillaceae.*

COBA was used as selective medium for Enterococci. Details, see text.

[c] No content was available from this pig.

L. crispatus by API 50CHL. They were respectively suggested to be *L. crispatus*, *L. lactis*, *L. crispatus*, and *L. gasseri* by the partial sequence of 16S rDNA. The colony morphotype of these isolates occupied from 9.4 to 100% of the total colony developed on the BL-plates from which they were isolated. The bacteria belonging to the *Enterobacteriaceae*, mostly *Escherichia coli*, were identified as the succinate producers

Table 3. Percentage of succinate producers in total colonies developed on BL and EG plates and the ratio of fermentation end products in vitro of the pig cecal digesta administered polymyxin B sulfate, enrofloxacin, or control.

Medium	Antimicrobials		Digestive tracts	Identification of succinate producer		Ratio in total colony (%)[c]	Organic acid products (ratio in concentration)[d]
				API[a]	BLAST[b]		
Eggerth Gagnon (EG) agar	Polymyxin B sulfate	Pig 1	Cecum	ND[e]	ND	ND	ND
			Gyri centripetales	ND	ND	ND	ND
			Gyri centrifugales	ND	ND	ND	ND
		Pig 2	Cecum	ND	ND	ND	ND
			Gyri centripetales	ND	ND	ND	ND
			Gyri centrifugales	ND	ND	ND	ND
	Enrofloxacin	Pig 3	Cecum	*Escherichia coli*	*E. coli*	69.2	ASF (4 : 3 : 3) pl
				Lactobacillus crispatus	*L. crispatus*	13.5	LS (5 : 1) ap
			Gyri centripetales	*E. coli*	*E. coli*	85.5	ASF (4 : 3 : 3) pl
				E. coli	*E. coli*	14.5	ASLF (6 : 5 : 5 : 4)
			Gyri centrifugales	*E. coli*	*E. coli*	50.0	ASF (4 : 3 : 3) pl
		Pig 4	Cecum	*E. coli*	*E. coli*	37.5	ASLF (2 : 2 : 2 : 1)
				E. coli	*E. coli*	37.5	ASF (4 : 3 : 3) pl
			Gyri centripetales	*E. coli*	*E. coli*	71.2	ASF (4 : 3 : 3) pl
			Gyri centrifugales	*E. coli*	*E. coli*	35.1	ASF (4 : 3 : 3) pl
				L. crispatus	*L. crispatus*	11.7	LS (4 : 1) ap
				Salmonella arizonae	*E. coli*	3.2	ASF (5 : 3 : 2) pl
	Control[f]	Pig 5	Cecum	*Serratia liquefaciens*	*Serratia marcescens*	71.4	ASBF (4 : 3 : 1 : 1) pl
			Gyri centripetales	*Serratia liquefaciens*	*S. marcescens*	61.5	ASBF (4 : 3 : 1 : 1) pl
			Gyri centrifugales	*Serratia liquefaciens*	*S. marcescens*	71.4	AS (4 : 3) pfl
				—[g]	*Actinomyces hyovaginalis*	14.3	SAFP (5 : 5 : 4 : 2)

Table 3. Continued.

Medium	Antimicrobials		Digestive tracts	Identification of succinate producer		Ratio in total colony (%)[c]	Organic acid products (ratio in concentration)[d]
				API[a]	BLAST[b]		
Blood Liver (BL) agar	Polymyxin B sulfate	Pig 1	Cecum	*L. crispatus*	*L. crispatus*	40.8	LS (4 : 1) ap
			Gyri centripetales	*L. crispatus*	*L. crispatus*	41.4	LS (4 : 1) ap
			Gyri centrifugales	*L. crispatus*	*L. crispatus*	100.0	LS (4 : 1) ap
		Pig 2	Cecum	*L. bucheneri*	*L. lactis*	15.8	LS (2 : 1) ap
			Gyri centripetales	*L. acidophilus*	*L. crispatus*	9.4	LS (2 : 1) ap
			Gyri centrifugales	*L. crispatus*	*L. gasseri*	66.7	LS (7 : 3) ap
	Enrofloxacin	Pig 3	Cecum	*E. coli*	*E. coli*	24.1	ASF (4 : 3 : 3) pl
				L. acidophilus	*L. reuteri*	1.9	LS (4 : 1) ap
			Gyri centripetales	*E. coli*	*E. coli*	20.0	ASF (4 : 3 : 3) pl
			Gyri centrifugales	*E. coli*	*E. coli*	31.0	ASF (4 : 3 : 3) pl
		Pig 4	Cecum	ND	ND	ND	ND
			Gyri centripetales	*L. bucheneri*	*L. crispatus*	13.6	LS (2 : 1) ap
				L. bucheneri	*L. lindneri*	4.5	LS (3 : 2) ap
			Gyri centrifugales	*L. bucheneri*	*L. lindneri*	11.5	LS (3: 2) ap
				L. crispatus	*L. gasseri*	9.8	LS (7 : 3) ap
	Control	Pig 5	Gyri centripetales	—	*Fusobacterium necrogenes*	10.9	ABPS (10 : 7 : 5 : 4) lvf
			Gyri centrifugales	—	*Bacteroides distasonis*	11.4	SAP (4 : 3 : 2)

[a] API 20E for Enterobacteria; API 50CHL for lactic acid bacteria.

[b] BLAST search results of 16S rDNA partial sequences. The name of the bacteria selected in the BLAST search is the most similar to the presently isolated strain of the bacteria that has an intestinal origin.

[c] The ratio (%) of the colony morphotype of isolates to the total colony number on a plate.

[d] S=succinate; L=lactate; A=acetate; P=propionate; B=*n*-butyrate; V=*n*-valerate; F=formate; lower case letters indicate minor products produced in GAM broth medium.

[e] Not detected.

[f] Isolates from pig #6 were not subjected to the detection of succinate producers due to the high lactate accumulation and larger water content in the cecum and gyri centripetales (see Tables 1 and 2).

[g] Not done, because these isolates were strict anaerobes not fit to API 20E and 50CHL.

Table 4. BLAST search results of 16S rDNA partial sequences cloned into pGEM-T vector of the pig cecal digesta administered polymyxin B sulfate, enrofloxacin, or control.

Antimicrobials	Clone group	Number of clones	BLAST search results	
			Nearest known family	Nearest known species
Polymyxin B sulfate[a] (Pig #2)	2–1	54	Bacillus/Clostridium group; Lactobacillaceae	*Lactobacillus crispatus*
	2–2	1	″	*Lactobacillus crispatus*
	2–3	1	″	*Lactobacillus crispatus*
	2–4	15	B/C group; Clostridiaceae	*Ruminococcus obeum*
	2–5	1	″	*Ruminococcus obeum*
	2–6	1	″	*Ruminococcus obeum*
	2–7	2	″	*Ruminococcus bromii*
	2–8	1	″	*Ruminococcus hydrogenotrophicus*
	2–9	1	″	*Ruminocuccus callidus*
	2–10	1	B/C group; Clostridiaceae	*Clostridium ramosum*
	2–11	1	B/C group; Clostridiaceae	*Eubacterium tortuosum*
	2–12	2	″	*Eubacterium sireaum*
	2–13	1	B/C group; Clostridiaceae	*Termitobacter aceticus*
	2–14	1	″	*Termitobacter aceticus*
	2–15	1	″	*Termitobacter aceticus*
	2–16	1	Actinobacteria; Coriobacteriaceae	*Atopobium oviles*
	2–17	1	″	*Atopobium oviles*
	2–18	2	Fusobacteria	*Fusobacterium prausnitzil*
	2–19	1	Cytophagales; Bacteroidaceae	*Prevotella oralis*
	2–20	1	″	*Prevotella oulora*
	2–21	1	—	Unidentified
Enrofloxacin[b] (Pig #3)	3–1	49	B/C group; Lactobacillaceae	*Lactobacillus crispatus*
	3–2	1	″	*Lactobacillus gasseri*
	3–3	7	″	*Lactobacillus acidophilus*
	3–4	1	″	*Lactobacillus crispatus*
	3–5	1	″	*Lactobacillus crispatus*
	3–6	4	B/C group; Clostridiaceae	*Clostridium* sp.
	3–7	1	″	*Clostridium colinum*
	3–8	1	B/C group; Clostridiaceae	*Eubacterium rectale*
	3–9	2	″	*Eubacterium rectale*
	3–10	1	″	*Eubacterium biforme*
	3–11	3	B/C group; Clostridiaceae	*Roseburia cericola*
	3–12	1	″	*Roseburia cericola*
	3–13	2	B/C group; Clostridiaceae	*Termitobacter aceticus*
	3–14	1	″	*Termitobacter aceticus*
	3–15	3	B/C group; Clostridiaceae	*Ruminococcus bromii*
	3–16	1	″	*Ruminococcus obeum*
	3–17	1	″	*Ruminococcus obeum*
	3–18	1	″	*Ruminococcus obeum*
	3–19	1	″	*Ruminococcus bromii*
	3–20	1	″	*Ruminococcus obeum*
	3–21	1	″	*Ruminococcus obeum*
	3–22	2	B/C group; Clostridiaceae	*Butyrivibrio fibrisolvens*
	3–23	7	B/C group; Sporomusa subbranch	*Anaerovibrio lypolytica*
	3–24	1	″	*Anaerovibrio lypolytica*
	3–25	1	Proteobacteria-γ; Enterobacteriaceae	*Escherichia* sp.

Table 4. Continued.

Antimicrobials	Clone group	Number of clones	BLAST search results	
			Nearest known family	Nearest known species
	3–26	1	Cytophagales; Bacteroidaceae	*Bacteroides stercolis*
	3–27	1	Cytophagales; Bacteroidaceae	*Prevotella veroralis*
	3–28	4	—	Unidentified
Control (Pig #5)	5–1	4	B/C group; Clostridiaceae	*Clostridium* sp.
	5–2	1	″	*Clostridium saccharolyticum*
	5–3	2	″	*Clostridium orbiscindens*
	5–4	1	″	*Clostridium* sp.
	5–5	1	″	*Clostridium methylpentosum*
	5–6	2	″	*Mitsuokella multiacidus*
	5–7	1	″	*Acidaminococcus fermentans*
	5–8	3	″	*Oscillospira guillermondii*
	5–9	4	″	*Veillonella ratti*
	5–10	3	″	*Streptococcus* sp.
	5–11	3	″	*Ruminococcus albus*
	5–12	2	″	*Anaerovorax odorimutans*
	5–13	2	″	*Phascolarctobacterium faecium*
	5–14	1	″	*Bacillus* sp.
	5–15	43	Proteobacteria-γ; Enterobacteriaceae	*Escherichia coli*
	5–16	2	″	*Escherichia coli*
	5–17	2	″	*Escherichia coli*
	5–18	1	″	*Shigella dysenteriae*
	5–19	1	Proteobacteria-γ; Succinivibrionaceae	*Succinivibrio dextrinosolvens*
	5–20	1	Proteobacteria-γ; Pasteurellaceae	*Actinobacillus rossii*
	5–21	3	Cytophagales; Bacteroidaceae	*Bacteroides distasonis*
	5–22	3	″	*Bacteroides merdae*
	5–23	2	″	*Bacteroides vulgatus*
	5–24	1	″	*Bacteroides splanchnicus*
	5–25	1	″	*Bacteroides* sp.
	5–26	4	″	*Prevotella oulora*
	5–27	2	″	*Prevotella bryantii*
	5–28	2	″	*Prevotella veroralis*
	5–29	2	″	*Prevotella oralis*
	5–30	1	″	*Prevotella buccae*
	5–31	1	″	*Prevotella ruminicola*
	5–32	1	″	*Prevotella tannerae*
	5–33	1	″	*Hallella seregens*
	5–34	1	Cytophagales; Flavobacteriaceae	*Riemerella anatipestifer*
	5–35	1	Cytophagales; Sphingobacteriaceae	*Flexibacter canadensis*
	5–36	1	Fusobacteria	*Fusobacterium necrogenes*

[a] Clones were groups by amplified ribosomal DNA restriction analysis using *Hae* III, *Hha* I, and *Sau* 3AI.

[b] Clones were groups by amplified ribosomal DNA restriction analysis using *Hae* I, *Hha* I, *Sau* 3AI, and *Rsa* I.

in the ERFX-treated pigs. In pig #s 3 and 4, 11 isolates from the EG plates produced succinate of which nine were suggested to be *Enterobacteriaceae.* The colony morphotype of these *Enterobacteriaceae* occupied from 3.2 to 85.5% of the total colony developed on the EG plates from which they were isolated. Eight strains were isolated as succinate producers by the BL plates in those pigs. Three of them were suggested to be *E.*

coli both by API 20E and 16S rDNA partial sequences. The remainders were identified as *Lactobacillaceae* that produced a smaller amount of succinate than *Enterobacteriaceae* did.

In the control pig (#5), three isolates of *Enterobacteriaceae* and one of Gram-positive bacteria were identified as succinate producers from among 13 isolates on the EG plates. In addition, two Gram-negative rods were isolated as succinate producers from among 10 isolates on the BL plates.

Sequence analyses on the ARDRA group of cecal microflora (Table 4)

In a PL-treated pig (#2), 21 ARDRA groups were obtained of which 92% belonged to Gram-positive low-GC bacteria. Particularly, 62% of the ARDRA group belonged to *Lactobacillaceae.* In an ERFX-treated pig (#3), 29 ARDRA groups were obtained of which 93% belonged to Gram-positive low-GC bacteria. Among them, 58% of the ARDRA group belonged to *Lactobacillaceae.* On the contrary, 35 ARDRA groups were obtained of which 47% were proteobacteria, 28% were Gram-positive low-GC bacteria, and 24% were in the Cytophaga-Bacteroides (CFB) group in a control pig (#5).

Discussion

A decrease in fecal SCFA concentration has been reported in AAD human subjects (Gustafsson et al., 1998; Högenauer et al., 1998; Hove, 1998; Høverstad et al., 1986; Mørtensen and Clausen, 1995). However, succinate or lactate were not measured in those reports. In this study, the concentration of total SCFA in digesta decreased in AAD-pigs, but the concentration of total organic acid was not necessarily low owing to the presence of succinate and lactate. Succinate and lactate are rarely detected in the hindgut digesta or feces of pigs under normal conditions. The abnormal accumulation of these acids can occur in rats fed on indigestible oligosaccharides or hi-amylose cornstarch (Hoshi et al., 1994; Morita et al., 1998). The succinate and lactate are very slowly absorbed by the epithelial cells (Umesaki et al., 1979), and, therefore, they readily accumulate in the lumen when a limited number of succinate- and lactate-utilizing bacteria are present. The low pH of the cecal digesta was presumably considered as a cause of succinate or lactate accumulation because a low pH could eliminate the acid-utilizing bacteria (Dawson and Allison, 1988). In contrast to SCFA, succinate and lactate do not promote water and electrolyte absorption (Bugaut, 1987; Engelhardt, 1995; Yajima and Sakata, 1987). Furthermore, succinate rather stimulates water secretion from the digestive tract (Shimazaki, 1992). Therefore, the luminal accumulation of these acids increases the moisture content of digesta and may induce diarrhea. The higher moisture content of hindgut digesta in ERFX-treated pigs than that observed in the PL-treated pigs (Table 1) may be related to the higher concentration of succinate and lactate and the lower concentration of SCFA in the digesta of the former pigs.

Substantial numbers (10^6 to 10^7 cfu/g) of Enterobacteria were detected in the ERFX-treated pigs (Table 2), although ERFX has a keen bactericidal effect on *Enterobacteriaceae* (Nakamura, 1994). Most of the colony-morphotypes developed on TS plates were large and irregular, and corresponded to the characteristics of *Enterobacteriaceae* (Ueno et al., 1982). The cfu determined on the DHL plates also indicated the survival of *Enterobacteriaceae* in these pigs (Table 2). Sequence analyses on an ARDRA group of cecal bacteria also indicated the presence of *Enterobacteriaceae* in an ERFX-treated pig (Table 4). The observation agrees with those that indicated the presence of fluoroquinolone-resistant *Escherichia coli* in humans (Deguchi et al., 1990) and livestock (Böttner et al., 1995; Orden et al., 1999; Pohl et al., 1991). The Enterobacteria isolated in this study were mostly identified as *E. coli* by API 20E, and their 16S rDNA partial sequences also indicated a close phylogenetical relationship to *E. coli* (Table 3). Unlike the ERFX-treated pigs, none of the colony was detected on the DHL plates in the PL-treated pigs (Table 2). The disappearance of *Enterobacteriaceae* in these pigs was further substantiated by the results on the TS medium (Table 2). The colonies that developed on TS were small and round, suggesting that they were not Enterobacteria but Enterococci (Ueno et al., 1982). The absence of *Enterobacteriaceae* was further substantiated by the sequence analyses on the ARDRA group of cecal bacteria in a PL-treated pig (Table 4). PL eliminates Gram-negative bacteria such as pseudomonads, *Enterobacteriaceae*, and *Bacteroidaceae* (Uete, 1987). PL is known to be effective against the antimicrobial-resistant Enterobacteria (Deguchi et al., 1993). Accordingly, the disappearance of *Enterobacteriaceae* in PL-treated pigs is reasonable.

Strains of Enterobacteria and Lactobacilli isolated from BL and EG plates were identified as succinate producers in ERFX-treated pigs (Table 3). Lactobacilli were the only bacteria identified as succinate producers in PL-treated pigs (Table 3). Lactobacilli are considered to produce lactate from sugars as the only or major end product with some minor products such as acetate, formate, or ethanol (Stanier et al., 1986). However, this does not necessarily exclude the potential of the wild strains for succinate production. Indeed, succinate was considered as one of the minor products of Lactobacilli grown on PYG medium (Holdeman et al., 1987). *E. coli*-like Enterobacteria, resistant against ERFX, produced a substantial amount of succinate. *E. coli* is known to ferment glucose under anaerobic conditions to acetate, formate, and ethanol with minor amounts of lactate and succinate (Blackwood et al., 1956). Industrial efforts have been made to increase succinate production by *E. coli* using genetically modified strains (Chatterjee et al., 2001; Millard et al., 1996; Stols and Donnelly, 1997). The presently isolated *E. coli*-like bacteria do not produce as much succinate as the engineered strains do; however, they still produced it as a major product (Table 3). In the control pig, *Enterobacteriaceae*, Gram-positive bacteria, and Gram-negative rods were isolated as succinate producers (Table 3). Sequence analyses on an ARDRA group also indicated the presence of a variety of succinate producers such as *Bacteroidaceae*, Fusobacteria, and *Succinivibrionaceae* (Table 4). Although bacteria from only one pig (#5) were subjected to microbiological assays, the results seem to be in accordance with the literature; these anaerobic bacteria such as Gram-negative rods (*Bacteroidaceae*) are the major succinate producers in a pure culture (Stewart and Bryant, 1988). They are common and constitute a major bacterial group in the large intestine of a healthy weaning pig (Robinson et al., 1981). These bacteria were apparently eliminated in AAD-pigs due to susceptibility to the PL or ERFX. Therefore, their contribution to succinate production in AAD-pigs can be ignored. In AAD, antimicrobial-resistant Enterobacteria close to *E. coli* in particular and Lactobacilli were the causative agents of an abnormal succinate accumulation and may be responsible for diarrhea due to the water malabsorption from the hindgut.

Acknowledgments

We thank Ms. Y. Azuma, Mr. T. Hirase, Ms. M. Maekawa and Ms. Y. Sakai for their technical assistance. Some of the experiments were financially supported by the Amano Pharmaceutical Co., Ltd., Nagoya, Japan.

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