

## A Shift from Colon- to Ileum-Predominant Bacteria in Ileal-Pouch Feces Following Total Proctocolectomy

Manami Hinata · Atsushi Kohyama · Hitoshi Ogawa · Sho Haneda · Kazuhiro Watanabe · Hideyuki Suzuki · Chikashi Shibata · Yuji Funayama · Ken-ichi Takahashi · Iwao Sasaki · Kouhei Fukushima

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### Abstract

**Background** We previously investigated fecal flora of the pouch after total proctocolectomy using terminal restriction fragment polymorphism analysis. Although the results of the cluster analysis demonstrated clearly that bacterial populations, including an unidentified bacteria generating a 213-bp PCR fragment, moved toward a colon-like community in the pouch, it did not track changes in the individual species of fecal bacteria.

**Aims** The aim of the present study was to estimate genome copy number of ten bacterial species, clusters, groups, or subgroups (including the bacteria generating 213-bp fragment in the previous study) in feces samples from pouches at various times following ileostomy closure.

**Methods** A total of 117 stool samples were collected from patients with ulcerative colitis after surgery as well as healthy volunteers. We used real-time polymerase chain reaction of the 16S rRNA gene to estimate genome copy numbers for the nine bacterial populations and the bacteria generating 213-bp fragment after identification by DNA sequencing.

**Results** We demonstrated a time-dependent increase in the number of anaerobic and colon-predominant bacteria (such as *Clostridium coccooides*, *C. leptum*, *Bacteroides fragilis* and *Atopobium*) present in proctocolectomy patients after stoma closure. In contrast, numbers of ileum-predominant bacterial species (such as *Lactobacillus* and *Enterococcus faecalis*) declined.

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M. Hinata · K. Fukushima (✉)  
Division of Molecular and Surgical Pathophysiology,  
Graduate School of Medicine, Tohoku University,  
2-1 Seiryomachi, Aoba-ku, Sendai 980-8575, Japan  
e-mail: kouhei@surg1.med.tohoku.ac.jp

A. Kohyama · H. Ogawa · S. Haneda · K. Watanabe ·  
H. Suzuki · C. Shibata · I. Sasaki  
Division of Biological Regulation and Oncology, Department  
of Surgery, Graduate School of Medicine, Tohoku University,  
1-1 Seiryomachi, Aoba-ku, Sendai 980-8574, Japan  
e-mail: akoyama@east.ncc.go.jp

H. Ogawa  
e-mail: hogawa@surg1.med.tohoku.ac.jp

S. Haneda  
e-mail: sho@surg1.med.tohoku.ac.jp

K. Watanabe  
e-mail: k-wata@surg1.med.tohoku.ac.jp

H. Suzuki  
e-mail: hideji@surg1.med.tohoku.ac.jp

C. Shibata  
e-mail: cshibata@surg1.med.tohoku.ac.jp

I. Sasaki  
e-mail: isasaki@surg1.med.tohoku.ac.jp

Y. Funayama · K. Takahashi  
Division of Colorectal and Anal Diseases, Tohoku Rosai  
Hospital, 4-3-21, Dainohara, Aoba-ku, Sendai 981-8563, Japan  
e-mail: yfunayama@tohokuh.rofuku.go.jp

K. Takahashi  
e-mail: kentaka@tohokuh.rofuku.go.jp

K. Fukushima  
Laboratory of Gastrointestinal Tract, Department  
of Regenerative Medicine, Graduate School of Biomedical  
Engineering, Tohoku University, Sendai, Japan

**Conclusions** Our data confirm previous findings that fecal flora in the pouch after total proctocolectomy changes significantly, and further demonstrate that the number and diversity of ileal bacteria decreases while a more colon-like community develops. The present data are essential for the future analysis of pathological conditions in the ileal pouch.

**Keywords** Ulcerative colitis · Restorative proctocolectomy · Pouchitis · Microbiota · Intestines

## Introduction

Total proctocolectomy (TPC) followed by ileal pouch-anal anastomosis (IPAA) is an established surgical treatment for ulcerative colitis (UC) and familial adenomatous polyposis (FAP). Removal of the entire colon enables patients to be cured of disease without construction of a permanent ileostomy. Postoperative adaptive change in the intestine, termed “intestinal adaptation,” is thought to be advantageous for maintaining homeostasis. A previous report involving microarray data derived from isolated epithelial cells described intestinal adaptation as a colon-like transformation of ileal epithelia (i.e. ileal epithelial cells assume a partial colonic phenotype and lose characteristics of the ileal phenotype) [1]. One functional result of adaptation is enhanced water and electrolyte absorption in the remnant small intestine over time, changing stool consistency from watery diarrhea to paste stool.

Beside changes in the water content of stool, intestinal adaptation also involves changes in the composition of fecal microbiota. In a previous study, we used terminal restriction fragment length polymorphism (T-RFLP) analysis to investigate changes in fecal flora at various times after total proctocolectomy, sampling both culturable and nonculturable fecal bacteria [2]. These T-RFLP data led us to define “ileal” DNA fragments as those that were both (a) detected in more than 70 % of ileostomy fecal samples and (b) present at a significantly greater frequency ( $p < 0.05$ ) in ileostomy samples relative to control feces. In contrast, we defined signature “colonic” DNA fragments as those (a) identified in more than 70 % of control samples and (b) present at a significantly greater concentration ( $p < 0.05$ ) in control feces relative to ileostomy samples [2]. T-RFLP patterns derived from ileal-pouch fecal DNA samples showed both a time-dependent decrease in the relative abundance of “ileal” fragments and a time-dependent increase in “colonic” DNA fragments (derived mainly from nonculturable bacteria). One specific 213-bp fragment that decreased in abundance after TPC/IPAA (an “ileal” signature) was sequenced and showed no matches within current sequence databases, suggesting

identification of a novel bacterium that may contribute to adaptation [2].

Traditional methods for determining the composition of intestinal microbiota require time-consuming and laborious culture techniques. While T-RFLP allows a more rapid and complete assessment of bacterial community diversity, this molecular approach does not accurately measure quantities of individual bacteria, and also can “miss” detecting DNAs from very small numbers of target bacteria. For these reasons, additional methods beyond T-RFLP are necessary to accurately detect and quantify populations of major and minor bacteria. While detection of specific-size molecular fragments provides clues to the presence of a bacterial species, procedures are required to compare and confirm the sequence of the fragment relative to the putative origin species. Alternative methods are also required for further analysis of any restriction fragment without a database match.

Real-time polymerase chain reaction (real-time PCR) has been used successfully to quantify small amounts of bacterial DNA from various samples, including feces [3, 4]. In this study, we estimated population sizes of fecal bacteria in UC patients after IPAA using real-time PCR analysis. We applied an extensive set of ten primer pairs designed to target 16S rRNA genes from species, genera, groups, and subgroups that are either (a) common in fecal flora, (b) predominant in the colon, (c) specific to “ileal” bacteria originating in the 213-bp “ileal” fragment after identification, or (d) found in conjunction with pouchitis (mucosal inflammation that develops in UC patients after IPAA).

In each case, detection of the genus *Desulfovibrios* served as a marker for pouchitis-associated flora. In a previous report, Ohge et al. found that release of hydrogen sulphide from feces increased and was significantly higher in patients with active pouchitis within the past year relative to patients in whom pouchitis never occurred or was inactive in the past year [5]. This is thought to be due to bacteria of the genus *Desulfovibrio* that reduce sulphate to sulphide, which is known to be toxic to colonic epithelial cells [6].

## Materials and Methods

### Samples

We obtained 117 stool samples from 69 patients and 20 healthy volunteers (Table 1). Diagnosis of UC was based on a combination of clinical symptoms, endoscopic findings and histological examination. All 69 UC patients underwent TPC followed by IPAA at Tohoku University Hospital, where two- or three-step surgeries were routine.

**Table 1** Summary of samples

Time since ileostomy	No. of samples	No. of patients (male:female)	Mean age (range)
Ileostomy	16	16 (9:7)	38.6 (21–70)
<50 days	12	9 (8:1)	33.1 (23–55)
51–100 days	11	9 (6:3)	39.5 (23–55)
101 days–1 year	14	8 (6:2)	33.6 (17–50)
>1 year	44	27 (15:12)	40.7 (24–62)
Healthy volunteer	20	20 (8:12)	32.8 (18–55)
Total	117	89 (52:37)	37.0 (17–70)

Upon complete closure of the covering loop-ileostomy, the ileal pouch generally becomes functional and stool can be excreted from the patient's anus. Of the 20 healthy volunteers, 19 were not treated with any medications and one took anti-hypertension drugs daily. As depicted in Table 1, we categorized stool samples into one of six groups based on their site and time of origin: Samples were either (1) from end- or loop-ileostomy (16 samples) at 14 or 15 days after the initial surgery, (2) from an ileal pouch within 50 days after stoma closure (12 samples), (3) from an ileal pouch more than 51 days and within 100 days after stoma closure (11 samples), (4) from an ileal pouch more than 101 days and within 1 year after stoma closure (14 samples), (5) from an ileal pouch over 1 year after stoma closure (44 samples), or (6) from healthy volunteer controls (20 samples).

Fourteen of 16 ileostomy samples were obtained from patients who were treated with prednisolone (10–30 mg/day) and antibiotics (cefotiam hydrochloride, 2 g/day) until the fourth post-operative day. All samples in this study originated from patients with an ileal pouch, but who also were free from surgical complications and any clinical symptoms that might indicate pouchitis. In an additional analysis, we compared stool samples taken from each of seven patients at two different times: the first sampling one or more years after ileostomy closure, and the second sampling 1 year later.

Fecal samples from hospital outpatients were collected at each visit following excretion into toilets designed for sample collection. The samples were frozen immediately and then stored at  $-80^{\circ}\text{C}$  until the time of DNA extraction. Fecal samples were obtained under informed consent, and the study was approved by the Ethics Committee of Tohoku University, Graduate School of Medicine.

#### DNA Extraction from Fecal Samples

Stool DNA was extracted using QIAamp DNA Stool Mini Kit (QIAGEN Co., Tokyo, Japan) according to the manufacturer's protocol. The DNA concentration of each sample was estimated from its spectrophotometric absorbance of 260-nm wavelength light.

#### Preliminary PCR Amplification and Cloning of Control Plasmids for Real-Time PCR

PCR with bacteria-specific primer pairs (Table 2) was used to amplify 16S rRNA fragments from target bacteria and control plasmid standards for real-time PCR. Each reaction mixture (12.5  $\mu\text{l}$ ) included 10 ng DNA,  $1\times$  buffer supplied by the manufacturer, 0.2 mM NTP, 0.6  $\mu\text{M}$  up- and downstream primers, and 0.3 unit TaKaRa Ex Taq (Takara Shuzo Co., Ltd., Otsu, Japan). DNA was initially denatured at  $94^{\circ}\text{C}$  for 2 min, and then proceeded through 35 thermocycles of  $94^{\circ}\text{C}$  for 30 s, 50 or  $55^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 30 s, with a final extension period at  $72^{\circ}\text{C}$  for 5 min. Resulting amplified products were resolved using gel electrophoresis and stained with ethidium bromide. Target DNAs of the expected size were cloned into pCR 2.1-TOPO (TOPO TA Cloning Kit, Invitrogen Co., Tokyo, Japan) according to the manufacturer's protocol. Plasmids from bacterial clones containing target DNA were purified using miniprep DNA Purification Kit (Takara Shuzo Co., Ltd.) and sequenced using BigDye Terminator v3.1 Cycle Sequence Kit (Applied Biosystems) during 25 thermocycles at  $96^{\circ}\text{C}$  for 10 s,  $50^{\circ}\text{C}$  for 5 s, and  $60^{\circ}\text{C}$  for 4 min. The resulting products were purified using BigDye XTerminator and analyzed using an ABI310 sequencer (Applied Biosystems Japan, Tokyo, Japan).

#### Quantification of Bacterial DNAs Using Real-Time PCR

Duplicate samples of 10-ng bacterial DNA were used for 16S rRNA gene quantification with QuantiTect SYBR Green PCR Kit (Qiagen K. K., Tokyo, Japan) (except for *Lactobacillus* species) and ABI 7500 Real-time PCR system (Applied Biosystems, Japan) according to the manufacturer's protocol. The amplification program consisted of one cycle of  $50^{\circ}\text{C}$  for 2 min, one cycle of  $95^{\circ}\text{C}$  for 10 min, 45 cycles of  $94^{\circ}\text{C}$  for 15 s,  $55^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 1 min. When *Enterococcus* species or *Enterococcus faecalis* was measured, the annealing temperature was 61 or  $57^{\circ}\text{C}$ , respectively. Quantification in duplicate of *Lactobacillus* species was performed using EagleTaq

**Table 2** Primer and probe sets used in this study

Target bacterial group	Primer sequence	Amplicon size (bp)	Reference
<i>All eubacteria</i>	TCCTACGGGAGGCAGCAGT GGACTACCAGGGTATCTATCCTGTT	466	[7]
<i>Clostridium coccooides</i> group	AAATGACGGTACCTGACTAA CTTTGAGTTTCATTCTTGCGAA	440	[3]
<i>Clostridium leptum</i> subgroup	GCACAAGCAGTGGAGT CTTCTCCGTTTTGTCAA	239	[3]
<i>Bacteroides fragilis</i> group	ATAGCCTTTCGAAAGRAAGAT CCAGTATCAACTGCAATTTTA	495	[3]
<i>Bifidobacterium</i>	CTCCTGGAAACGGGTGG GGTGTCTTCCCGATATCTACA	550	[3]
<i>Atopobium</i> cluster	GGGTTGAGAGACCGACC CGGRGCTTCTTCTGCAGG	190	[3]
<i>Prevotella</i>	CACRGTAACGATGGATGCC GGTCGGGTTGCAGACC	513	[3]
<i>Desulfovibrios</i>	CCGTAGATATCTGGAGGAACATCA ACATCTAGCATCCATCGTTTACAGC	135	[8]
<i>Lactobacillus</i> species	TACATYCCAACHCCAGAACG AAGCAACAGTACCACGACCA Probe: FAM- AAGCAACAGTACCACGACCA- TAMRA	71	[9]
<i>Enterococcus</i> species	CCCTTATTGTTAGTTGCCATCATT ACTCGTTGTACTTCCCATTGT	144	[4]
<i>Enterococcus faecalis</i>	AACCTACCCATCAGAGGG GACGTTCAAGTTACTAACG	358	[10]

Master Mix with ROX (Roche Diagnostics Co., Tokyo, Japan). The amplification program consisted of one cycle of 50 °C for 2 min, one cycle of 95 °C for 10 min, 45 cycles of 95 °C for 15 s, 60 °C for 1 min, and 72 °C for 1 min. Copy number per microgram stool DNA was calculated relative to plasmid DNA controls, and median and percentile values in each group were evaluated.

#### Isolation and Identification of the 213-bp Fragment

We used the AccuPrime Taq DNA polymerase system (Invitrogen) to amplify the 213-bp fragment from 10 ng DNA derived from stool samples from the ileostomy or the pouch within 50 days after stoma closure with 16S rRNA gene primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (the same as the primers used in the previous T-RFLP analysis) [2]. DNA was initially denatured at 94 °C for 2 min, and then passed through 37 cycles of 94 °C for 30 s, 50 °C for 30 s, and 68 °C for 90 s. Amplification products were purified using Wizard PCR Preps DNA purification system (Promega Co., Tokyo, Japan) and digested with *Cfo* I (Roche Diagnostics Co.), an isozyme of *Hha* I that yielded

in the 213-bp ileal fragment in the previous report. Digested DNA was then electrophoresed on a 6 % acrylamide gel and visualized using SYBR Green I (FMC, Rockland, USA). Gel sections corresponding to fragments that span the 213-bp-size region of the gel were visualized with UV and excised. DNA was recovered from the gel, precipitated with ethanol, and self-ligated using DNA ligase (Roche Diagnostics Co.). Subsequent product (equivalent to 1 µl of the original PCR reaction) was used as a template and re-amplified using only the 27F primer through 37 cycles of 94 °C for 20 s, 60 °C for 20 s, and 68 °C for 20 s with a final extension period at 68 °C for 5 min. The resulting amplification products (before and after *Cfo* I digestion) were resolved by polyacrylamide electrophoresis, cloned and sequenced; the resulting sequence served as the probe for DNA homology searches within the BLAST network service (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

#### Statistical Analysis

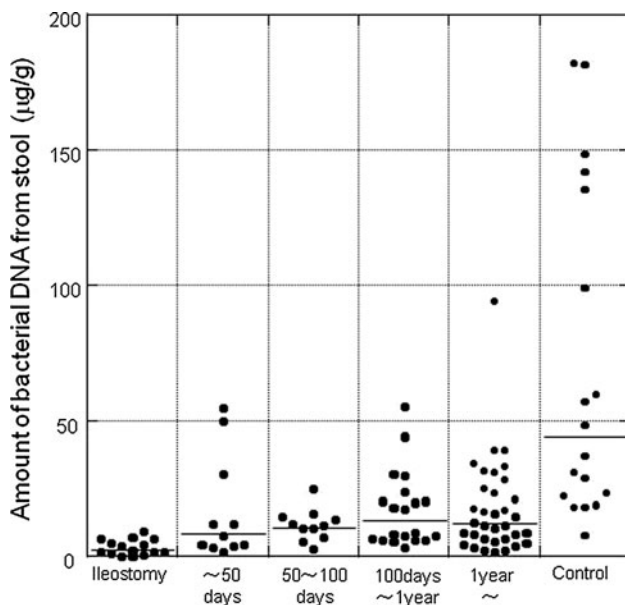
Relative copy number values estimated with real-time PCR are presented as median and percentile values within each

group. A Kruskal-Wallis rank test was used to determine if there was a significant correlation among the six sample groups. A Mann-Whitney test was used to compare two independent groups. A Wilcoxon signed-ranks test was used to compare paired groups, with significance at  $p < 0.01$ .

## Results

### Recovery of Fecal DNA

Adequate quantity and quality of DNA samples were obtained from both firm and watery stool samples (Fig. 1). Median concentrations of sample DNA included 2.3  $\mu\text{g/g}$  stool in the ileostomy group, 7.7  $\mu\text{g/g}$  in patients with an ileal pouch within 50 days of ileostomy closure, 11.9  $\mu\text{g/g}$  from 50 to 100 days, 13.3  $\mu\text{g/g}$  from 100 days to 1 year, and 12.4  $\mu\text{g/g}$  in patients with an established ileal pouch (more than 1 year since ileostomy closure). In contrast, DNA concentrations from control group samples averaged 42.9  $\mu\text{g/g}$ . Because the amount of DNA recovered per gram of wet stool varied and was not necessarily proportional to stool weight, we estimated DNA copy number (number of 16S rRNA genes per  $\mu\text{g}$  stool DNA) median and range from the 25th and 75th percentile values within each group. Control plasmids for real-time PCR were obtained by amplification of bacterial 16S rRNA genes with the primer pairs listed in Table 2, followed by cloning of the resulting fragments into plasmid vectors and verification of sequence (data not shown).



**Fig. 1** DNA recovery from stool samples ( $\mu\text{g}$  DNA per gram wet stools) in the six study groups

### Time-Dependent Changes in 16S rRNA Gene Copy Number in Feces

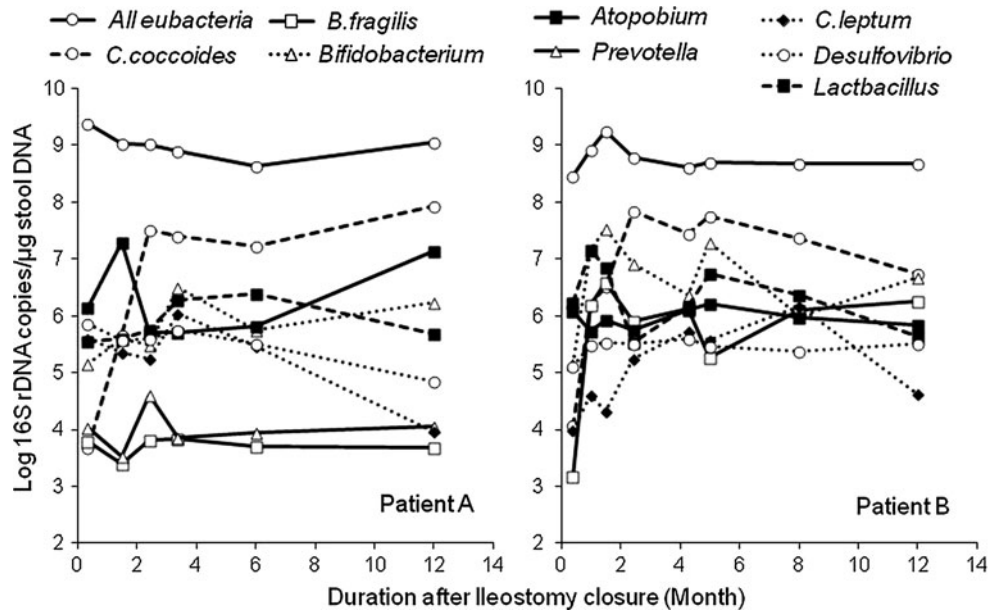
In preliminary experiments, we estimated 16S rRNA gene copy number from stool samples obtained from two patients (A and B) at various time intervals since ileostomy closure (Fig. 2). While the total amount of all eubacteria (expressed by 16S rRNA gene copy number per  $\mu\text{g}$  stool DNA) was stable, changes in the relative copy numbers of different bacteria were observed within 2–3 months after ileostomy closure. Early changes included an increase in *Atopobium*, *C. coccoiodes*, and *Bifidobacterium* in patient A, and an increase in *C. coccoiodes*, *B. fragilis*, and *Bifidobacterium* in patient B. The amount of DNA detected for each strain correlated positively with the number of days since ileostomy closure. We observed the greatest change within approximately 50 days, less change between 50 and 100 days, and almost no change between 100 days and 1 year. Based on these preliminary results, we classified pouch samples in the larger group study into four groups based on the time since ileostomy closure (<50, 50–100 days, 100 days–1 year, >1 year).

Significant changes in relative copy number over time (using a Kruskal-Wallis rank test) were detected in the *C. coccoiodes* group, *C. leptum* subgroup, *B. fragilis* group, *Atopobium* cluster, *Lactobacillus* species, and not in *Bifidobacterium*, *Prevotella*, and *Desulfovibrios* (Fig. 3, Table 3). Anaerobic bacteria in the *C. coccoiodes* group, *C. leptum* subgroup, *B. fragilis* group, and *Atopobium* cluster were less abundant in samples just after ileostomy closure and showed a time-dependent increase in copy number; however, even in pouches more than 1 year old, the levels of these anaerobes never exceeded those from healthy controls. Levels of specific bacteria, particularly *C. coccoiodes* and *B. fragilis* groups, were more consistent in control group samples than in post-surgical samples. In contrast, the quantity of *Lactobacillus* species was most abundant in ileostomy samples and then decreased after ileostomy closure to levels comparable to controls. We found no significant difference in bacteria levels between samples from pouches at 1 year after ileostomy closure and those after an additional year (Table 4).

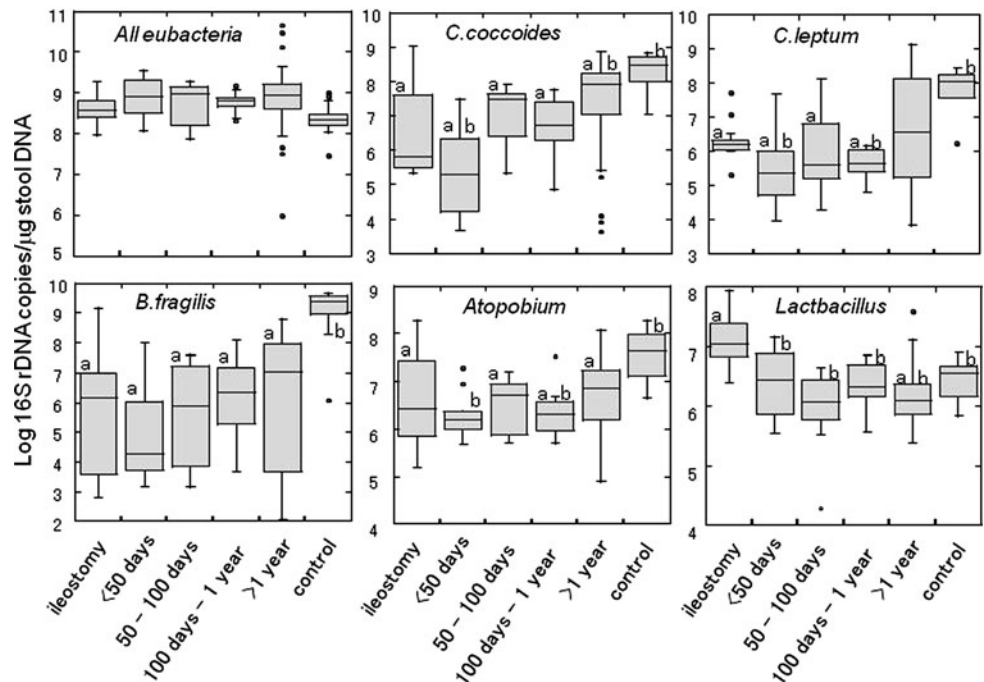
### Identification and Quantification of the 213-bp “Ileal” Fragment

In a previous study, we detected a 213-bp PCR fragment preferentially in samples taken at the time of ileostomy and from early-stage pouches [2]. In this study, we PCR-amplified 213-bp fragments of DNA from one patient’s stool samples taken at the time of ileostomy closure (Fig. 4a, lane 1), and 14 days after ileostomy closure (Lane 2). We used electroelution to recover these fragments and then

**Fig. 2** Progressive changes in copy numbers of fecal bacteria species and groups measured at increasing time points since the time of ileostomy closure in two patients (A and B). Plotted points mark the logarithmic number of 16S rRNA gene copies per  $\mu\text{g}$  stool DNA relative to the number of months since ileostomy closure



**Fig. 3** Estimated logarithmic number of 16S rRNA gene copies per  $\mu\text{g}$  stool DNA. The copy number of fecal bacteria types in stool samples from ileostomies, pouches within 50 days of closure, pouches from 50 to 100 days, pouches from 100 days to 1 year after ileostomy closure, and controls are illustrated. Shaded bars and lines represent 25/75 % percentile and median values, respectively. Error bars indicate 10/90 % percentile values. *p*-values were calculated by Kruskal–Wallis rank test, with significance at  $p < 0.01$ . *a* and *b* indicate a statistically significant difference ( $p < 0.01$  by Mann–Whitney–Wilcoxon test) in the copy number of samples relative to the control (*a*) or ileostomy group (*b*), respectively



self-ligated them for use as template in further amplifications. Among the products re-amplified with only the 27F primer, bands of approximately 430-bp were detected in both samples (Fig. 4b). Similarly, PCR amplified products from both templates yielded two restriction fragments (approximately 200- and 220-bp long) when digested with *Cfo I* (Fig. 4c). Among the 11 plasmid clones recovered with these DNA fragments, insert sizes were 107, 192, 202, 218, and 238 bp, respectively. The 218-bp DNA fragment encoded a *Cfo I* restriction site at its 3'-end and its sequence was strictly

homologous to a partial sequence of the *Enterococcus faecalis* 16S ribosomal RNA gene (AB530699.1 etc.).

Upon identifying the origin of the 213-bp fragment, we measured the amount of *Enterococcus* species and *Enterococcus faecalis* using specific 16S ribosomal RNA gene primers (Fig. 5), and found that *Enterococcus* species and *Enterococcus faecalis* were most abundant in samples from pouches within 50 days after ileostomy closure, and least abundant in the control group. When we compared levels of *Enterococcal* bacteria between samples from

**Table 3** Changes in relative number over time

Bacterial group	Ileostomy	<50 days	50–100 days	100 days–1 year	>1 year	Healthy volunteer	<i>p</i> -value
<i>Bifidobacterium</i>	7.08	6.22	5.91	6.43	6.49	6.60	<i>p</i> = 0.0785
	6.26/7.83	5.16/7.11	5.72/6.20	5.99/6.74	5.73/7.24	6.00/8.26	
<i>Prevotella</i>	4.57	3.96 <sup>a</sup>	3.86	3.90	4.55 <sup>b</sup>	4.42	<i>p</i> = 0.3971
	3.93/5.81	3.58/4.91	3.67/5.10	3.64/4.28	3.78/5.55	4.03/4.86	
<i>Desulfovibrios</i>	5.64	5.53	5.50	5.61	5.61	5.80	<i>p</i> = 0.3131
	5.58/5.81	5.41/4.91	5.49/5.62	5.49/5.72	5.42/5.73	5.65/6.01	

Values are the median logarithmic number of rDNA copies per  $\mu\text{g}$  stool DNA, and 25/75 % percentile values. Samples yielding values below the sensitivity threshold were excluded from the statistical analysis (<sup>a</sup> 2 samples, <sup>b</sup> 14 samples excluded). *p*-values were calculated by Kruskal–Wallis rank test

**Table 4** Bacteria levels long after ileostomy closure

Bacterial group	Time point over 1 year	Time point plus an additional 1 year	<i>p</i> -value
<i>All Eubacteria</i>	9.15	8.86	<i>p</i> = 0.1094
	10.34/8.62	8.65/9.05	
<i>C. coccoides</i>	8.23	8.07	<i>p</i> = 0.4375
	8.88/6.71	7.99/8.15	
<i>B. fragilis</i>	6.87	7.69	<i>p</i> = 0.5781
	8.04/5.60	6.66/8.25	
<i>Bifidobacterium</i>	7.02	7.01	<i>p</i> = 0.6875
	7.84/5.41	6.52/7.50	
<i>Lactobacillus</i>	6.20	6.06	<i>p</i> = 0.6721
	6.52/5.86	5.94/6.24	
<i>Atopobium</i>	6.75	7.11	<i>p</i> = 0.4687
	7.45/6.00	6.23/7.61	
<i>Prevotella</i>	5.12	5.37	<i>p</i> = 0.375
	5.88/4.50	4.96/5.71	
<i>C. leptum</i>	8.17	7.18	<i>p</i> = 0.2188
	9.15/6.21	6.37/8.37	
<i>Desulfovibrio</i>	5.86	5.58	<i>p</i> = 0.9489
	8.61/4.61	5.24/5.73	

Values are the median logarithmic number of rDNA copies per  $\mu\text{g}$  stool DNA, and 25/75 % percentile values. *p*-values were calculated by Wilcoxon signed-ranks test

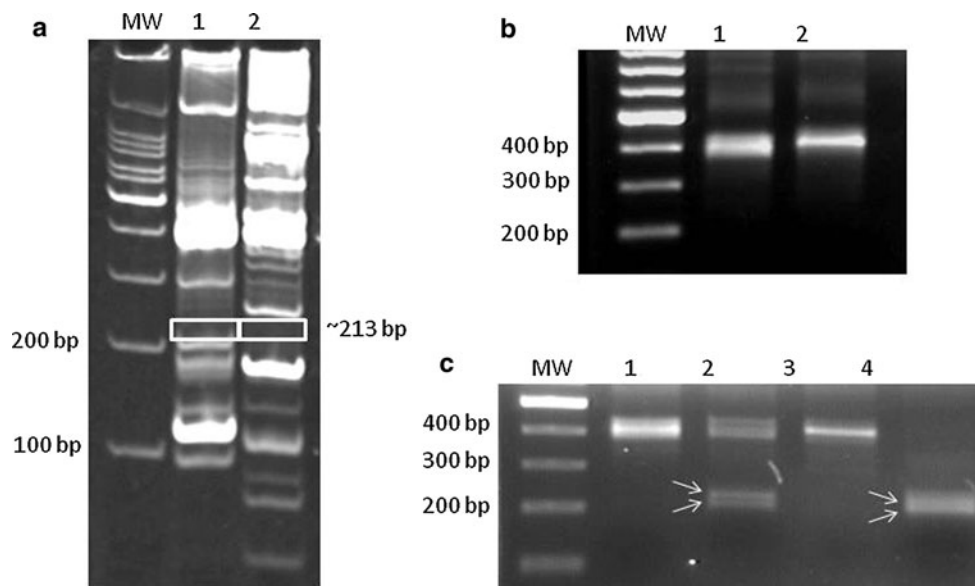
pouches of various times since closure, we found that there were fewer of these bacteria as the time since ileostomy closure increased. In addition, there was a significantly higher frequency of fecal samples with low-detectability levels of *Enterococcus faecalis* among patients 1 year after ileostomy closure relative to control subjects. These findings suggest that *Enterococcus* species predominate transiently during the first 50 days of post-surgical adaptation.

## Discussion

The human gastrointestinal tract is postulated to harbor a complex community of over  $10^{14}$  microorganisms. This community has the power to influence gut physiology and

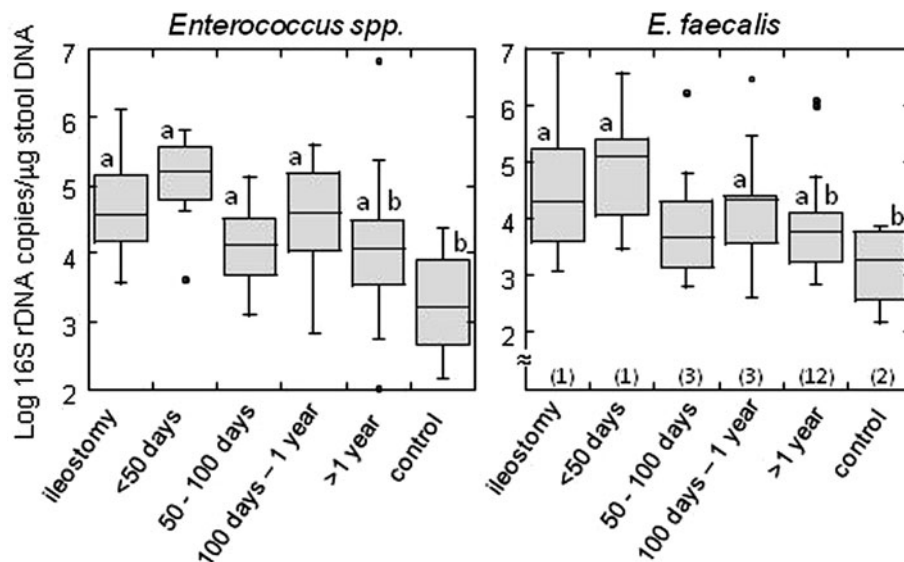
health via a number of activities, including fermentation of dietary components, production of short-chain fatty acids, modulation of the immune system, transformation of bile acids, production of vitamins and health-protective substances, and provision of a barrier against pathogenic bacteria [11]. Gut flora also affects host immunity and may be an important contributor to altered immune responses after total proctocolectomy.

Pouchitis is a non-specific mucosal inflammation in a pouch. It has been suggested to be the most frequent complication with a pelvic pouch, as well as with a Kock continent ileostomy at late stage [12]. The fact that antibiotics, including metronidazole and ciprofloxacin, are effective in treating pouchitis indicates a direct or indirect link of pouch microbiota to this mucosal inflammation with unknown etiology. Floral changes in the ileal pouch may



**Fig. 4** **a** Gel electrophoresis of PCR-amplified samples using the universal 16S rDNA primers. *Lane 1*: ileostomy sample, *Lane 2*: pouch sample 14 days after ileostomy closure. *White rectangles* mark boundaries of the gel slab that was excised for DNA extraction. **b** Extracted DNAs from (**a**) were self-ligated, PCR-amplified with only the 27F primer of the universal 16S rDNA primer set, and resolved by gel electrophoresis. *Lane 1*: ileostomy sample;

*Lane 2*: a sample from the pouch at 14 days after ileostomy closure. **c** Gel electrophoresis before (*Lanes 1, 3*) and after (*Lanes 2, 4*) treatment of PCR products in (**b**) with *Cfo* I. *Lanes 1* and *2*: ileostomy sample; *Lanes 3* and *4*: sample from the pouch at 14 days after ileostomy closure. *Arrows* indicate two molecules with slightly different mobilities



**Fig. 5** Estimated copy number of the 16S rDNA gene from *Enterococcus* species (*left panel*) and *E. faecalis* (*right panel*) in samples from ileostomies, pouches within 50 days, pouches from 50 to 100 days, pouches from 100 days to 1 year, pouches over 1 year after ileostomy closure, and control stool samples. The y-axis represents the logarithmic number of 16S rDNA copies per  $\mu\text{g}$  stool DNA. *Shaded bars* and *lines* represent 25/75 % percentile and median values, respectively. *Error bars* indicate 10/90 % percentile values.

*p*-values were calculated by Kruskal–Wallis rank test, with significance at  $p < 0.01$ . *a* and *b* indicate a statistically significant difference ( $p < 0.01$  by Mann–Whitney–Wilcoxon test) in the copy number of samples relative to the control (*a*) or ileostomy group (*b*), respectively. For *E. faecalis* data, the number with a parenthesis at the bottom of the chart mark indicate the number of samples that yielded DNA levels below the threshold sensitivity and were not analyzed

be associated with triggering and/or amplifying mucosal inflammation of the pouch. For these reasons, evaluation of the pouch flora is essential.

Since the water content in stool after total proctocolectomy is generally very high and easily affected by meals or enteritis, DNA recovery per gram wet stool is highly



variable and not necessarily proportional to wet weight of stool samples. Therefore, we compared flora density and diversity using real-time-PCR of 16S rRNA genes to estimate the numbers of bacteria present per microgram stool DNA. Data from this study expand our understanding of the time-dependent progression in fecal flora (from “ileal” to “colonic”) after ileostomy closure [2] by providing explicit values for specific species, genera, group, or subgroup densities in samples collected at various times since ileostomy closure. Overall, we found increased numbers of colon-predominant anaerobic bacteriae and decreased numbers of ileum-predominant species. In addition, the relative numbers of these bacteria were observed to stabilize within 1 year after ileostomy closure.

Using conventional culture techniques, Nasmyth et al. compared fecal flora from 11 pouches and 12 ileostomies and found a significant increase in numbers of anaerobic bacteria (such as *Bacteroides* and *Bifidobacteria*) in pouch-derived samples [13]. Smith et al. also investigated individual strains of fecal anaerobic bacteria in seven ileostomies, nine ileal pouches with UC, and five ileal pouches with FAP using conventional culture methods [14] and found that the ratio of strict to facultative anaerobes within the UC pouch was maintained between sample groups. In this study, we investigated changes in common fecal bacteria using molecular techniques that permit analyses of bacterial DNAs extracted from both cultivable and uncultivable bacteria. Molecular data demonstrate a similar increase in anaerobic bacteria such as *C. coccides* group, *C. leptum* subgroup, *B. fragilis* group, and *Atopobium* cluster with time. Although our methods detected major populations of fecal bacteria, it is certainly possible that low-abundance taxa not detected using quantitative PCR may also contribute significantly to changes in microbial population structures following total proctocolectomy.

There are several possible limitations to the methodologies used in this study. First, efficiencies of DNA extraction in gram-positive versus -negative bacteria can differ due to different cell-wall components. Second, DNAs from living bacteria and dead bacteria that are sufficiently intact for amplification will both yield amplification products. Finally, the fact that patients typically receive antibiotics until the fourth post-operative day must be taken into consideration in that this may bias the bacterial composition of ileostomy samples. Despite these limitations, results from conventional culture and molecular studies are similar and indicate that increased numbers of anaerobic bacteria are highly relevant in pouches after total proctocolectomy, and that quantification of fecal bacteria using real-time PCR is appropriate and useful.

Although the mechanism for altering bacterial composition during intestinal adaptation is speculative, a “colonic transformation” includes both of the acquisition of colonic

flora phenotypes as well as the loss of small intestinal phenotypes after total proctocolectomy. Postoperative changes in the host include activation of the rennin-angiotensin-aldosterone system, altered phenotype of the remnant small intestine epithelia, stasis due to pouch formation, and possible alterations in mucosal immune response [1, 15]. Consistent with the concept of “colonic transformation,” we observed that the abundance of *Lactobacillus* and *Enterococcus* species that predominate in the small intestine decreases progressively after ileostomy closure.

The changes that occur during intestinal adaptation also depend on other variables that affect bacterial composition after total proctocolectomy, including original flora before surgery, meal components, hygiene environment, and genetic background of the host. In order to study variation in fecal flora, repeated sampling from the same individuals over a course of time is necessary. We measured bacteria in feces because it is feasible to collect repeated samples from both healthy individuals and patients. While both mucosa-associated microbiota and those attached to epithelial cells may also have a strong impact on gut physiology and pathology after total proctocolectomy [16–18], previous DNA-based approaches have reported a similarity index of approximately 85 % between fecal microbiota and mucosa-associated microbiota [19], suggesting that analysis of fecal bacteria is a relevant measure. Regardless of the variation that is observed in the bacterial composition of feces between individuals, increased numbers of anaerobic bacteria and decreased numbers of small-intestine bacteria appear to be consistent and relevant postoperative phenomena following total proctocolectomy.

In a previous study, an approximately 213-bp terminal restriction fragment was detected in more than 70 % of ileostomy samples, a significantly greater frequency ( $p < 0.05$ ) than in controls [2]. Because this predominantly “ileal” fragment exhibited time-dependent decreases in detection after ileostomy closure, we hypothesized decreased numbers of “ileal” bacteria and increased numbers of “colonic” bacteria were hallmarks of the mucosal immune response after ileostomy closure. In this study, we continued our analysis by successfully cloning a 218-bp fragment with sequence identical to a partial sequence of the *Enterococcus faecalis* 16S ribosomal RNA gene. With only a five base-pair difference between the expected and identified fragments, and since *Enterococcus faecalis* decreased in a time-dependent fashion after ileostomy closure, we considered the 218-bp fragment to be our target. The five-base misreads in the previous T-RFLP study might have led to our previous failure in hitting in the database.

Inflammation of the ileal pouch, or “pouchitis,” has been hypothesized to be linked with the presence of large numbers of sulfate-reducing bacteria of the genus

*Desulfovibrio* in stools [5]. Sulfide, a product of sulfate reduction, has been shown to inhibit butyrate metabolism in colonocytes and to induce epithelial abnormalities such as hyperproliferation [20]. In a comparison of cultures of sulfate-reducing bacteria derived from UC pouches versus familial adenomatous polyposis (FAP) pouches, Duffys et al. found sulfate-reducing bacteria in 80 % of UC pouches, but none in FAP pouches [21]. In the present study, we found that the amount of *Desulfovibrios* throughout the postoperative term was both stable and comparable to that of control cases. Although samples in this study were all obtained from pouches without inflammation, it is essential to monitor *Desulfovibrios* levels before and after therapies when patients are at risk for pouchitis. In future analyses, samples from patients with familial adenomatous polyposis could provide additional information about pouchitis development.

In conclusion, our molecular quantification of fecal bacteria clearly demonstrates a time-dependent shift in fecal flora from ileal to colonic bacteria after total proctocolectomy. Although the study is still descriptive, postoperative alteration of fecal flora will be one essential element in future investigations of pouchitis development.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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