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Scientific Research

Diversity of Microflora in Colonic Mucus from Severe Ulcerative Colitis Patients Analyzed by Terminal Restriction Fragment Length Polymorphism and Clone Libraries of Bacterial 16S rRNA Gene Sequences

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Abstract

Although the gut microflora is thought to be an essential factor in the development of ulcerative colitis (UC), the entire gut microflora occurring in UC remains unknown. Most studies use feces to represent the microflora distribution; however, here we analyzed the bacterial diversity in colonic mucus from UC patients receiving colectomy surgery and control patients. The diversity of microflora was investigated using a combination of terminal restriction fragment length polymorphism (T-RFLP) and clone library analyses of the 16S rRNA gene sequences. In the T-RFLP analysis, the number of terminal restriction fragments (T-RFs) decreased significantly in UC patients when compared to control samples. Also in the clone library analysis, the number of operational taxonomic units (OTU) and the Shannon diversity index were reduced significantly in UC patients. These molecular analyses reveal an overall dysbiosis in UC patients. No specific pathogen was found, and a strong negative correlation in relative abundance of bacterial populations was

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observed between the phyla Bacteroidetes and Firmicutes in the UC patients. This is the first report showing a significant correlation between these two phyla, which may be important characteristics in the pathogenesis of UC.

Keywords

Ulcerative Colitis, Microflora, Terminal Restriction Fragment Length Polymorphism, 16*S* rRNA Gene Clone Library

1. Introduction

Ulcerative colitis (UC) is an intractable disease where erosion and ulcers occur in the colon with chronic inflammation. Although medical therapy, e.g. antibiotics and 5-aminosalicylic acid (5-ASA) treatments, can suppress the inflammation of UC, colectomy surgery is required in some cases. The etiology of UC is associated with genetic and environmental factors causing immunological disorders and chronic inflammation; however, the mechanism remains unclear. Genetic factors, such as *ECM1* [1] and *HLA-DR* [2] were reported to be strongly associated with an excessive immune response in UC. However, because the increase in UC patients in Asian nations began when the life style changed to western with a western dietary style, the change in the microflora of the intestine was considered to be an important environmental factor in the pathogenesis of UC [3] [4].

The commensal gut flora contributes to the control of the immune system by promoting the growth of regulatory T cells that maintain the homeostasis in the gut [5]. Without the commensal flora in the intestine, immune-associated tissues, such as Peyer's patches, mesenteric lymph nodes [6], and isolated lymphoid follicles [7] remain immature, indicating the normal flora in the gut influences the growth of the immune systems. Moreover, immunodeficient mouse models, such as the interleukin (IL)-2 knockout mouse [8], IL-10 knockout mouse [9], and TCR-α knockout mouse [10] show similar inflammatory symptoms to human inflammatory bowel disease (IBD). The symptoms disappeared when the mice were bred in sterile conditions. This supports the hypothesis that the presence of microflora is necessary for development of UC symptoms.

Due to the importance of the microflora in UC, there are a large number of reports that investigate gut microflora in UC patients using feces or biopsy by culture-dependent methods and culture-independent methods [11]-[15]. Some bacteria, such as *Bacteroides* [16], pathogenic *Escherichia coli* [17], and *Fusobacterium varium* [18] have been implicated in the pathogenesis of UC. A positive correlation between the expressed proportion of sulfomucin and sulfate-reducing bacteria was observed in the gut microflora of healthy subjects [19], whereas sulfomucin was decreased in UC [20]. However, consensus has not been achieved where severe UC patients undergoing colectomy surgery have lower quality of life and their gut microflora has not been investigated. Therefore, the purpose of this study was to investigate the diversity of gut microflora from the colonic mucus collected from UC patients receiving colectomy surgery. We applied culture-independent molecular approaches to combine terminal restriction fragment length polymorphism (T-RFLP) and clone library analyses of the 16S rRNA gene sequences. T-RFLP is a molecular technique that provides rapid comparison of community structures and diversity of complex bacterial flora and has been applied to characterize the endodontic microflora [21], soil bacterial communities [22], gut microflora [23], and bacterial communities in other environments [24]. The clone analysis of 16S rRNA gene sequences is a powerful tool for determination of exact species of bacteria, and the fragments in the T-RFLP analysis can be assigned to corresponding bacterial species. The diversity of microflora and the correlation between different taxa of bacteria were also investigated in this study. Using analysis of the gut microflora in UC patients (especially in serious cases), our observations may contribute to explain the pathogenesis of UC, and provide new therapeutic strategies for UC.

2. Materials and Methods

2.1. Preparation of Mucus Samples for DNA Extraction

This study was approved by the ethics committee of Tohoku University Graduate School of Medicine. Before we began this study, informed consent was obtained from all patients. The patients were not treated with anti-

biotics. The human colonic mucus was prepared from four UC patients who were diagnosed with UC based on clinical symptoms and required colectomy surgery at Tohoku University Hospital. As a control, the mucus from the normal portions of six colorectal cancer patients was used (**Table 1**). The mucus was collected from colon specimens by scraping and stored at −80˚C until used.

2.2. DNA Extraction and PCR Amplification of the 16*S* rRNA Gene

DNA was extracted from colonic mucus using MORA-EXTRACT (Kyokuto Pharmaceutical Industrial Co., Japan) according to the manufacturer's instructions. The total DNA was dissolved in 100 µl of TE buffer (pH 8.0, Nippon Gene Co., Japan) and stored at −30˚C until used. The 16S rRNA gene was amplified using 35F (5'-CCTGGCTCAGGATGAACG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The primers with or without a fluorescent label were used for T-RFLP analysis and clone library analysis, respectively. Primer 35F was labeled at 5' end with 6'-carboxyfluorescein (6-FAM). Amplification reactions were performed in a total volume of 50 µl containing dissolved DNA (<100 ng), 0.25 µl TaKaRa Ex Taq (Takara Shuzo, Japan), 5 µl 10× Ex Taq buffer, 4 µl dNTP mixture (each 2.5 mM), and 10 pmol each primer. The 16S rRNA gene was amplified using 10 min at 95˚C for initial denaturation, followed by 30 cycles: 95˚C for 30 s; 50˚C for 30 s; 72˚C for 1.5 min; and a final extension of 10 min at 72˚C. The products were electrophoresed in 1% agarose using 1× TAE as the reservoir buffer. After electrophoresis, the gel was visualized using ethidium bromide staining. Further, PCR products were purified with the QIAquick PCR purification kit (Qiagen, Germany).

2.3. T-RFLP Analysis

T-RFLP analysis was performed using the method from Sakamoto *et al*. [21]. In brief, purified PCR products were digested with *Hha*I, *Msp*I, *Alu*I, *Hae*III, or *Rsa*I (20 U each; TaKaRa Shuzo) in a total volume of 10 µl at 37˚C for 2.5 h. The restriction digest product (1 µl) was mixed with 8 µl of Hi-Di Formamide (Applied Biosystems, USA) and 1µl of GS1200LIZ® Size Standard (Applied Biosystems) as an internal standard. After denaturation at 95˚C for 2 min and cooling on ice, the lengths of the terminal restriction fragments (T-RFs) were analyzed using an ABI PRISM 3130x/genetic analyzer (Applied Biosystems) in Genescan mode. To remove background and small peaks, T-RFs whose relative areas were less than 2.0% of the total area were deleted. Fragments ranging from three to five base pairs (bp) were grouped into one operational taxonomic unit (OTU). T-RF length of the 16S rRNA gene of known bacterial species was predicted *in silico* using the Genetyx program (version10, Genetyx Corporation, Japan).

2.4. 16*S* rRNA Gene Clone Library Analysis

The purified PCR products were cloned into *Escherichia coli* DH5*α* using the pGEM-T Easy vector system (Promega, USA). 96 recombinant colonies were selected for direct PCR by the blue-white selection. The vector-specific primers T7 (5'-TAATACGACTCACTATAGGG-3') and Sp6 (5'-ATTTAGGTGACACTATAGA-

Table 1. Patient samples used in this study.

Sample	Mucus area	Age (year)	Male/Female	Blood type	Treatment of antibiotics
UC1	Colon	29	M	O+	No
UC3	Colon	51	F	A+	No
UC4	Rectum	17	M	B+	No
UC9	Colon	16	F	A+	No
Cont1	Rectum	70	M	O+	No
Cont2	Rectum	84	M	O+	No
Cont3	Right colon	72	F	O+	No
Cont4	Transverse colon	72	M	O+	No
Cont5	Colon	60	M	O+	No
Cont7	Colon	65	M	O+	No

AT-3') were used. Amplification was performed in a 20 µl reaction mixture containing 10 µl TaKaRa Ex Taq (Takara Shuzo), 200 µl 10× Ex Taq buffer, 160 µl dNTP mixture (2.5 mM each), each primer (10 pmol), and bacterial cells. The samples were amplified using a C1000™ Thermal Cycles (Bio-Rad, USA) and the following: 95˚C for 3 min; 30 cycles of 95˚C for 30 s, 50˚C for 30 s, and 72˚C for 1.5 min; and finally 72˚C for 10 min. After electrophoresis and purification with ExoSAP-IT (GE Healthcare, UK), approximately 500 bp of the 5' end of the 16S rRNA gene containing hypervariable regions V1 to V3 was sequenced using the 35F primer, a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). Sequences were aligned with the Clustal X 2.0.12 program [25], and corrected by manual inspection. Libraries were clustered using the Dist program of Mothur [26], and compared with similar sequences to the reference organisms using EzTaxon server 2.1 (http://www.ezbiocloud.net/eztaxon) or a BLAST search [27]. 16S rRNA gene sequence similarity of 98% was used as the cut-off for positive identification of a taxon (OTU). The coverage of the sequence was expressed as a percentage representing sampling intensity [28]. Com- munity diversity was measured using the Shannon [29] and Simpson [30] index. The Chao1 index was used to measure community richness [31]. The fraction of shared OTUs was represented in Venn diagrams using the Venn program of Mothur [26].

2.5. Statistical Analysis

The average number of fragments generated using digestion with each restriction enzyme was expressed as mean ± SE. Differences between two groups were examined using the Student's t-test. The test of population proportion was performed for the related ratios of gut bacteria in UC patients compared to the controls using the SAS computer program, the GLM procedure.

Correlations among the related ratios of microflora detected by the clone library were examined, and significances of their coefficients were estimated by the SAS computer program, the CORR procedure.

3. Results

3.1. T-RFLP Analysis

T-RFLP analysis was performed using four UC patients and six control patients. The typical T-RFLP patterns are shown in **Figure 1**. The number of T-RFs having an area >2% of the total fragment area was counted, and computed to compare the average value for each group. The number of total T-RFs decreased significantly in UC patients (8.3 ± 2.9) compared to controls (10.4 ± 3.9) ($p < 0.05$). Although the number of *Hae*III, *Hha*I, or *Msp*I-digested T-RFs in UC patients (7.5 ± 2.1, 8.0 ± 1.4, 7.5 ± 2.4, respectively) was significantly larger than in controls (11.7 ± 3.8, 11.3 ± 1.9, 12.7 ± 2.0, respectively) ($p < 0.05$), there was no significant difference using *Alu*I or *Rsa*I (**Figure 2**).

3.2. 16S rRNA Gene Clone Library Analysis

We constructed 16S rRNA gene clone libraries and obtained sequences from 93, 89, 94, 92, 93, 94, 58, and 94 clones representing cont1, cont2, cont3, cont4, UC1, UC3, UC4, and UC9, respectively. The coverage of UC and control group libraries was found to be 88.7% (range 84.5% - 93.5%) and 75.9% (range 62.9% - 87.2%), respectively. No significant differences in sampling coverage were evident between the two groups, thus permitting meaningful comparison of the samples.

The 16S rRNA clones with >98% sequence similarity were grouped into the same OTU. The number of OTUs decreased significantly in UC patients (16.5 ± 5.7) compared to the controls (34.5 ± 12.8), and the Shannon index also decreased significantly in UC patients (1.7 ± 0.6) as compared to the controls (2.9 ± 0.6) (**Figure 3**). This suggests decreased microflora diversity in UC patients; as was also shown in the T-RFLP analysis where the number of fragments in UC was less than the controls. Although the average of the Chao1 richness estimators was lower in UC patients (28.5 ± 9.9) than in controls (76.3 ± 44.5), there was no significant difference between the two groups ($p = 0.13$). Additionally, there was no significant difference in the Simpson index (data not shown).

Here, a total of 707 clones from both groups were analyzed, and finally 146 OTUs were identified, with 51 from the UC and 111 from the control group (**Figure 4**). Based on the sequence similarity with reference bacteria, the identified OTUs were classified into 32 genera. Specific bacterial species that generally exists in the UC

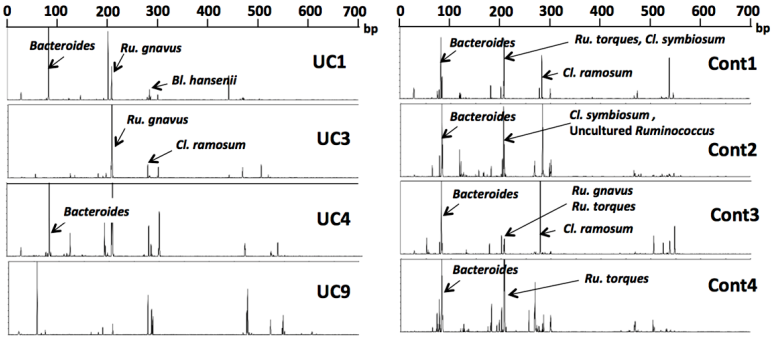


Figure 1. T-RFLP patterns of 16S rRNA genes from UC and control samples generated after digestion with *Msp*I restriction enzyme. 16S rRNA genes were amplified with universal primers 35F and 1492R.*Cl*., *Clostridium*; *Ru*., *Ruminococcus*.

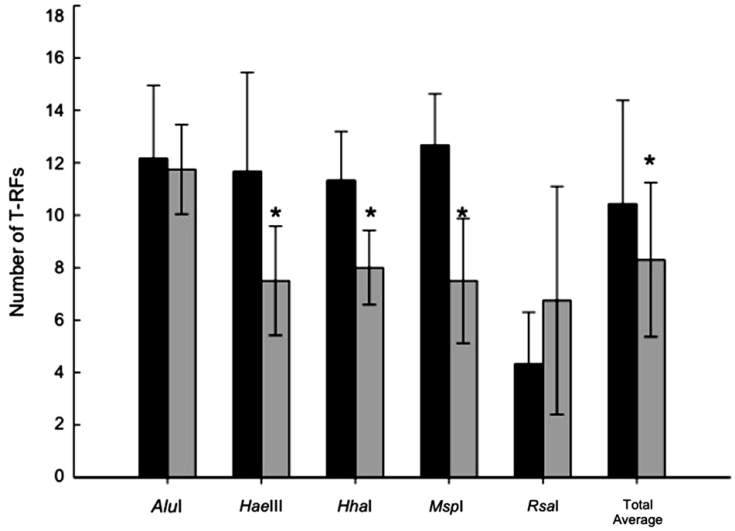


Figure 2. Average number of T-RFs detected in T-RFLP analysis of control (black bars) and UC (gray bars) groups digested with five restriction enzymes. *p < 0.05.

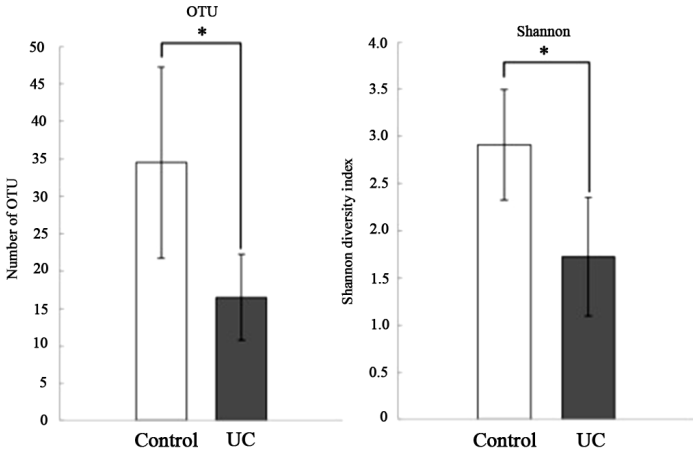
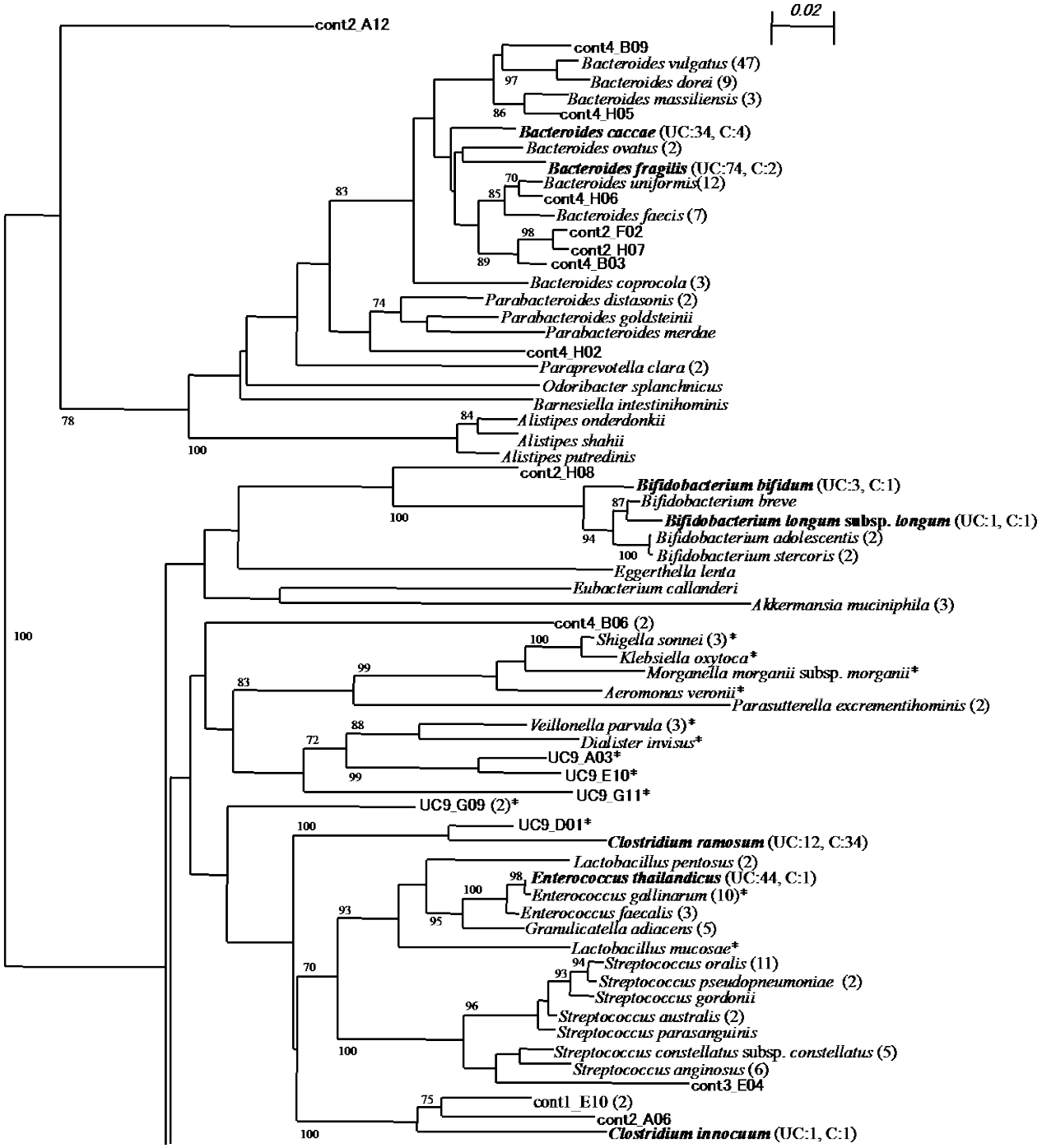


Figure 3. Comparison of the number of OTUs and Shannon diversity index in the 16S rRNA gene clone library between controls (white bars) and UC patients (gray bars). *p < 0.05.

groups was not detected, suggesting there was no unique pathogen causing the UC pathogenesis. *Bacteroides*, *Clostridium* and *Ruminococcus* were abundant in both groups. *Bacteroides* was abundant in UC1 and UC4. Although the most abundant bacteria in UC3 and UC9 belonged to Firmicutes, they were different genera (*Ruminococcus* and *Enterococcus*, respectively) (**Table 2**). At the phylum level, the averages of the clone numbers of Firmicutes, Bacteroidetes, and Proteobacteria in the control group were about 47%, 26%, and 0.5%, respectively. The bacteria in Firmicutes were more than Bacteroidetes in all control samples. Bacteroidetes occupied about 70% with few Firmicutes in UC1 and 4, whereas Firmicutes was about 70% and no Bacteroidetes was detected in UC3 and 9 (**Figure 5**). The commonality of detected OTUs among individual patients was also analyzed in this



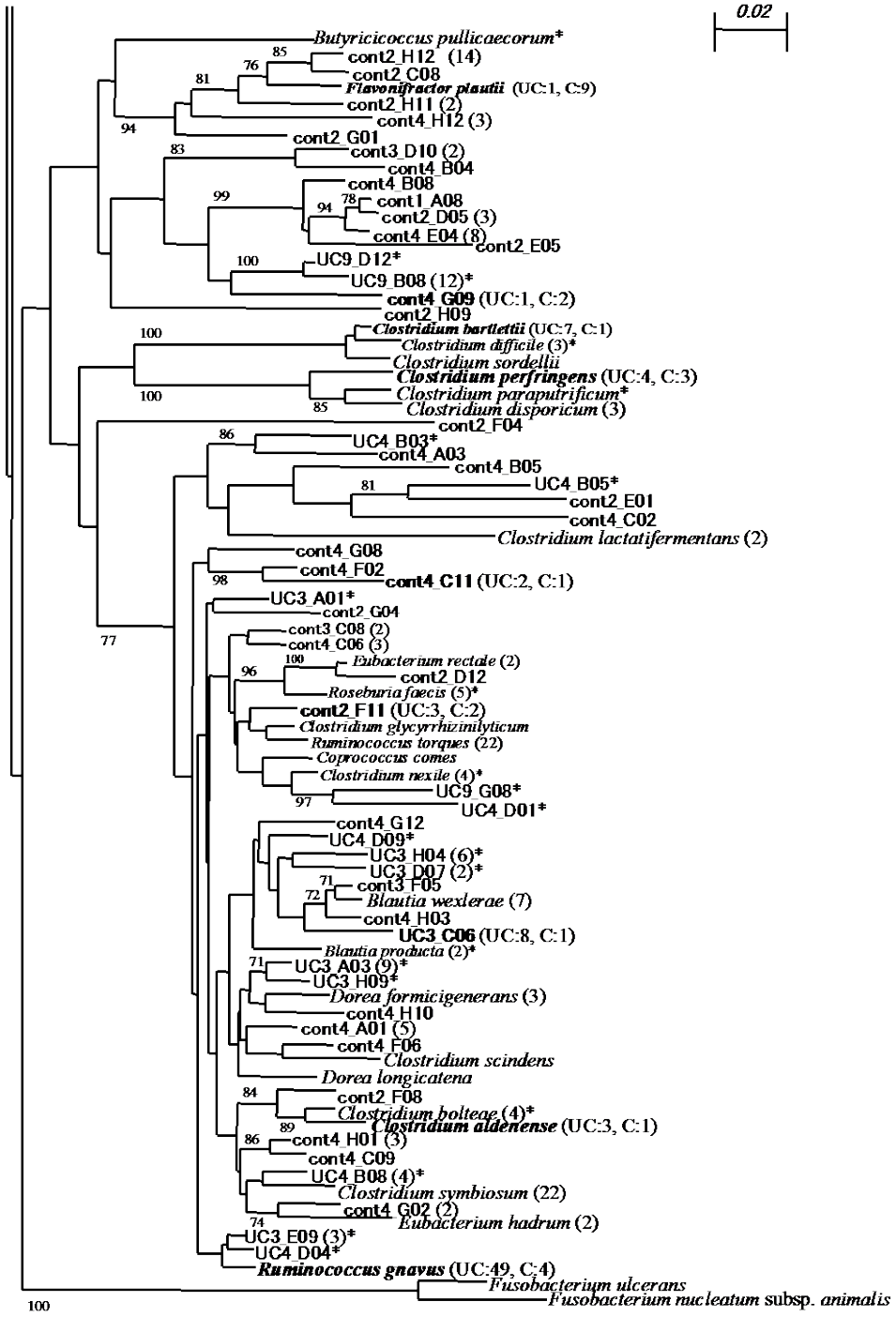


Figure 4. Phylogenetic tree showing clones detected in the eight libraries. The tree was constructed by the neighbor-joining method based on 16S rRNA gene sequence comparisons. The scale bar represents 0.05 substitutions per nucleotide position. The numbers at the nodes of the tree indicate bootstrap values for each node out of 100 bootstrap resampling. The numbers at the end of species name represent the number of clones detected in UC and control group for each species. * Only detected in UC group. The species name without * means only detected in control group. The species without number at the end means only detected in one clone.

study as shown in the Venn diagrams (**Figure 6**). The common OTUs were very poor among individuals both in the UC and control groups.

Table 2. Bacterial OTUs in UC and control groups identified by 16S rRNA gene clone library analysis.

Bacteria			No. of clones							
			Control				UC			
Phylum	Class	Genus	1	2	3	4	1	3	4	9
Actinobacteria	Actinobacteria	*Bifidobacterium*	4	3			2	2		
		Eggerthella		1						
Bacteroidetes	Bacteroidia	*Alistipes*		2		2				
		Bacteroides	29	14	23	19	74		34	
		Barnesiella				1				
		Odoribacter	1							
		Parabacteroides	2		1					
		Paraprevotella		2		1				
Firimicutes	Bacilli	*Enterococcus*			4			2		52
		Granulicatella			5					
		Lactobacillus		2				1		
		Streptococcus	15	4	9					
	Clostridia	*Blautia*	1		3	3	1	1		
		Butyricicoccus					1			
		Clostridium	25	2	37	5	5	16	3	15
		Coprococcus		1						
		Dorea	1	3						
		Eubacterium		5		1		2		
		Faecalibacterium	1			8				
		Flavonifractor	8	1			1			
		Roseburia						5		
		Ruminococcus	1	11	4	12	1	36	12	
	Negativicutes	*Dialister*						1		
		Veillonella						1		2
Fusobacteria	Fusobacteria	*Fusobacterium*	1		1					
Proteobacteria	Gammaproteobacteria	*Aeromonas*								1
		Klebsiella								1
		Morganella								1
		Shigella					1			2
	Betaproteobacteria	*Parasutterella*		1		1				
Verrucomicrobia	Verrucomicrobiae	*Akkermansia*				3				
Uncultured bacterium			4	37	7	36	7	27	9	20
Total			93	89	94	92	93	94	58	94

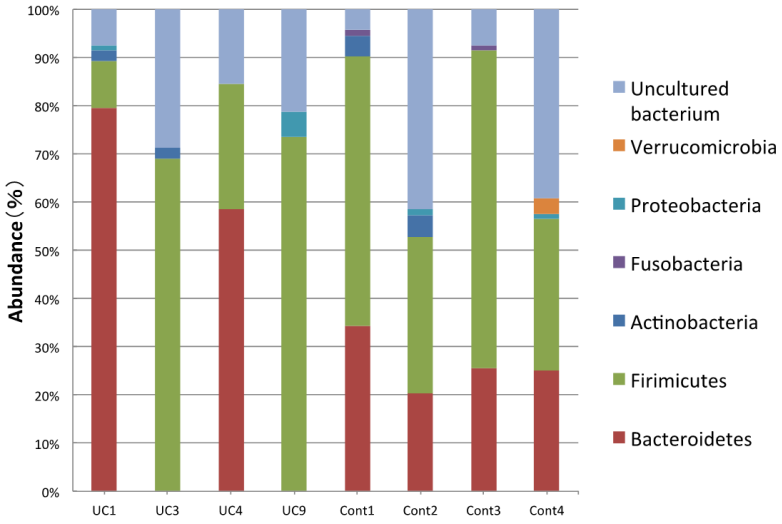


Figure 5. Relative abundance of bacterial phyla identified in the 16S rRNA gene clone library analysis. Each bar represents the percent contribution of the phylum level profiles for each individual.

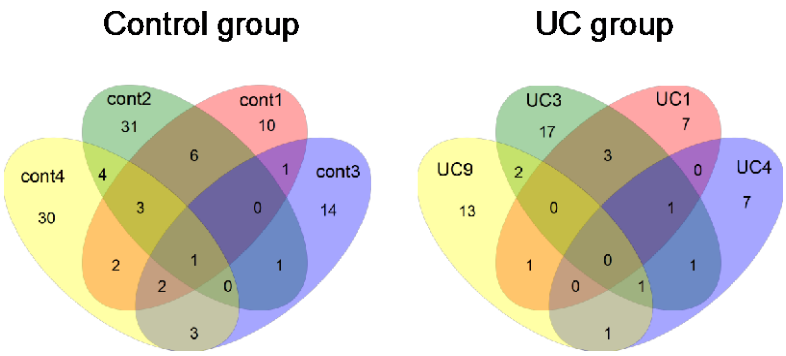


Figure 6. Venn diagrams showing the number of OTUs shared with each samples. The criterion used for grouping the sequences into OTU was 2% dissimilarity.

Because UC patients showed inconsistent diversity in microflora, the correlation of abundance ratio of each species (in phylum level) was analyzed. In the control group, significant positive correlations were found between Firmicutes and Fusobacteria ($p < 0.05$) and between Proteobacteria and uncultured bacterium ($p < 0.01$), whereas significant negative correlations were observed between Firmicutes and Proteobacteria ($p < 0.05$), Firmicutes and uncultured bacterium ($p < 0.05$), Fusobacteria and Proteobacteria ($p < 0.01$), and Fusobacteria and uncultured bacterium ($p < 0.01$). In contrast, only a strong negative correlation between Bacteroidetes and Firmicutes was significantly found in the UC group ($p < 0.01$) (**Table 3**).

3.3. Computer-Simulated T-RFLP Analysis

Based on the results from the 16S rRNA gene clone library analysis, we enabled to match the bacterial genus and/or species to T-RFs in the T-RFLP patterns of the four UC samples and six controls. Most of the T-RFs were presumed to represent corresponding OTUs detected by the clone library analysis. There were 5 OTUs obtained from *Alu*I, *Hae*III, or *Msp*I-digested T-RFs that showed significant changes between UC and controls ($p < 0.05$). The OTUs from *Hae*III-digested T-RFs (70-bp, 226-bp, and 303-bp) and *Msp*I-digested T-RFs (72-bp) were significantly decreased in the UC patients as compared to the controls. However, only the 76-bp OTU predicting *Bacteroides vulgatus* and *Clostridium disporicum* from *Alu*I-digested T-RFs was significantly increased in UC samples (**Table 4**).

Table 3. Statistical relationship between abundance of each phylum in (a) control and (b) UC group respectively. $^{*}p < 0.05$, $^{**}p < 0.01$. (a) Control group; (b) UC group.

(a)

	Actinobacteria	Bacteroidetes	Firmicutes	Fusobacteria	Proteobacteria	Uncultured bacterium
Actinobacteria	1.0000					
Bacteroidetes	0.1697	1.0000				
Firmicutes	−0.1681	0.5427	1.0000			
Fusobacteria	−0.0167	0.7229	0.9689*	1.0000		
Proteobacteria	0.0389	−0.7258	−0.9699*	−0.9997**	1.0000	
Uncultured bacterium	0.0135	−0.7717	−0.9509*	−0.9971**	0.9976**	1.0000

(b)

	Actinobacteria	Bacteroidetes	Firmicutes	Proteobacteria	Uncultured bacterium
Actinobacteria	1.0000				
Bacteroidetes	0.1542	1.0000			
Firmicutes	−0.1923	−0.9985**	1.0000		
Proteobacteria	−0.4826	−0.4368	0.4834	1.0000	
Uncultured bacterium	−0.0248	−0.9246	0.9045	0.0660	1.0000

Table 4. Significant change in OTUs when compared to controls in T-RFLP analysis.

OTU	Predicted bacteria (genus or species)	Control (n = 6)	UC (n = 4)	p value
***Alu*I**				
76	*Bacteroides vulgatus, Clostridium disporicum*	0	5.2 ± 3.7	0.03
***Hae*III**				
70	*B. vulgatus, B. massilensis, Bifidobacterium*	13.2 ± 10.4	0	0.01
226	*Flavonifractor plautii, Eubacterium hallii, Clostridium*	9.3 ± 8.6	1.3 ± 2.6	0.04
303	*Ruminococcus torques, Cl. symbiosum*	15.7 ± 8.6	4.8 ± 3.7	0.01
***Msp*I**				
72	*Bifidobacterium, Parabacteroides*	3.1 ± 2.1	0	<0.01

The OTUs were quantified as the percentage values of individual OTU per total OTU areas. Values (%) were expressed as means ± SD. $p < 0.05$ was considered significant.

4. Discussion

Although it is evident that there is a strong relationship between UC and gut microflora, the entire picture remains unclear. Using molecular techniques, we analyzed the bacteria existing in colonic mucus that were considered to be influencing the inflammation of UC to clarify the relationship between gut inflammation and microflora diversity in UC. Instead of using feces, the colonic mucus from severe UC patients who had not been treated with antibiotics and received colectomy surgery were analyzed in this study.

As a control group, we collected colonic mucus from the normal portions of colorectal cancer patients who were older than the UC patients (**Table 1**). Decreased Firmicutes in elders [32] and increased Firmicutes in young people [33]-[35] were usually observed when investigating human gut microflora. In mucosal tissues from healthy adults, previous reports show the bacteria from the phylum Firmicutes was about 62%, Bacteroidetes about 33%, and Proteobacteria about 5% [36]. Bibiloni *et al*. [37] also reported that Firmicutes was about 53%, Bacteroidetes about 27%, and Proteobacteria about 6.4% in intestinal biopsies of healthy subjects. Additionally, Frank *et al*. [13] indicated that Firmicutes was about 58%, Bacteroidetes about 33%, and Proteobacteria

about 7% in intestinal tissue of healthy subjects. Compared with the results above, although Firmicutes was lower in this study (**Figure 5**), the microflora composition was similar in our data and in an acceptable range; thus our control group was comparative in this study.

Molecular techniques investigating sequence diversity of the 16S rRNA gene were employed to estimate the microflora composition in the gut. Using T-RFLP analysis, a culture-independent tool that provides a rapid overview of microbial communities, the 16S rRNA genes from the mucosa-adherent microflora of UC patients and controls were analyzed after digestion with five restriction enzymes (**Figure 2**). *Hha*I and *Msp*I-digested T-RFs from mucosa-associated microflora were reported to decrease in UC patients [15]. The decreased number of total T-RFs was also confirmed in feces sample of UC patients [38], suggesting the diversity of the microflora was reduced in UC.

From the 16S rRNA gene clone library, the gut microflora was further clarified. To determine if each simulation was representative of the bacterial community in each group, coverage values were calculated. The average value of coverage in this study was in an acceptable range and corroborated previous findings that coverage averaged 80% [39], thus offering a valid basis for inter-group comparisons. A decreased number of OTUs, Shannon (**Figure 3**), and Chao1 indices in UC samples were observed in this study and other similar studies [36] [40], suggesting the bacterial diversity and richness were reduced in UC pathogenesis. However, Bibiloni *et al*. [37] indicated that there was a significant difference in the Shannon index between Crohn's disease and healthy subjects but not between UC and healthy subjects, showing some conflicting results remain.

After sequences were compared with reference bacteria, we found the commonality of bacterial species was low between each sample, which was supported by the Venn diagrams of the control and UC groups (**Figure 6**). The data suggested individual differences in gut microflora exist in both groups. It is a common phenomenon globally that the difference in microflora between human individuals was large, whether in UC patients or healthy individuals [36] [41] [42]. Moreover, a significant negative correlation was observed between the phylum Bacteroidetes and Firmicutes in the UC group (**Table 3**). *Bacteroides vulgatus*, a member of Bacteroidetes, was thought to be the most predominant bacterial species in the gut of both UC and healthy people [12] [43]. Bacteroidetes was reported to decrease in gut flora of UC [14] [40], but inconsistent reports indicated Bacteroidetes increased [12] [36] [37] or showed no significant change [44] in UC microflora. Similarly, some reports indicated Firmicutes increased [44] or decreased [36] in UC microflora. Although previous studies gave different results, we found a significant negative correlation of Bacteroidetes and Firmicutes in UC patients but not in the control microflora.

With a combination of T-RFLP and 16S rRNA gene clone library, most of the T-RFs were assigned to corresponding species or genera. There were five OTUs significantly changed in UC samples in this study. Except the *Alu*I-digested T-RF 76-bp that was absent in controls, other OTUs decreased significantly in UC patients, and most of the corresponding bacteria were classified into *Bacteroides*, *Clostridium*, or *Bifidobacterium* (**Table 4**). The Clostridium [45] and Bacteroides [46] cluster decreased significantly in fecal samples from UC patients, whereas T-RFs were determined after digestion with *Bsl*I. Similar results were also confirmed by quantitative real-time PCR [38]. However, comparing *Hha*I-digested T-RFs, the T-RFs derived from *Bacteroides* and *Bifidobacterium* were detected in fecal sample from UC patients not healthy individuals, whereas the T-RFs derived from the class Clostridia and *Lactobacillus* were detected in healthy individuals not UC patients [47]. These findings showed different bacterial growth and decline, but indicated the same conclusion that the diversity of gut microflora collapsed in UC patients.

In this study, no specific pathogen was observed in the microflora of UC patients. The bacterial species found in UC were known as commensal bacteria, and also found in healthy people's intestinal bacterial flora (**Table 2**). Mucus under inflammatory conditions and the changed environment in the gut influenced growth and adhesion ability of bacteria selectively in the mouse [48] [49]. It was reported that the thickness of the human gut mucus layer decreases with inflammation [50], suggesting that reduction of the mucin, which serves as the scaffold for growth and the source of nutrition for microflora, may cause loss of microflora diversity in the gut. Moreover, using Interleukin (IL)-10 deficient mice infected with *Enterococcus faecalis* or *Escherichia coli* respectively, toll-like receptor mediated NF-κB was activated and secretion of IL-23 increased, causing intestinal inflammation [51]. The pathogenesis of UC may be induced by non-pathogenic commensal bacteria in the host with special genetic factors, but not induced by a specific pathogen.

Although the diversity of gut microflora in UC decreased in both analyses of this study and other reports [11] [38], it is noted higher bacterial concentrations were prevalent in mucus biopsies from UC patients as compared

to healthy subjects [43] [52], and there was no significant difference between microflora from inflamed and non-inflamed gut tissue from the same individual [53]. However, the mechanism of decreased diversity in UC is still unclear. A strong correlation between Bacteroidetes and Firmicutes in UC was shown here that may be one of the indicators in UC progression, and may contribute to the development of new therapeutic strategies for UC patients.

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