The Journal of Microbiology (2012) Vol. 50, No. 2, pp. 218–225
DOI 10.1007/s12275-012-1362-8

Microbial Fingerprinting Detects Unique Bacterial Communities in the Faecal Microbiota of Rats with Experimentally-Induced Colitis[§]

Ashis K. Samanta[1], Valeria A. Torok[2], Nigel J. Percy[2], Suzanne M. Abimosleh[3,4], and Gordon S. Howarth[4,5]*

[1]*National Institute of Animal Nutrition and Physiology, Bangalore, Karnataka 560030, India*
[2]*South Australian Research and Development Institute, Plant and Soil Health, Waite Campus, Urrbrae, South Australia 5064, Australia*
[3]*Discipline of Physiology, School of Health Sciences, The University of Adelaide, South Australia 5001, Australia*
[4]*Gastroenterology Department, Women's and Children's Hospital, North Adelaide, South Australia 5006, Australia*
[5]*School of Animal and Veterinary Sciences, Roseworthy Campus, The University of Adelaide, South Australia 5371, Australia*

(Received July 25, 2011 / Accepted December 19, 2011)

An abnormal composition of the gut microbiota is believed to be associated with the pathogenesis of inflammatory bowel disease (IBD). We utilized terminal restriction fragment length polymorphism (T-RFLP) analysis to quantify faecal bacterial communities from rats with experimental colitis. Male Sprague Dawley rats (n=10/group) ingested 2% dextran sulfate sodium (DSS) or water for up to 7 days. Rats were killed and colonic tissues collected for histological analysis. Damage severity score in the distal colon was significantly greater (P<0.001) following DSS consumption compared to controls. T-RFLP faecal bacterial profiles generated with either *Msp*I or *Cfo*I revealed a significant difference (P<0.001) in community composition between healthy and colitic rats, with bacterial composition in healthy rats more variable than in rats with colitis. Operational taxonomic units (OTU: taxonomically related groups of bacteria) associated with either the healthy or colitic state were identified. OTU (116, 226, 360, and 948; *Cfo*I) and (118 and 188; *Msp*I) were strongly associated with untreated healthy rats, while OTU (94, 98, 174, and 384; *Cfo*I) and (94 and 914; *Msp*I) were predominantly associated with DSS-treated colitic rats. Phylogenetic OTU assignment suggested that *Bacteroidales* and *Lactobacillus* sp. were predominantly associated with the colitic and healthy rats, respectively. These results show that faecal bacterial profiling is a rapid, sensitive and non-invasive tool for detecting and identifying changes in gut microbiota associated with colitis. Restoring microbial homeostasis by targeting colitis-associated OTU through specific microbiological interventions could form the basis of novel therapeutic strategies for IBD.

*For correspondence. E-mail: gordon.howarth@adelaide.edu.au; Tel.: +61-8-8303-7885; Fax: +61-8-8303-7972
[§]Supplemental material for this article may be found at http://www.springerlink.com/content/120956.

Keywords: faecal bacterial profiling, inflammatory bowel disease, T-RFLP, experimental colitis, rat

Introduction

Inflammatory bowel disease (IBD) is the collective term for a group of idiopathic gut disorders of unknown etiology. Although the precise etiology of IBD remains undefined, immunological abnormalities triggered by genetic and environmental factors are thought to be contributing factors to disease pathogenesis (Fiocchi, 1998). Symptoms include malaise, weight loss, abdominal pain, diarrhea, anorexia and the presence of blood in the stools (Munkholm *et al.*, 1995). Importantly, gut microbes are believed to be intrinsically involved in IBD pathogenesis (Takaishi *et al.*, 2008; Xenoulis *et al.*, 2008). Moreover, breakdown of gut homeostasis as a result of defective host-microbe interplay may also contribute to the pathogenesis of IBD (Strober *et al.*, 2007). An impediment in studying the gut microbiota has been that the growth requirements of many gut inhabiting microbes are as yet unknown (Bjerketorp *et al.*, 2008), with only 20% of bacterial species successfully cultivated to date (Eckburg *et al.*, 2005). However, molecular techniques targeting the 16S rRNA have enabled investigation of the uncultivable bacterial communities, contributing to our understanding of the gastrointestinal microbiota in gut health. Use of such molecular techniques may ultimately give further insight into the basic biology and pathophysiology of IBD (Bousvaros *et al.*, 2008). The microbial community changes associated with IBD are poorly understood, and it remains a subject of intense investigation to determine whether these changes are responsible for disease etiology, or alternatively, an indirect consequence of IBD (Steed *et al.*, 2008; Xenoulis *et al.*, 2008).

The faecal microbiota of IBD patients has been a focus of attention during the last decade (Steed *et al.*, 2008) and has been demonstrated to differ between healthy individuals and IBD patients (Tannock, 2007). Investigations using molecular techniques have demonstrated decreased microbial diversity in intestinal tissues from subjects with active Crohn's disease, with a numerical increase of *Enterobacteriaceae* and a decrease in members of the phyla *Bacteroidetes* and *Firmicutes* (Manichanh *et al.*, 2006; Frank *et al.*, 2007; Xenoulis *et al.*, 2008).

Animal models have contributed greatly to our understanding of IBD pathogenesis (Tamboli *et al.*, 2004), revealing that gut inflammation occurs only in the presence of the gut microbiota (Taurog *et al.*, 1994). Moreover, animals raised under germ free conditions do not develop colitis

(Sellon *et al*., 1998). Knowledge of the community composition of the gut microbiota in IBD is therefore of utmost importance to our understanding of the condition and the subsequent development of new treatment strategies. Both human and animal model studies have indicated changes of faecal bacterial composition with the onset of colonic inflammation or infection (Scanlan *et al*., 2006; Xenoulis *et al*., 2008). However, despite significant advances in the field of gut microbiology, our knowledge of the gastrointestinal microbiota is far from complete (Dethlefsen *et al*., 2007).

In order to better define the composition of the intestinal microbiota, small subunit ribosomal RNA (16S rRNA) based terminal restriction fragment length polymorphism (T-RFLP) techniques have been applied to studies in humans (Andoh *et al*., 2007; Dicksved *et al*., 2007; Sepehri *et al*., 2007), poultry (Torok *et al*., 2008, 2009) and termites (Egert *et al*., 2004) as the technique is rapid and highly reproducible. This technique enables a "snap shot" view of the complex bacterial population of the gut at any particular time; enabling comparative analysis (Torok *et al*., 2008). However, to date, few attempts have been made to unravel the microbial community under colitic conditions through T-RFLP analysis (Andoh *et al*., 2007; Sepehri *et al*., 2007). Accordingly, the principal aim of the current study was to define the faecal bacterial community changes in rats with experimentally-induced colitis by application of T-RFLP analysis.

Materials and Methods

Animal trial protocol

Male Sprague Dawley rats (n=20) 4–5 weeks of age were obtained from Laboratory Animal Services, University of Adelaide, Adelaide. Faecal samples were obtained from two sets of experimental rat trials. In set I, four rats were untreated healthy controls and four rats ingested 2% dextran sulfate sodium (DSS, ICN Biomedicals, USA) for 7 days to induce colitis. The DSS-colitis model in rodents is utilized widely as a model of the ulcerative colitis variant of inflammatory bowel disease, typified by superficial mucosal erosion, crypt loss and polymorphonuclear cell infiltration of the mucosa (Geier *et al*., 2007). Set II comprised six healthy control rats and six DSS-treated rats (6 days DSS ingestion). The experiments were conducted with the approval of the Animal Ethics Committee of the Children, Youth and Women's Health Service and the University of Adelaide. Rats were housed in individual metabolism cages (Tecniplast, USA) at a room temperature of 22±1°C with 12 h light dark cycle (Geier *et al*., 2007). During the trial period, rats were maintained on a standard 18% casein based diet (Tomas *et al*., 1991) with *ad libitum* access to food and water. Rats with DSS colitis had 2% DSS substituted for water for 6 days (set II) or 7 days (set I) to simulate progression of colonic inflammation, after which rats were killed by carbon dioxide overdose and cervical dislocation. The two studies were conducted eleven months apart. The severity of colitis was assessed daily, in a blinded fashion, utilizing a disease activity index (DAI). The DAI scored body weight loss, rectal bleeding and stool consistency increasing in severity on a scale of 0–3 for each parameter (Murthy *et al*., 1993). This was combined with a score (0–3) for the general condition of the animal, considering grooming, appearance and mobility; summed to achieve an overall DAI (maximum score 12). Faecal samples were collected on day 5 (set II) and day 6 (set I), one day before animals were killed and stored at -20°C for later analysis.

Histological analyses

Proximal and distal colon samples were routinely processed and embedded in paraffin wax (Yazbeck *et al*., 2008). Sections (4 μm) were then stained with haematoxylin and eosin. Damage severity of intestinal sections was assessed using a semi-quantitative analysis based on parameters including: crypt area; enterocyte disruption; crypt disruption; crypt cell disruption; reduction in goblet cell numbers; lymphocytic and polymorphonuclear infiltration; thickening/oedema of the submucosa; and thickening/oedema of muscularis externa (Yazbeck *et al*., 2008). A score from 0 (unaffected) to 3 (severe) was recorded for each of the eight parameters to provide a maximum damage severity score of 24.

Nucleic acid extraction

Total nucleic acid was extracted from frozen faecal material (approximately 200 mg) obtained from individual healthy and colitic rats (n=10/treatment group) using a modification (Torok *et al*., 2008) of a proprietary extraction method developed by the South Australian Research and Development Institute (Stirling *et al*., 2004). Total nucleic acid from samples was analyzed for total faecal bacterial community composition by T-RFLP.

T-RFLP

T-RFLP analysis was done following the technique described by Torok *et al*. (2008). Bacterial 16S rRNA gene was amplified with universal bacterial primers 27F (Lane, 1991) and 907R (Muyzer *et al*., 1995). The forward primer (27F) was 5′-labeled with 6-carboxyfluorescein (FAM) to enable subsequent detection of terminal restriction fragments (T-RFs). The PCR reactions were done in duplicate in 50 μl volumes according to Torok *et al*. (2008). Approximately 200 ng PCR product was digested with 2 U *Cfo*I (Roche Applied Science, Australia) or *Msp*I (New England Biolabs, Australia) in duplicate following manufacturer's instructions. DNA fragments were separated by capillary electrophoresis on an ABI 3730 automated DNA analyzer (Applied Biosystems, Australia) at the Australian Genomics Research Facilities, Adelaide Node. The lengths of fluorescently labeled T-RFs were determined by comparison with an internal standard (GeneScan 1200 LIZ®; Applied Biosystems) and data analyzed using GeneMapper software (Applied Biosystems). Data points generated by the GeneMapper software were further analyzed using a custom built database containing queries to validate data points and generate outputs for statistical analysis (Torok *et al*., 2008). T-RF were defined as peaks with a size of x±2 bp within pseudo replicates of samples and rounded to the nearest even number between samples to produce operational taxonomic units (OTU).

Statistical analysis

Statistical comparisons for histological damage severity were performed using SPSS version 16.0 for Windows (SPSS Inc., USA). Damage severity and DAI were expressed as median score (range) and compared by a Kruskal Wallis test with Dunn's *post hoc* test.

The OTU obtained from the faecal material of 10 healthy control and 10 DSS-induced colitic rats were analyzed using multivariate statistical techniques (PRIMER 6, PRIMER-E Ltd., UK). These analyses were used to examine similarities in rat faecal bacterial communities. Bray-Curtis measures of similarity (Bray and Curtis, 1957) were calculated to examine similarities among faecal bacterial communities of rats from the T-RFLP generated (OTU) data matrices, after standardization and fourth root transformation. One-way analysis of similarity (ANOSIM; Clarke, 1993) was used to test if faecal bacterial communities were significantly different between healthy and colitic states, as well as, between the two sets of experimental trials. The R-statistic value describes the extent of similarity between each pair in the ANOSIM analyses, with values close to unity indicating that the two groups are entirely separate and a zero value indicating no difference between the groups.

Similarity percentages (SIMPER; Clarke, 1993) analyses were calculated to determine the overall average similarity in faecal bacterial community composition among either the healthy or colitic rats, as well as to determine which OTU contributed greatest to the dissimilarity between the healthy and colitic groups. The overall average dissimilarity (δ) between faecal bacterial communities of healthy and colitic rats was calculated and the average contribution of the ith OTU ($\bar{\delta}i$) to the overall dissimilarity determined. The average abundance of important OTU in each of the groups was determined and OTU contributing significantly to the dissimilarity between groups identified [$\delta i/SD(\delta i)>1$]. The percent contributions of individual OTU and the cumulative percent contribution to the top 50% of the average dissimilarities were also calculated.

Unconstrained ordinations using non-metric multidimensional scaling (nMDS) were done to illustrate the relationships graphically between control and DSS-induced rats, as well as between sets of experimental animals (Shepard, 1962; Kruskal, 1964). The nMDS ordinations show the relationship among samples using the ranks of similarities.

T-RFLP phylogenetic assignment tool (PAT)

T-RFLP PAT enables phylogenetic assignments based on data from a series of restriction enzyme digests. A custom database for analysing our T-RFLP data was generated using the Microbial Community Analysis III Virtual Digest (http://mica.ibest.uidaho.edu/digest.php) interface (Shyu *et al.*, 2007). The *in-silico* database was constructed with the 27F and 907R primer pair, *Msp*I and *Cfo*I restriction enzymes and RDP (R10, U12) 776,206 bacterial SSU 16S rRNA sequences. The resulting custom database output file contained 81,365 sequences. This database was uploaded along with our T-RFLP data onto the T-RFLP PAT interface (https://secure.limnology.wisc.edu/trflp/index.jsp). Data were analyzed using default settings as outlined by Kent *et al.* (2003). Identified 16S rRNA sequences were assigned a classification using the Ribosomal Database Project (RDP) Release 10 Classifier (http://rdp.cme.msu.edu/misc/about.jsp) with a threshold of 80%. Closest related bacterial species were also identified using the National Centre for Biotechnology Information (NCBI) BLASTn interface (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with nucleotide collection (nr/nt) databases and Megablast algorithm.

Results

In the current study, clinical disease activity (DAI) was significantly greater (P=0.04) in DSS-treated animals [1 (0-4); median (range)] compared to controls [0 (0–1)] with a total DAI of 10 in the DSS-treated group compared to 1 in the water treated controls (Table 1). Four rats with DSS-induced colitis exhibited a DAI of zero. Three of these rats were from experimental set II (rat 15, 17, and 19) whilst one was from experimental set I (rat 7). Furthermore, one rat from the control group (rat 12) had a DAI score of one. Histological examination of these rats revealed that rats with DSS-induced colitis had significantly greater damage severity scores for the distal colon (ranging from 7 to 17) than the control rats (ranging from 0 to 2) (Table 1). Moreover, damage severity score in the distal colon was significantly greater (12-fold) following DSS consumption, compared to healthy controls (P<0.001; Fig. 1).

T-RFLP with two different restriction enzymes (*Msp*I and *Cfo*I) was used to investigate faecal bacterial community

Table 1. DAI and damage severity score for water control and DSS treated rats

	Treatment									
	Water control group									
Rat ID[a]	1	2	3	4	9	10	11	12	13	14
DAI	0	0	0	0	0	0	0	1	0	0
Damage severity score	1	0	-[b]	1	1.5	2	1	1.5	1.5	0
	DSS treated group									
Rat ID	5	6	7	8	15	16	17	18	19	20
DAI	1	2	0	1	0	1	0	4	0	1
Damage severity score	7	7	17	13.5	12	15	13	13.5	9.5	16

[a] Rats 1–8 are from experimental set I and rats 9–20 are from experimental set II.
[b] Sample could not be scored due to mechanical damage.

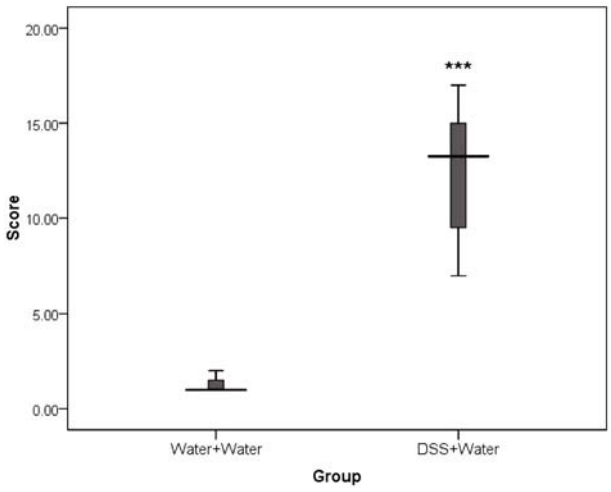

Fig. 1. Histological damage severity of distal colon in control (Water +Water n=9) and DSS-induced colitis (DSS+Water n=10) rats. Data are expressed as median damage severity score (range). *** indicates P<0.001 compared to control (Water+Water).

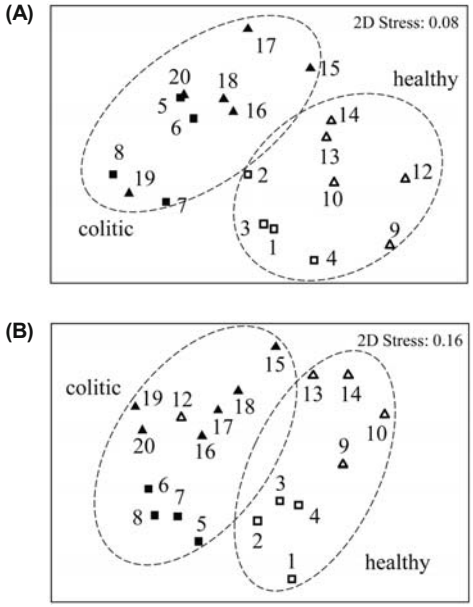

Fig. 2. nMDS ordination of faecal bacterial communities identified by experimental set and disease state. Faecal bacterial communities are identified by healthy (open symbol) and DSS-induced colitic states (filled symbol), as well as, by experimental set I (open and filled square) and set II (open and filled triangle). Numbers indicate rat identification. (A) Faecal bacterial communities generated with *Msp*I. (B) Faecal bacterial communities generated with *Cfo*I. The ordination is based on Bray-Curtis similarities calculated from fourth-root transformed OTU abundances (29 OTU for *Msp*I and 50 OTU for *Cfo*I). nMDS ordinations attempt to place all samples in an arbitrary two-dimensional space such that their relative distances apart match the corresponding pairwise similarities. Hence, the closer two samples are in the ordination, the greater the similarity of their overall faecal bacterial communities.

composition in healthy and colitic rats. Multivariate statistical analysis of OTU generated with either restriction enzyme showed that the composition of the faecal bacterial community was significantly different between healthy and colitic rats (Global R=0.702, P<0.001; *Msp*I and Global R= 0.598, P<0.001; *Cfo*I). Furthermore, faecal bacterial communities were significantly different between rats from the two sets of experiments, regardless of restriction enzyme used (Global R=0.307, P=0.002; *Msp*I and Global R=0.629, P<0.001; *Cfo*I). The differences between disease states and between experimental sets are illustrated in the nMDS ordination for both the *Msp*I (Fig. 2A) and *Cfo*I (Fig. 2B) generated bacterial profiling data. When rats were identified by disease state, the overriding influence of DSS treatment on faecal bacterial community composition was apparent. Faecal microbial profiles of DSS-treated rats (7, 15, 17, and 19) with a DAI of zero (Table 1) grouped closer to the microbial profiles of other DSS-treated rats than the healthy control rats (Fig. 2). The faecal microbial profile of the control rat (12) with a DAI=1 (Table 1) grouped closer to the other control rats when analyzed with *Msp*I (Fig. 2A), but closer to the DSS-treated rats when analyzed with *Cfo*I (Fig. 2B).

Similarities in faecal bacterial community composition among the healthy and colitic rats were calculated with SIMPER for both the *Msp*I and *Cfo*I bacterial profiling analysis. Amongst the healthy rats, the average similarities in faecal bacterial communities were 33.0% and 35.2% for *Msp*I and *Cfo*I, respectively. SIMPER analysis of the faecal microbiota in colitic rats showed a higher similarity of 41.9% with *Cfo*I, but not *Msp*I (33.8%). The average dissimilarity of OTU between healthy and colitic rats was 90.5% and 71.8% with *Msp*I and *Cfo*I, respectively. The OTU identified as contributing significantly to the dissimilarity in faecal bacterial composition between healthy and DSS-induced colitic rats with *Msp*I were 94, 118, 188, and

Table 2. OTU contribution to the dissimilarity in faecal bacterial communities between healthy and DSS-induced colitic rats (*Msp*I analysis)

OTU	Average abundance		$\bar{\delta}i$	$\bar{\delta}i/SD(\delta i)$	Individual contribution (%)	Cumulative contribution (%)
	Healthy	Colitic				
188*	1.75	0.00	9.45	1.59	10.44	10.44
94*	0.24	1.86	9.15	1.55	10.11	20.55
914*	0.00	1.48	7.94	1.11	8.77	29.32
118*	1.58	0.58	7.75	1.21	8.57	37.89
120	1.03	0.21	5.38	0.89	5.95	43.83
898	0.49	0.63	4.67	0.77	5.16	48.99

Average abundances of OTU in faecal bacterial communities of healthy control rats and rats with DSS-induced colitis are presented. OTU are listed in order of their contribution ($\bar{\delta}i$) to the average dissimilarity (δ=90.5%) between the healthy and colitic state. The percentage contribution of the individual OTU and the cumulative percentage contribution to the top 50% of average dissimilarities are depicted. OTU identified as good discriminators between healthy and colitic states are marked with an asterisk.

Table 3. OTU contribution to the dissimilarity in faecal bacterial communities between healthy and DSS-induced colitic rats (*Cfo*I analysis)

OTU	Average abundance		$\bar{\delta}i$	$\bar{\delta}i$/SD (δi)	Individual contribution (%)	Cumulative contribution (%)
	Healthy	Colitic				
360*	1.54	0.73	3.46	1.23	4.82	4.82
94*	0.58	1.39	3.20	1.34	4.46	9.28
116*	1.10	0.15	3.15	1.25	4.39	13.67
98*	1.38	2.37	3.04	1.15	4.24	17.91
362	1.04	0.30	3.03	0.98	4.22	22.13
174*	0.70	1.49	3.03	1.31	4.22	26.35
948*	1.00	0.00	2.83	1.01	3.94	30.30
226*	1.40	0.81	2.67	1.18	3.72	34.01
384*	0.47	1.04	2.57	1.25	3.58	37.59
192	0.65	0.64	2.53	0.98	3.53	41.12
188	0.35	0.68	2.27	0.91	3.16	44.28
206	0.75	0.16	2.15	0.90	2.99	47.28

Average abundances of OTU in faecal bacterial communities of healthy control rats and rats with DSS-induced colitis are presented. OTU are listed in order of their contribution ($\bar{\delta}i$) to the average dissimilarity (δ=71.8%) between the healthy and colitic state. The percentage contribution of the individual OTU and the cumulative percentage contribution to the top 50% of average dissimilarities are depicted. OTU identified as good discriminators between healthy and colitic states are marked with an asterisk.

914 (Table 2). OTU 118 and 188 were mainly associated with faecal specimens from healthy rats; whereas 94 and 914 were more closely associated with faecal samples from rats with induced colitis. The OTU identified as contributing significantly to the dissimilarity in faecal bacterial composition between healthy and DSS-induced colitic rats with *Cfo*I were 94, 98, 116, 174, 226, 360, 384, and 948 (Table 3). OTU 116, 226, 360, and 948 were mainly associated with faeces from healthy rats; whereas 94, 98, 174, and 384 were more closely associated with faecal samples from rats with induced colitis.

Individual faecal bacterial profiles of rats varied within a treatment, but the differences between disease states were greater for both restriction enzymes analyzed. The contribution that OTU made to the overall bacterial profiles of individual animals is shown for *Msp*I (Supplementary data Fig. S1) and *Cfo*I (Supplementary data Fig. S2). The closer association of OTU 118 and 188 with healthy animals and OTU 94 and 914 with colitic animals for the *Msp*I analysis was evident in both sets of experiments (Supplementary data Fig. S1). Similarly, the close association of OTU 116, 226, 360, and 948 with healthy rats and OTU 94, 98, 174, and 384 with colitic rats in both experimental sets is apparent with *Cfo*I (Supplementary data Fig. S2). The number of OTU characterized by *Msp*I (n=29) were lower than those characterized by *Cfo*I (n=50).

PAT was used to assign potential species matches to our T-RFLP data. Total potential matches identified were to 5,875 bacterial sequences, of which 5,165 were uncultured bacteria. Table 4 summarizes the possible identity of OTU determined by SIMPER analysis as being significantly different between the healthy control and DSS-induced colitic rat groups. Only bacteria identified by both the *Msp*I and *Cfo*I SIMPER analysis, as differing between treatment groups, and of faecal or gut origin, are listed. OTU 98 (*Cfo*I) and 94 (*Msp*I) were mainly associated with the DSS-induced colitic rats. PAT assignment of this OTU combination identified potential *Allistipes*, *Bacteroides*, *Butyricimonas*, *Odoribacter*, and *Parabacteroides* species, all belonging to the order Bacteroidales. OTU 360 (*Cfo*I) and 188 (*Msp*I) were associated with healthy control rats and potentially represented an uncultured rumen bacterium belonging to the order

Table 4. T-RFLP PAT output for faecal samples collected from healthy control and DSS-induced colitic rats[a]

T-RFLP PAT match and accession number	OTU		Detected in rat[†]	RDP classification	Closest culturable bacteria (NCBI BLASTn identity)
	*Cfo*I	*Msp*I			
Alistipes sp. (EU728719)	98	94	2, 5-8, 16, 18-20	*Alistipes*	*Alistipes finegoldii* DSM 17242 (99%)
Bacteroides intestinalis (AB214328)	98	94	2, 5-8, 16, 18-20	*Bacteroides*	*Bacteroides intestinalis* DSM 17393 (100%)
Butyricimonas synergistica (AB443948)	98	94	2, 5-8, 16, 18-20	*Butyricimonas*	*Butyricimonas synergistica* JCM 15148 (100%)
Butyricimonas virosa (AB443949)	98	94	2, 5-8, 16, 18-20	*Butyricimonas*	*Butyricimonas virosa* JCM 15149 (100%)
Bacteroides splanchnicus (EU728709)	98	94	2, 5-8, 16, 18-20	*Odoribacter*	*Odoribacter splanchnicus* DSM 20712 (99%)
Porphyromonadaceae bacterium (EU728718)	98	94	2, 5-8, 16, 18-20	*Parabacteroides*	*Parabacteroides distasonis* JCM 13400 (93%)
uncultured rumen bacterium (DQ394701)	360	188	2, 4, 10, 13-14	Clostridiales	*Syntrophococcus sucromutans* DSM 3224 (89%)
uncultured bacterium (EU452462)	948	188	2, 4, 9-10, 12-14	*Lactobacillus*	*Lactobacillus johnsonii* FI9785 (99%)
uncultured bacterium (EU458027)	948	188	2, 4, 9-10, 12-14	*Lactobacillus*	*Lactobacillus johnsonii* FI9785 (99%)
uncultured bacterium (EU504607)	948	188	2, 4, 9-10, 12-14	*Lactobacillus*	*Lactobacillus johnsonii* FI9785 (99%)
uncultured bacterium (EF096341)	948	188	2, 4, 9-10, 12-14	*Lactobacillus*	*Lactobacillus johnsonii* FI9785 (99%)

[a] The phylogenetic assignments are sorted by *Cfo*I OTU size. The output indicates which two OTU generated with *Msp*I and *Cfo*I were used to determine phylogenetic assignment. Only OTU identified with both *Msp*I and *Cfo*I SIMPER analysis as being significantly associated with a particular treatment are listed.
[†] Rats containing significant OTU are listed. Rats 1–8 are from experimental set I and rats 9–20 are from experimental set II. Rats 1–4 and 9–14 are healthy control rats and 5–8 and 15–20 are from the DSS-induced colitic group.

Clostridiales. OTU 948 (*Cfo*I) and 188 (*Msp*I), associated with healthy rats, potentially represented uncultured bacteria belonging to the genus *Lactobacillus* and showed 99% sequence identity with *Lactobacillus johnsonii*.

Discussion

Colitis was induced in rats to determine whether changes in the gut microbiota associated with the colitic state could be detected in faeces. We used T-RFLP analysis to investigate changes in overall faecal bacterial community composition between healthy control and DSS-induced colitic rats. From the histological assessment of damage severity in the colon it was apparent that all rats from the healthy control group showed no overt features of colitis, whilst all DSS-induced colitic rats exhibited pathological features consistent with colitis. T-RFLP profiling of the faecal bacterial communities of healthy control and DSS-induced rats showed significant differences between these two groups. Interestingly, faecal bacterial profiles from four DSS-induced rats with a DAI score of zero were more similar to the faecal bacterial profiles of the other DSS-treated rats which exhibited physical symptoms consistent with colitis. These four DSS-treated rats were confirmed to have histological features consistent with colitis. This indicates that faecal T-RFLP profiling may be a more sensitive non-invasive tool than DAI scoring for investigating changes associated with colitis.

In addition to the differences observed in faecal bacterial community composition between healthy controls and colitic rats, there were also significant differences between the experimental sets from which faecal samples were sourced. This suggests other factors were also at play in influencing the gut microbiota composition. These could have included genetics (litter differences between the two experimental sets), as well as variations in environmental rearing conditions and the microbiological status of feed and water. Despite these differences, the same core OTU could be identified as being associated with the healthy and colitic state among both experimental sets of rats.

In the present investigation there was significant inter-individual variation in faecal bacterial composition among both healthy and colitic rats. Others have also noted that gut microbial T-RFLP profiles vary substantially between individuals, as each sample exhibits a unique community profile, consisting of a different assemblage of OTU (Nagashima *et al*., 2003; Dicksved *et al*., 2007; Li *et al*., 2007). This inter-individual variation has been proposed to be responsible for the inability to clearly cluster samples based on treatment (Zoetendal *et al*., 2002). Indeed, studies using cluster analysis of T-RFLP data have been unable to discriminate differences in faecal microbiota between healthy patients and those with Crohn's disease or ulcerative colitis (Sepehri *et al*., 2007) or to identify differences in faecal microbiota associated with lifestyle or environmental changes (Dicksved *et al*., 2007). However, using nMDS we have been able to successfully show differences in faecal microbial communities using T-RFLP data, despite the high inter-individual variability. Unlike clustering analysis, nMDS does not group samples into discrete clusters, but instead, displays their interrelations on a continual scale (Clarke and Warwick, 2001).

Interestingly, in the current study, the similarity in faecal microbiota composition amongst the colitic animals was higher (41.9%) than for healthy rats (35.2%) with *Cfo*I but not with *Msp*I. This may have been a consequence of the superior resolving power of T-RFLP with *Cfo*I, as it generated greater numbers of OTU compared with *Msp*I (50 as compared with 29). Decreased richness or diversity of bacterial species has been reported widely in human patients with ulcerative colitis and Crohn's disease, as well as in dogs with IBD (Manichanh *et al*., 2006; Andoh *et al*., 2007; Frank *et al*., 2007; Xenoulis *et al*., 2008) which would be consistent with our observation of a higher faecal microbial similarity in the colitic rats. However, in the current study it should be noted that the faecal bacterial profile generated with *Cfo*I digestion from one healthy control rat was aligned more closely with the colitic group, which would have contributed to the lower similarity in microbiota composition among the healthy rats. Closer examination of this rat to eliminate a subclinical colitis, later confirmed its healthy status based on histological parameters. It could therefore be hypothesized that, from its faecal microbial community composition, this healthy rat may have been more susceptible to the development of colitis under appropriate environmental and physiological conditions. If confirmed, this could have important implications for the potential identification of individuals at risk of developing IBD.

Our report that the faecal microbiota varied significantly between DSS-treated colitic and healthy control rats is consistent with observations made by others when comparing faecal microbial communities from healthy individuals with patients suffering from either IBD or colitis (Andoh *et al*., 2007; Takaishi *et al*., 2008). Although many reports have suggested individual bacterial species are linked to the clinical manifestation of IBD or colitis; there is little consistent evidence for the involvement of specific microbial groups (Marteau *et al*., 2003; Steed *et al*., 2008). *Gammaproteobacteria* have been reported to be increased in the microbiota of both experimentally induced colitic animals and IBD patients, as a result of more favorable conditions for these bacteria during inflammation (Mai *et al*., 2006; Heimesaat *et al*., 2007; Lupp *et al*., 2007; Xenoulis *et al*., 2008), while increased numbers of *Bacteroides* spp. and *E. coli* have been reported in IBD, ulcerative colitis and Crohn's disease patients (Gorbach *et al*., 1968; Hartley *et al*., 1992; Swidsinski *et al*., 2005; Takaishi *et al*., 2008). Our *in-silico* results for phylogentic assignment of significant OTU identified between healthy and colitic rats indicated that OTU combination 98 (*Cfo*I) and 94 (*Msp*I), predominantly associated with the DSS-induced colitic rats, may have represented *Bacteroides* spp. This is consistent with previous reports in IBD (Hartley *et al*., 1992; Swidsinski *et al*., 2005; Takaishi *et al*., 2008). However, this OTU combination may also represent other bacterial species (*Allistipes*, *Butyricimonas*, *Odoribacter*, and *Parabacteroides* spp.) within the order Bacteroidales, or as yet unidentified bacteria. OTU combination 948 (*Cfo*I) and 188 (*Msp*I) were predominantly associated with healthy rats and could represent an uncultured *Lactobacillus* sp. Indeed, the probiotic formu-

lation, VSL#3, which has demonstrated therapeutic utility in IBD, includes four different lactobacilli: *L. acidophilus*, *L. plantarum*, *L. casei*, and *L. bulgaricus*. These lactobacilli presumably contribute to the restoration of homeostasis to the dysbiosis characteristic of IBD (Bibiloni *et al.*, 2005).

We propose that OTU combinations, and not the single OTU, are the major determinants of the healthy or colitic state. OTU (94, 98, 116, 174, 226, 360, 384, and 948; *Cfo*I) and (94 and 118; *Msp*I) were identified in both healthy and colitic rats but with different relative abundance while OTU 948 (*Cfo*I) and 188 (*Msp*I) were specific to healthy rats and OTU 914 (*Msp*I) was solely detected in DSS-induced colitic rats. Thus, changes in the proportion of common OTU are most likely to be associated with the development of IBD. Future research should expand studies of these typical OTU in an attempt to unravel the underlying mechanism of colitis. Using T-RFLP and multivariate statistical analyses we have identified potential OTU driving differences in bacterial community composition. OTU (116, 226, 360, and 948; *Cfo*I) and (118 and 188; *Msp*I) were predominantly associated with the healthy state while OTU (94, 98, 174, and 384; *Cfo*I) and (94 and 914; *Msp*I) were predominantly associated with the colitic state. The current study did not seek to definitively identify the faecal bacterial genera or species; instead detecting OTU associated with the healthy or colitic states. Future studies could address definitive identification of OTU identified in this study using novel strategies for the extraction of specific phylogenetic sequence information from community T-RFLP data based on targeted isolation, cloning and sequencing of terminal restriction fragments (Widmer *et al.*, 2006; Lee *et al.*, 2008).

In conclusion, T-RFLP analysis has proven to be a highly sensitive and non-invasive tool for detecting microbial changes in faeces of rats with experimentally induced colitis. More specifically, the dissimilarity in faecal bacterial community composition was greater in healthy rats, compared to those with colitis, in which the faecal bacterial composition was more homogenous. Potential OTU were identified as being more closely associated with either a healthy or diseased state. These OTU could be species or taxonomically related groups of bacteria, which could form the basis of future investigations. Targeting the colitis associated OTU by specific antibiotic and/or probiotic intervention could form the basis of a novel therapeutic strategy for IBD. Combining determinations of faecal microbial community composition through T-RFLP, with other morphological, biochemical or molecular analyses, provides a powerful approach to better understand the etiology and pathogenesis of IBD.

Acknowledgements

The authors are grateful to Haydn Atkins for providing experimental rat faecal samples for the T-RFLP analysis. Professor Gordon S. Howarth is supported by the Sally Birch Cancer Council Australia Research Fellowship in Cancer Control. Dr. Ashis Samanta was supported by an Endeavour Fellowship. We would like to acknowledge the South Australian Research and Development Institute for use of laboratory facilities.

References

Andoh, A., Sakata, S., Koizumi, Y., Mitsuyama, K., Fujiyama, Y., and Benno, Y. 2007. Terminal restriction fragment length polymorphism analysis of the diversity of fecal microbiota in patients with ulcerative colitis. *Inflamm. Bowel Dis.* **13**, 955–962.

Bibiloni, R., Fedorak, R.N., Tannock, G.W., Madsen, K.L., Gionchetti, P., Campieri, M., De Simone, C., and Sartor, R.B. 2005. VSL#3 probiotic-mixture induces remission in patients with active ulcerative colitis. *Am. J. Gastroenterol.* **100**, 1539–1546.

Bjerketorp, J., Chiang, A.N.T., Hjort, K., Rosenquist, M., Liu, W.T., and Jansson, J.K. 2008. Rapid lab-on-a-chip profiling of human gut bacteria. *J. Microbiol. Methods* **72**, 82–90.

Bousvaros, A., Morley, F.A., Pensabene, L., and Cucchiara, S. 2008. Research and clinical challenges in pediatric inflammatory bowel disease. *Dig. Liver. Dis.* **40**, 32–38.

Bray, J.R. and Curtis, K.R. 1957. An ordination of the upland forest communities of southern Wisconsin. *Ecol. Monogr.* **27**, 325–349.

Clarke, K.R. 1993. Non-parametric multivariate analyses of changes in community structure. *Austr. J. Ecol.* **18**, 117–143.

Clarke, K.R. and Warwick, R.M. 2001. Changes in marine communities: an approach to statistical analysis and interpretation. 2nd ed. Primer F Ltd., Plymouth, UK.

Dethlefsen, L., Mcfall-Ngai, M., and Relman, D.A. 2007. An ecological and evolutionary perspectives on human microbe mutualism and disease. *Nature* **449**, 811–818.

Dicksved, J., Floistrup, H., Bergstrom, A., Rosenquist, M., Pershagen, G., Scheynius, A., Roos, S., Alm, J.S., Engstrand, L., Braun-Fahrlander, C., and *et al.* 2007. Molecular fingerprinting of the fecal microbiota of children raised according to different lifestyles. *Appl. Environ. Microbiol.* **73**, 2284–2289.

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S.R., Nelson, K.E., and Relman, D.A. 2005. Diversity of the human intestinal microbial flora. *Science* **308**, 1635–1638.

Egert, M., Marhan, S., Wagner, B., Scheu, S., and Friedrich, M.W. 2004. Molecular profiling of 16S rRNA genes reveals diet-related differences of microbial communities in soil, gut, and casts of *Lumbricus terrestris* L. (Oligochaeta: Lumbricidae). *FEMS Microbiol. Ecol.* **48**, 187–197.

Fiocchi, C. 1998. Inflammatory bowel disease: etiology and pathogenesis. *Gastroenterol.* **155**, 182–205.

Frank, D.N., Amand, A.L.S., Feldman, R.A., Boedeker, E.C., Harpaz, N., and Pace, N.R. 2007. Molecular phylogenetic characterization of microbial community imbalance in human inflammatory bowel diseases. *Proc. Natl. Acad. Sci. USA* **104**, 13780–13785.

Geier, M.S., Butler, R.N., Giffard, P.M., and Howarth, G.S. 2007. *Lactobacillus fermentum* BR11, a potential new probiotic, alleviates symptoms of colitis induced by dextran sulfate sodium (DSS) in rats. *Int. J. Food Microbiol.* **114**, 267–274.

Gorbach, S.L., Nahas, L., Plaut, A.G., Weinstein, L., Patterson, J.F., and Levitan, R. 1968. Studies of intestinal microflora. V. Faecal microbial ecology in ulcerative colitis and regional enteritis: relationship to severity of disease and chemotherapy. *Gastroenterol.* **54**, 575–587.

Hartley, M.G., Hudson, M.J., Swarbrick, E.T., Hill, M.J., Gent, A.E., Hellier, M.D., and Grace, R.H. 1992. The rectal mucosa associated microflora in patients with ulcerative colitis. *J. Med. Microbiol.* **36**, 33–39.

Heimesaat, M.M., Fischer, A., Siegmund, B., Kupz, A., Niebergall, J., Fuchs, D., Jahn, H.K., Freudenberg, M., Loddenkemper, C., Batra, A., and *et al.* 2007. Shifts towards proinflammatory intestinal bacteria aggravate acute murine colitis via toll like receptors 2 and 4. *PLoS One* **2**, e662.

Kent, A.D., Smith, D.J., Benson, B.J., and Triplett, E.W. 2003. A

web-based phylogenetic assignment tool for analysis of terminal restriction fragment length polymorphism profiles of microbial communities. *Appl. Environ. Microbiol.* **69**, 6768–6776.

Kruskal, J.B. 1964. Multidimensional scaling by optimizing a goodness of fit to a nonmetric hypothesis. *Psychometrics* **29**, 1–28.

Lane, D.J. 1991. 16S/23S rRNA sequencing, pp. 115–175. *In* Stackebrandt, E. and Goodfellow, M. (eds.), Nucleic acid techniques in bacterial systematics. John Wiley and Sons Inc., New York, USA.

Lee, H.K., Kim, H.R., Mengoni, A., and Lee, D.H. 2008. Modified T-RFLP methods for taxonomic interpretation of T-RF. *J. Microbiol. Biotechnol.* **18**, 624–630.

Li, F., Hullar, M.A.J., and Lampe, J.W. 2007. Optimization of terminal restriction fragment polymorphism (TRFLP) analysis of human gut microbiota. *J. Microbiol. Methods* **68**, 303–311.

Lupp, C., Robertson, M.L., Wickham, M.E., Sekirov, I., Champion, O.L., Gaynor, E.C., and Finlay, B.B. 2007. Host mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of *Enterobacteriaceae*. *Cell. Host Microbe* **2**, 119–129.

Mai, V., Braden, C.R., Heckendorf, J., Pironis, B., and Hirshon, J.M. 2006. Monitoring of stool microbiota in subjects with diarrhea indicates distortions in composition. *J. Clin. Microbiol.* **44**, 4550–4552.

Manichanh, C., Rigottier-Gois, L., Bonnaud, E., Gloux, K., Pelletier, E., Frangeul, L., Nalin, R., Jarrin, C., Chardon, P., Marteau, P., and *et al.* 2006. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. *Gut* **55**, 205–211.

Marteau, P., Seksik, P., and Shanahan, F. 2003. Manipulation of the bacterial flora in inflammatory bowel disease. *Best Pract. Res. Clin. Gastroenterol.* **17**, 47–61.

Munkholm, P., Langholz, E., Davidsen, M., and Binder, V. 1995. Disease activity courses in a regional cohort of Crohn's disease patients. *Scand. J. Gastroenterol.* **30**, 699–706.

Murthy, S.N., Cooper, H.S., Shim, H., Shah, R.S., Ibrahim, S.A., and Sedergran, D.J. 1993. Treatment of dextran sulphate sodium induced murine colitis by intracolonic cyclosporine. *Dig. Dis. Sci.* **38**, 1722–1734.

Muyzer, G., Teske, A., Wirsen, C.O., and Jannasch, H.W. 1995. Phylogenetic relationships of *Thiomicrospira* species and their identification in deep sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Arch. Microbiol.* **164**, 165–172.

Nagashima, K., Hisada, T., Sato, M., and Mochizuki, J. 2003. Application of new primer-enzyme combination to terminal restriction fragment length polymorphism profiling of bacterial populations in human feces. *Appl. Environ. Microbiol.* **69**, 1251–1262.

Scanlan, P.D., Shanahan, F., O'Mahony, C., and Marchesi, J.R. 2006. Culture independent analyses of temporal variation of the dominant fecal microbiota and targeted bacterial subgroups in Crohn's disease. *J. Clin. Microbiol.* **44**, 3980–3988.

Sellon, R.K., Tonkonogy, S., Schultz, M., Dieleman, L.A., Grenther, W., Balish, E., Rennick, D.M., and Sartor, R.B. 1998. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin 10 deficient mice. *Infect. Immun.* **66**, 5224–5231.

Sepehri, S., Kotlowski, R., Bernstein, C.N., and Krause, D.O. 2007. Microbial diversity of inflamed and noninflamed gut biopsy tissues in inflammatory bowel disease. *Inflamm. Bowel. Dis.* **13**, 675–683.

Shepard, R.N. 1962. The analysis of proximities: multidimensional scaling with an unknown distance function: parts I and II. *Psychometrics* **27**, 125–140.

Shyu, C., Soule, T., Bent, S.J., Foster, J.A., and Forney, L.J. 2007. MiCA: A web-based tool for the analysis of microbial communities based on terminal-restriction fragment length polymorphisms of 16S and 18S rRNA Genes. *J. Microb. Ecol.* **53**, 562–570.

Steed, H., Macfarlane, G.T., and Macfarlane, S. 2008. Prebiotics, synbiotics and inflammatory bowel disease. *Mol. Nutr. Food Res.* **52**, 898–905.

Stirling, G.R., Griffin, D., Ophel-Keller, K., McKay, A., Hartley, D., Curran, J., Stirling, A.M., Monsour, D., Winch, J., and Hardie, B. 2004. Combining an initial risk assessment process with DNA assays to improve prediction of soilborne diseases caused by root-knot nematode (*Meloidogyne* spp.) and *Fusarrium oxysporum* f. sp. *lycopersici* in the Queensland tomato industry. *Aus. Plant Pathol.* **33**, 285–293.

Strober, W., Fuss, I., and Mannon, P. 2007. The fundamental basis of inflammatory bowel disease. *J. Clin. Invest.* **117**, 514–521.

Swidsinski, A., Loening-Baucke, V., Lochs, H., and Hale, L.P. 2005. Spatial organization of bacterial flora in normal and inflamed intestine: a fluorescence *in situ* hybridization study in mice. *World J. Gastroenterol.* **11**, 1131–1140.

Takaishi, H., Matsuki, T., Nakazawa, A., Takada, T., Kado, S., Asahara, T., Kamada, N., Sakuraba, A., Yajima, T., Higuchi, H., and *et al.* 2008. Imbalance in intestinal microflora constitution could be involved in the pathogenesis of inflammatory bowel disease. *Int. J. Med. Microbiol.* **298**, 463–472.

Tamboli, C.P., Neut, C., Desreumaux, P., and Colombel, J.F. 2004. Dysbiosis in inflammatory bowel disease. *Gut* **53**, 1–4.

Tannock, G.W. 2007. What immunologists should know about bacterial communities of the human bowel. *Semin. Immunol.* **19**, 94–105.

Taurog, J.D., Richardson, J.A., Croft, J.T., Simmons, W.A., Zhou, M., Fernandez-Sueiro, J.L., Balish, E., and Hammer, R.E. 1994. The germfree state prevents development of gut and joint inflammatory disease in HLA-B27 transgenic rats. *J. Exp. Med.* **180**, 2359–2364.

Tomas, F.M., Knowles, S.E., Owens, P.C., Read, L.C., Chandler, C.S., Gargosky, S.E., and Ballard, F.J. 1991. Increased weight gain, nitrogen retention and muscle protein synthesis following treatment of diabetic rats with insulin like growth factor (IGF)-1 and des (1-3) IGF-1. *Biochem. J.* **276**, 547–554.

Torok, V.A., Hughes, R.J., Ophel-Keller, K., Ali, M., and MacAlpine, R. 2009. Influence of different litter materials on cecal microbiota colonization in broiler chickens. *Poult. Sci.* **88**, 2474–2481.

Torok, V.A., Ophel-Keller, K., Loo, M., and Hughes, R.J. 2008. Application of methods for identifying broiler chicken gut bacterial species linked with increased energy metabolism. *Appl. Environ. Microbiol.* **74**, 783–791.

Widmer, F., Hartmann, M., Frey, B., and Kolliker, R. 2006. A novel strategy to extract specific phylogenetic sequence information from community T-RFLP. *J. Microbiol. Methods* **66**, 512–520.

Xenoulis, P.G., Palculict, B., Allenspach, K., Steiner, J.M., Van House, A.M., and Suchodolski, J.S. 2008. Molecular phylogenetic characterization of microbial communities imbalances in the small intestine of dogs with inflammatory bowel disease. *FEMS Microbiol. Ecol.* **66**, 579–589.

Yazbeck, R., Howarth, G.S., Geier, M.S., Demuth, H.U., and Abbott, C.A. 2008. Inhibiting dipeptidyl peptidase activity partially ameliorates colitis in mice. *Front. Biosci.* **13**, 6850–6858.

Zoetendal, E.G., von Wright, A., Vilpponen-Salmela, T., Ben-Amor, K., Akkermans, A.D.L., and de Vos, W.M. 2002. Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from faces. *Appl. Environ. Microbiol.* **68**, 3401–3407.