

## Effect of Obstructive Jaundice and Nitric Oxide on the Profiles of Intestinal Bacterial Flora in Wild and iNOS<sup>-/-</sup> Mice

Ji-Young Hong, Eisuke F. Sato\*, Tomoko Nishikawa, Keiichi Hiramoto, and Masayasu Inoue

Department of Biochemistry and Molecular Pathology, Osaka City University Medical School,  
1-4-3 Asahimachi, Abeno, Osaka 545-8585, Japan

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**Summary** We previously reported that the plasma level of endotoxin and colonic expression of IgA in the mouse increased with obstructive jaundice induced by bile duct ligation (BDL). To elucidate the mechanism of the BDL-induced increase, we analyzed the effect of BDL on intestinal flora in wild type and inducible nitric oxide synthase (iNOS)-deficient mice (iNOS<sup>-/-</sup>) using the terminal restriction fragment length polymorphism analysis (T-RFLP) and 16S rDNA clone libraries. The amounts of bacterial DNA detected in fecal samples from both animal groups pretreated with antibiotics were extremely low as compared with untreated groups. We found that the profiles of enteric bacteria changed markedly after BDL. The bacterial composition is significantly different between not only wild type and iNOS<sup>-/-</sup> mice but also those before and after BDL, respectively. Among enteric bacteria examined, *Lactobacillus murinus* was found to increase markedly after BDL in rectum of both animal groups. However, *Escherichia coli* markedly increased after BDL in the iNOS<sup>-/-</sup> mice. These findings suggest that profiles of enteric flora change markedly in animals during obstructive jaundice by some mechanism that is affected by bile constituents and iNOS-derived NO.

**Key Words:** intestinal flora, endotoxemia, obstructive jaundice, nitric oxide, T-RFLP

### Introduction

The composition of the intestinal microbial flora is relatively stable and unique for each species and individual [1]. The commensal microbial flora in gastrointestinal tract plays important roles in the maintenance and regulation of animal health and nutrition, respectively. Under physiological conditions, the presence of enteric microbiota is important for the protection of animals against potential pathogens [2]. Hence, disruption of the ecological balance in intestinal lumen gives rise to clinical complications [3, 4].

It has been suggested that bile acids are one of the important factors that affect the ecological balance of intestinal flora. In fact, the translocation of intestinal bacteria across

the mucosal layer increases markedly and sometimes causes lethal endotoxemia in patients with obstructive jaundice [5–8]. Changes in intestinal flora sometimes modulate the population of lymphocytes that secrete Th2-type cytokines and antibodies, such as IL-10 and IgA, respectively [9]. We previously demonstrated that obstructive liver injury was inhibited by pretreatment of animals with antibiotics [10]. Kinetic analysis revealed that disturbance of the ecological balance of enteric bacteria played critical roles in the regulation of immunological network among liver, spleen and intestine, and in the pathogenesis of liver injury during obstructive jaundice. However, the pathological significance of the bile duct ligation (BDL)-induced changes in the profiles of bacterial flora remains unknown. We also reported that BDL increased endotoxin levels in plasma more markedly with inducible nitric oxide synthase deficient (iNOS<sup>-/-</sup>) mice than with wild-type animals and this increase was strongly inhibited by antibiotics [10]. Since nitric oxide (NO) has a potent antibacterial activity, bacterial composi-

\*To whom correspondence should be addressed.  
Tel: +81-6-6645-3722 Fax: +81-6-6645-3721  
E-mail: sato@med.osaka-cu.ac.jp

tion in the intestinal lumen would be affected by luminal occurrence of NO. To examine the role of iNOS-derived NO in the profiles of bacterial flora, we used iNOS<sup>-/-</sup> mice in this study.

It has been practically difficult for a long time to characterize the composition of intestinal microbiota predominantly due to its large variations among individuals and a lack of appropriate methods. For example, classic methods based on cell culture are useful for the detection of less than 40% of the microorganisms predominantly due to the difficulty to handle anaerobic bacteria in the intestine [11]. However, the recent development of molecular techniques for the analysis of complex microbial communities bypassed the necessity of cell culture and made considerable progress in the characterization of intestinal bacteria [1, 3, 12, 13]. The terminal restriction fragment length polymorphism analysis (T-RFLP) is a useful method for the assessment of the differences in the composition of intestinal microbial communities [3, 14–16].

To elucidate the mechanism underlying the pathological changes induced by BDL, we analyzed the profiles of fecal bacteria in C57BL and iNOS<sup>-/-</sup> mice before and after BDL using TRFLP and 16S rDNA clone libraries.

## Materials and Methods

### Reagents

A Kit for the isolation of DNA from mouse feces and primers 7F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTGTACGACTT-3') were obtained from MO BIO laboratories Inc. (Carlsbad, CA) and SIGMA Genosis (Japan), respectively. TaKaRa Ex Taq<sup>TM</sup> (1,000 U) and TA Cloning<sup>®</sup> Kit and One Shot TOP 10 Chemically Competent *E. coli* were obtained from Takara Bio (Shiga, Japan) and Invitrogen<sup>TM</sup> (Carlsbad, CA), respectively. Wizard Plus SV Minipreps DNA Purification System and Montage<sup>TM</sup> PCR was obtained from Promega (Madison, WI) and Millipore (Bedford, MA), respectively. Mouse monoclonal antibody against iNOS (610432), rabbit anti-iNOS polyclonal antibody (KAS-NO001) were obtained from BD Transduction Laboratories (San Jose, CA) and Stressgen Biotechnologies (Victoria, Canada), respectively. Other reagents used were of the highest grade commercially available.

### Animal experiments

Male C57BL/6J mice (20–25 g) and their iNOS<sup>-/-</sup> strain were purchased from SLC (Shizuoka, Japan) and Jackson Laboratories (Caldspring Harbor, MA), respectively. Animals were allowed free access to laboratory chow (CE-2, Oriental Yeast Co., Tokyo) and water *ad libitum* during the experiments. All experiments were approved by the Institutional Animal Care and Use Committee of Osaka City University

Medical School. Obstructive jaundice was elicited by the common BDL as described previously [17]. Under light ether anesthesia, animals were subjected to BDL. In the control sham group, sham-operation was performed. At indicated times after performing BDL, animals were sacrificed to obtain blood and feces. The collected fecal samples were stored at -80°C until analysis.

### DNA extraction from fecal samples

Extraction and purification of DNA were carried out according to the methods described by Clement and Kitts [18] using an Ultra Clean Soil DNA isolation kit as described previously [19].

### PCR amplification for TRFLP analysis

A pair of universal primers, 27F and 1492R [20] was used for PCR amplification and 27F was labeled with 6-carboxyfluorescein (6-FAM). PCR was performed with a Thermocycler T Gradient (Biometra, Göttingen, Germany) in 50 µl of reaction mixture containing 5 µl of dissolved DNA (100 ng), 1.25 U of TaKaRa *Ex Taq*, 10 × *Ex Taq* buffer, 4 µl of dNTP mixture (2.5 mM each), and 10 pmol of each primer. The amplification program used was as follows: preheating at 95°C for 3 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1.5 min; terminal extension at 72°C for 10 min [19, 21]. The amplified DNA was verified by 1.5% agarose gel electrophoresis. PCR products were purified using a Montage<sup>TM</sup> PCR (Millipore). The purified PCR products were stored at -20°C until analysis.

### TRFLP analysis

The restriction enzymes used were selected according to the methods of Liu *et al.* [15] and Moyer *et al.* [22]. Purified PCR products (4 µl) were digested with 20 U of either *HhaI* or *MspI* (Takara Bio) in a total volume of 10 µl at 37°C for 24 h. The lengths of the terminal restriction fragments (T-RFs) were determined using a standard size marker GS500 ROX and 1000 ROX (Applied Biosystems, Carlsbad, CA) in an ABI PRISM<sup>TM</sup> 3100 genetic analyzer (Applied Biosystems) and GeneScan<sup>®</sup> Analysis Software (Applied Biosystems). The peak of T-RFs was determined using TRFLP analysis program (TAP) [23].

### PCR amplification of 16S rDNA gene sequences and cloning

The samples used for cloning and sequencing were obtained from mice before and 7 day after BDL. The 16S rDNA gene was amplified using two universal primers 27F and 1492R [19, 20]. PCR was performed as described previously [24] and [19]. The amplified 16S rDNA genes were purified using an Ultra Clean PCR Clean-up Kit (Mo Bio Laboratories). The purified amplicons were cloned into pCR<sup>®</sup> 2.1 vectors (Invitrogen, San Diego, CA), and One

Shot® INVaF' competent cells were transformed [19]. The transformants were randomly picked and purified with Wizard® Plus SV Minipreps DAN Purification System (Promega).

#### DNA sequencing and phylogenetic analysis

Cycle sequencing was performed with a Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), 27F or 1492R primers, and ABI PRISM™ 3100 Genetic Analyzer (Applied Biosystems). All sequences were compared with similar sequences of the reference organisms by using BLAST search [25].

#### Histological analysis

The colon specimens were rapidly frozen in an OCT embedding medium (Tissue-Tek, Elkhart, IN) and stored at  $-80^{\circ}\text{C}$  until use. Cryostat sections (6  $\mu\text{m}$  thickness) were fixed in ice-cold acetone for 10 min. The expression of iNOS was evaluated immunohistochemically under a fluorescent microscope as described previously [26, 27]. Rabbit anti-iNOS polyclonal antibody (KAS-NO001) was obtained from Stressgen Biotechnologies (Victoria, Canada).

#### Western blot analysis

The colon was rinsed with saline and homogenized in a lysis buffer containing 0.5% Nonidet P-40, 10% glycerol, 137 mM NaCl, 2 mM ethylenediamine-tetraacetic acid, and 50 mM Tris-HCl buffer (pH 8.0). After centrifugation at  $3,000 \times g$  for 10 min, the supernatant was separated and stored at  $-80^{\circ}\text{C}$ . The stored specimens were subjected to 7.5% polyacrylamide gel electrophoresis (PAGE) in the presence of 0.1% SDS. The electrophoresed proteins in the gel were transferred to an Immobilon membrane (Millipore). The membrane was blocked with 5% skim milk at  $4^{\circ}\text{C}$  for overnight, subsequently incubated with primary antibodies at  $25^{\circ}\text{C}$  for 1 h and then with horseradish peroxidase-conjugated secondary antibodies. Immune complexes thus formed were detected with ECL reagents (Amersham Bioscience, Buckinghamshire, UK).

## Results

#### Analysis of fecal bacterial community by TRFLP

Although bacterial DNA was easily detected in fecal samples from wild and iNOS<sup>-/-</sup> mice, it was practically difficult to detect DNA from both animal groups pretreated with antibiotics (Fig. 1). TRFLP patterns of the wild and iNOS<sup>-/-</sup> mice were compared using two different restriction enzymes before and after BDL (Fig. 2 and 3). The profiles of bacterial community in the feces from wild and iNOS<sup>-/-</sup> mice changed markedly after BDL. The profiles of bacterial distribution in the feces from the rectum and the cecum also differed significantly after BDL. After digestion with *HhaI*,

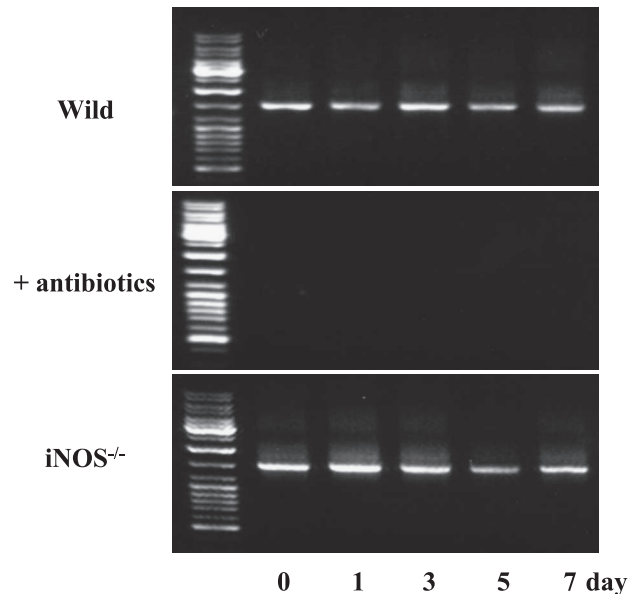


Fig. 1. Bacterial DNA extraction from fecal samples. DNA was extracted from 50 mg of the fecal sample. A pair of universal primers, 27F (5'-AGAGTTTGATCCTGGCT-CAG-3') and 1492R (5'-GGTACCTTGTTACGACTT-3'), were used for PCR amplification. The amplified DNA was verified by 1.5% agarose gel electrophoresis.

terminal restriction fragments (T-RFs) at bp 249 and 370, and bp 370 were detected in the feces from rectum and cecum of wild type mice after BDL. In contrast, *HhaI* digestion revealed T-RFs at bp 233, 249, and 370, and bp 197 and 208 in the feces from the rectum and cecum of iNOS<sup>-/-</sup> mice that had been subjected to BDL (Fig. 2). *MspI* digestion revealed T-RFs at bp 491, 492 and 565, and bp 491 in the feces from the rectum and cecum of BDL-treated wild type mice. *MspI* digestion revealed T-RFs at bp 492, 568 and 583, and bp 223 and 269 in the fecal sample from the rectum and cecum of BDL-treated iNOS<sup>-/-</sup> mice (Fig. 3).

#### BDL induced expression of iNOS in the colon

Colonic expression of iNOS markedly increased after BDL. As shown in Fig. 4 and 5, the expression of iNOS in the wild type mice increased after BDL, peaked on day 3, and decreased thereafter. Naturally, no iNOS was induced by BDL in the knockout mice.

#### Identification of bacterial 16S rDNA gene

The sequences of 16S rDNA clones were used to identify enteric bacterial profiles in the feces from the wild type and iNOS<sup>-/-</sup> mice before and after BDL (Table 1). Among fifty clones from the inoculum analyzed, no exact 16S rDNA gene similarity limits exist to define specific taxa, such as genus and species. The definition of species generally requires sequence similarities of greater than 98% [28]. The

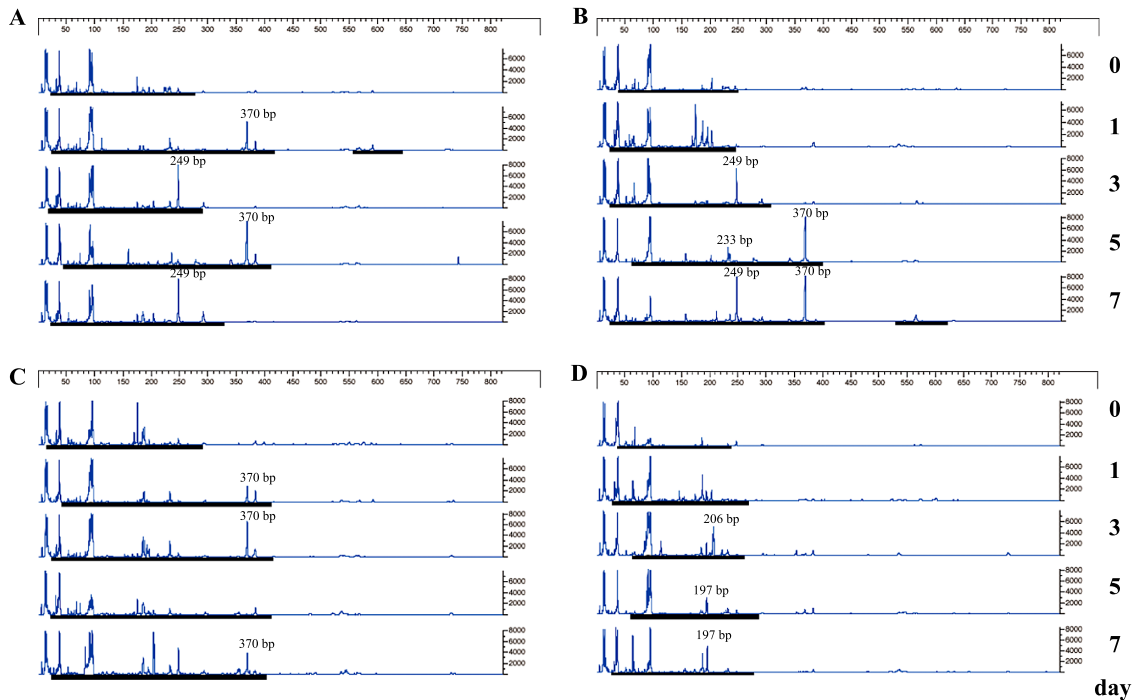


Fig. 2. T-RFLP analysis of intestinal microbiota before and after BDL. Data shows T-RFLP patterns of 16S rDNA from mouse feces (day 0~7) digested with *HhaI*. 16S rDNA were amplified using universal primers 27F and 1492R. The minimum and maximum values of the ordinate of each T-RFLP pattern are 0 to 800 fluorescence intensity (arbitrary units) for *HhaI*. Fecal samples from the rectum of (A) wild and (B) *iNOS*<sup>-/-</sup> mice, and the cerum of (C) wild and (D) *iNOS*<sup>-/-</sup> mice

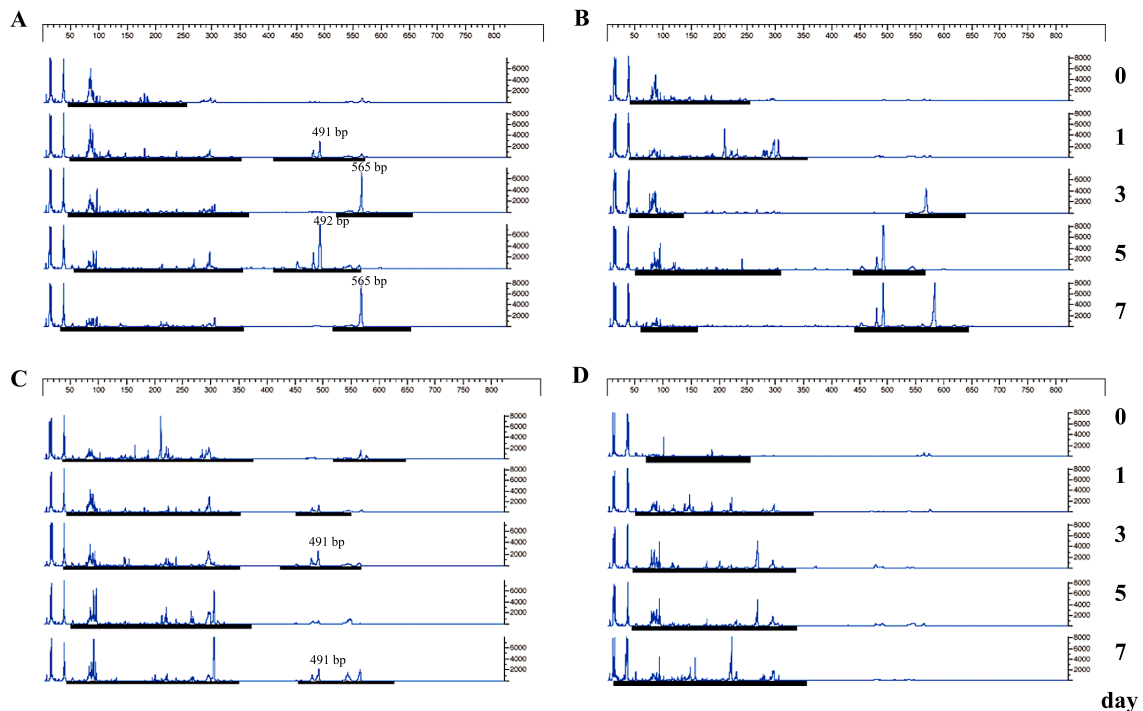


Fig. 3. T-RFLP analysis of intestinal microbiota before and after BDL. Data shows T-RFLP patterns of 16S rDNA from mouse feces (day 0~7) digested with *MspI*. Other conditions were same as in Fig. 2.

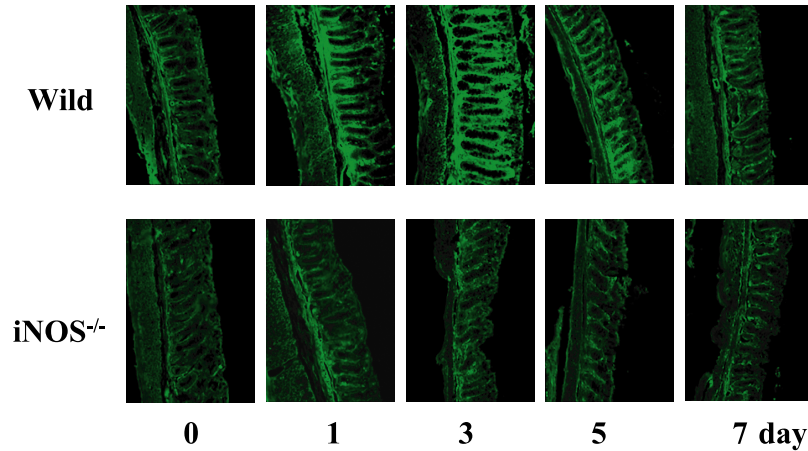


Fig. 4. Histological examination of the expression of iNOS in the colon. At the indicated times after BDL, colon specimens were frozen, cut into thin sections, treated with anti-iNOS antibody and then stained with FITC-conjugated second antibody. Scale bar = 50  $\mu$ m

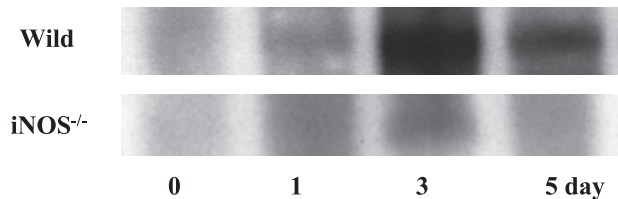


Fig. 5. Western blotting analysis of expression of iNOS in the colon. At the indicated times after BDL, colon specimens were collected. Each amount of protein (30  $\mu$ g/lane) from colon was subjected to a 7.5% SDS-PAGE, and the electrophoresed proteins were transferred onto Immobilon-P membranes, and analyzed using anti-iNOS antibody. The bands with 130 kDa responsible for iNOS were seen in colon specimens from BDL mice. Data show one typical result out of 3 representative experiments.

compositions of enteric bacteria from wild type mice differed significantly from that of iNOS<sup>-/-</sup> mice; both of them changed markedly after BDL. Among various bacteria observed, *Lactobacillus murinus* in the rectum markedly increased after BDL both in wild type and iNOS<sup>-/-</sup> mice. On day 7 after BDL, total numbers of *Lactobacillus murinus* in the rectum were found to be about 35.0% and 30.2% of the flora in wild type and iNOS<sup>-/-</sup> mice, respectively. *Bacteroides acidofaciens* in the rectum of wild animals showed no detectable changes even after BDL. In contrast, *Escherichia coli* in the rectum of iNOS<sup>-/-</sup> mice markedly increased after BDL (Table 1). On day 7 after BDL, *Bacteroides intestinalis*, Clone aab24h05, and *Bacteroides acidofaciens* in the cecum of wild animals increased to 30%, 12%, and 10% of the total number of enteric bacteria, respectively (Table 1).

## Discussion

The present work shows that bacterial composition in the intestinal lumen was different in wild type and iNOS<sup>-/-</sup> mice, and both of them changed markedly after BDL. Bile acids have been known to inhibit bacterial overgrowth and their translocation across the intestinal mucosa [5, 6, 29]. Since NO has a potent antibacterial activity, bacterial composition in the intestinal lumen would be affected by luminal occurrence of NO.

The population of *E. coli* in the intestine increased after BDL in iNOS<sup>-/-</sup> mice but not in wild type mice. Although, the lifetime of NO has been postulated to be extremely short (several seconds) particularly under air atmospheric conditions, it is fairly stable under low oxygen tensions [30–32]. It should be noted that oxygen is the substrate for NOS and, hence, the rate of NO synthesis may decrease under physiological low oxygen tensions in the intestinal lumen. Thus, the enhancement of NO action by low oxygen tensions may occur minimally in anaerobic compartments, such as in the large intestinal lumen. In this context, the  $K_m$  value of iNOS for molecular oxygen has been reported to be about  $6.3 \pm 0.9 \mu$ M [33]. Therefore, macrophages and neutrophils not only in the circulation but also in the intestinal mucosa might generate reactive oxygen species including NO [34, 35]. Because of a gaseous nature of NO, it easily diffuses from a mucosal site of generation into intestinal lumen. Thus, NO generated by the activated leukocytes in an inflammatory lesion of the intestine might reach to its luminal space where a large number of enteric bacteria live. We previously reported that endotoxin levels in mouse plasma increased after BDL; its increase was more marked in iNOS<sup>-/-</sup> than in the wild mice [10]. The increased endotoxin in BDL-treated iNOS<sup>-/-</sup> mice might reflect the change

Table 1. Identification by 16S rDNA sequences analysis

	Wild	(%)	iNOS <sup>-/-</sup>	(%)	
rectum	Clone Adhufec 33 ml	10.4	Clone R-1264 (2)	16.7	
	Uncultured rumen bacterium gene	5.1	Clone aab23g09	11.1	
	Bacteroides acidofaciens	5.1	Clone F3	11.1	
	Day 0	Clone R-1264(2)	5.1	Clone aab21h12	8.3
	Clone ratBN020109C	5.1	Clone M1-g02-3	8.3	
	The others (each less than 4%)	69.2	Clone aab42c05	5.1	
			Lactobacillus johnsonii NCC 533	5.1	
			The others (each less than 4%)	33.3	
	Day 7	Lactobacillus murinus	35.0	Lactobacillus murinus	30.2
	Clone aab24h05	7.5	Escherichia coli	25.6	
	Clone M3-c08-3	5.0	Shigella boydii strain 5216-70	7.0	
	Bacteroides acidofaciens	5.0	The others (each less than 4%)	37.2	
	The others (each less than 4%)	47.5			
	cecum	Clone aab22a11	7.3	Clone M2-a05	11.4
Lactobacillus johnsonii NCC 533		7.3	Clone aab23f05	8.6	
Bacteroides acidofaciens		4.9	Mucispirillum schaedleri strain HRI I 17	5.7	
Day 0		Clone M1-b06-1	4.9	The others (each less than 4%)	74.3
Clone aab21h12		4.9			
The others (each less than 4%)		70.7			
Day 7		Bacteroides intestinalis	30.0	Clone 28-2 small subunit	12.9
Clone aab24h05		12.0	Clone aab 24a06	6.5	
Bacteroides acidofaciens		10.0	The others (each less than 4%)	80.6	
Uncultured Gram-positive bacterium		6.0			
Lactobacillus murinus		4.0			
Escherichia coli		4.0			
Clone abc 38b10.x1		4.0			
The others (each less than 4%)		30.0			

Values show the percentage of bacterial species obtained from the rectum and cecum of wild and iNOS<sup>-/-</sup> mice.

in intestinal flora, such as increased *E. coli*.

Interestingly, *Lactobacillus murinus* markedly increased after BDL both in wild type and iNOS<sup>-/-</sup> mice. Since the growth of *Lactobacilli* and *Bifidobacteria* were inhibited by bile acids [36], it is not surprising that *L. murinus* increased markedly after BDL. *Lactobacilli* are a major component of the commensal microbial flora in the intestine both of humans and animals [37]. Such probiotic bacteria have been shown to give beneficial effects including improvement of intestinal health, enhancement of the immunological activity, synthesis of nutrients and reduction of lactose intolerance, allergic reactions, and risk of cancers [37–39]. Although the mechanisms by which such probiotics exert their beneficial effects are largely unknown, it may involve regulation of luminal pH, and competition for available nutrients and growth factors with pathogens. We previously reported that obstructive liver injury was inhibited by the administration of antibiotics and that enteric bacteria play critical roles in the pathogenesis of liver injury induced by

obstructive jaundice [10]. However, the role of *L. murinus* in the pathological events in the intestine of BDL-treated animals remains obscure.

It should be noted that large numbers of unknown bacteria not cultured successfully are living in the intestine and play a role in the modulation of intestinal diseases. Thus, TRFLP is a useful method for the assessment of the changes in intestinal microbial flora under physiological as well as pathological conditions.

The present work suggests that bacterial composition in the intestinal lumen changes significantly after BDL by a mechanism that is affected by iNOS-derived NO.

Recent studies revealed that metagenome analysis is also an efficient and convenient method for the analysis of enteric bacteria. Pathophysiological significance of BDL-induced changes in intestinal flora should be studied further using more convenient methods.

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## Abbreviations

T-RFLP, terminal restriction fragment length polymorphism analysis; BDL, bile duct ligation; iNOS, inducible nitric oxide synthase; NO, nitric oxide.

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