***Bifidobacterium bifidum* BF-1 suppresses *Helicobacter pylori*-induced genes in human epithelial cells**

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ABSTRACT

Helicobacter pylori infection alters gene expression in host cells. Specifically, inflammatory chemokines such as IL-8 are upregulated in the gastric mucosa during *H. pylori* infection. Although the mechanism by which *H. pylori* causes inflammation of the gastric mucosa is not yet understood, many studies have suggested that nuclear factor kappa B (NF- κ B) plays a key regulatory role in host cells. We have shown that preincubation with *Bifidobacterium bifidum* strain BF-1, a probiotic strain known to improve *H. pylori*-associated gastritis, suppresses induction of IL-8 by the pathogen. To investigate how BF-1 affects gene expression in *H. pylori*-infected cells, we performed microarray analysis to assess gene expression in epithelial cells, which had been preincubated with BF-1 and infected with *H. pylori*. We found that preincubation with BF-1 suppresses the expression of *H. pylori*-induced genes in human cells and that most of the affected genes are related to the NF- κ B signaling pathways. These results suggest that BF-1 can affect the regulatory mechanism of the NF- κ B signaling pathways.

Key words: *Bifidobacterium bifidum*, *Helicobacter pylori*, probiotics

INTRODUCTION

Chronic *Helicobacter pylori* infection of the gastric mucosa affects approximately half of the world's population, leads to chronic gastritis and peptic ulcer disease, and is linked to gastric carcinoma (Morris and Nicholson, 1987; Uemura et al., 1997). The oral administration of 3 or 4 antibiotics and a proton pump inhibitor is currently recommended to eradicate *H. pylori*; however, increasing antimicrobial resistance has diminished the efficacy of standard eradication regimens (Selgrad et al., 2009). A combination therapy, including both antibiotics and probiotics, is proposed as an alternative to standard eradication therapy. For patients who are

infected with *H. pylori* but remain asymptomatic, the risk of therapeutic failure and adverse events associated with standard antibiotic therapy may make coexistence with the pathogen the preferred option, particularly if the pathogenic effects of *H. pylori* can be suppressed by probiotics.

Probiotics are defined as live microorganisms that confer a health benefit on the host; they are considered a useful alternative to avoid overuse of antibiotics. Thus far, most studies on probiotics have focused on the intestinal flora, but studies of probiotic effects on other organs have begun to appear, particularly in the field of gastric diseases. Several evaluation trials of probiotic therapies on *H. pylori*-infected humans have been performed (Sakamoto et al., 2001; Wang et al., 2004; Lesbros-Pantoflickova et al., 2007; Boonyaritichai et al., 2009; Worona-Dibner, 2009). Multi-species probiotic preparations have been shown to have beneficial effects against *H. pylori* infections (Myllyluoma et al., 2008). Pretreatment with *Lactobacillus*- and *Bifidobacterium*-containing yogurt improved the efficacy of quadruple antibiotic therapy in eradicating residual *H. pylori* infection after failed triple therapy (Sheu et al., 2006). Systematic reviews and meta-analyses have shown that fermented milk-based probiotic preparations improve *H. pylori* eradication rates by 5 to 15% (Sachdeva and Nagpal, 2009).

Along with lactic acid bacteria, *Bifidobacteria* are used in the production of yogurt and are known to have probiotic effects (Shimakawa et al., 2003; Picard et al., 2005). *Bifidobacteria* are known to adhere to epithelial cells (Bernet et al., 1993; Gueimonde et al., 2005; Guglielmetti et al., 2008). The anti-ulcer effects of *Bifidobacterium bifidum* YIT 4007 have been shown using acetic acid-induced gastric ulcer and ethanol-induced erosion models in rats (Nagaoka et al., 1994). The administration of *B. bifidum* dry powder has been successfully used to treat *H. pylori*-positive gastric ulcer patients; in 3 out of 10 cases, *H. pylori* was successfully eradicated from the gastric mucous membrane (Nagaoka et al., 2003). *Bifidobacterium bifidum* BF-1 (BF-1) was isolated from *B. bifidum* YIT 4007 as an oxygen-resistant strain; a randomized, double-blind, placebo-controlled comparative study has shown that

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consumption of BF-1-fermented milk improved *H. pylori*-induced gastric symptoms. It was also shown that *H. pylori*-induced IL-8 secretion was suppressed by preincubation of a human gastric carcinoma cell line, GCIY, with BF-1 (Miki et al., 2007). The inflammatory cytokine IL-8 is induced by *H. pylori* infection, and is known to be specifically involved in the recruitment and activation of neutrophils, thus playing an important role in the gastric inflammation that accompanies neutrophil infiltration (Crabtree et al., 1994). In addition to IL-8, many other inflammatory cytokines and cellular adhesive factors seem to be involved in gastric inflammation (Yamaoka et al., 1998). It is believed that nuclear factor- κ B (**NF- κ B**) plays a key role in the regulation of these genes (Sharma et al., 1998). The mechanism by which *H. pylori* causes inflammation is not yet understood, but many reports have shown that various inflammatory responses in the gastric mucosa are mediated by NF- κ B (Maeda et al., 2001).

In this study, we analyzed the suppression mechanism of BF-1 by investigating the whole gene expression profile of BF-1 pretreated and *H. pylori*-infected cells using a 2-color microarray method. Quantitative analyses were performed using real-time PCR to confirm the microarray results.

MATERIALS AND METHODS

Bacterial Culture

Helicobacter pylori YIT 10238 (ATCC 43504), YIT 11123 (ATCC 700392), and YIT 10057 (clinical isolate) strains were obtained from the culture collection of Yakult Central Institute of Microbiological Research (Tokyo, Japan) and cultured in Brucella broth (Becton Dickinson Co., Sparks, MD) with 10% heat-inactivated horse serum under microaerobic conditions using AnaeroPack Helico (Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan) with mild shaking (140 rpm) at 37°C for 48 h. *Bifidobacterium bifidum* BF-1 was cultured in modified International Lactobacilli Shirota (mILS) broth (Shimakawa et al., 2003) under anaerobic conditions using AnaeroPack (Mitsubishi Gas Chemical Co. Inc.).

Cell Culture

Cells from a human gastric epithelial cell line GCIY (RCB0555; Nozue et al., 1991) were obtained from Riken Cell Bank (Tokyo, Japan) and cultured in Eagle's minimal essential medium (**MEM**; Nissui, Osaka, Japan) containing 15% fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA) with 100 U/mL of penicillin G and 100 μ g/mL of streptomycin sulfate at 37°C in humidified air containing 5% CO₂.

Infection Procedures

Helicobacter pylori YIT 10238 and BF-1 were cultured as described above, washed, and resuspended in MEM without antibiotics. *Helicobacter pylori* infection studies were performed with GCIY cells in vitro. The GCIY cells, which were maintained in MEM supplemented with 15% fetal serum, were seeded into collagen-coated, 6-well tissue culture plates and grown to confluence at 37°C. Then, BF-1 at a concentration of 10⁹ cfu/mL or control medium was added to the tissue culture plates and co-incubated with GCIY cells. After 3 h of incubation, cells were washed 3 times with medium; then, *H. pylori* at 10⁷ cfu/mL or control medium was added to the cells and incubated for 4 or 6 h.

RNA Extraction

Infected cells were washed 3 times with MEM and treated with RNeasy lysis buffer (Qiagen, Düsseldorf, Germany) for 16 h to stabilize RNA. After removing the RNeasy lysis buffer, total RNA was extracted using the RNeasy Mini kit (Qiagen). Quality of RNA was evaluated with the Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA) and RNA yield was determined spectrophotometrically.

Microarray Procedures and Data Analysis

A whole human genome oligo DNA microarray kit 4x44k (Agilent Technologies), which contains 4 independent microarrays on one slide, and 60-mer oligo probe sets were used to monitor the expression of more than 41,000 human transcripts per microarray. Total RNA was reverse-transcribed and labeled using the Agilent Low RNA Input Linear Amplification Kit PLUS, Two-Color to produce cyanine 3(**Cy3**)- or cyanine 5(**Cy5**)-labeled cRNA. The cRNA were purified with RNeasy columns and quantified. The sample was fragmented and suspended in Hybridization Buffer HI-PRM (Agilent) before application to the microarray for hybridization. The microarray was incubated for 17 h at 65°C with 10 rpm rotation in a hybridization chamber. After hybridization, the microarray was washed with 37°C prewarmed Gene Expression Wash Buffer (Agilent), acetonitrile (Sigma-Aldrich Co., St. Louis, MO), and Stabilization and Drying solution (Agilent). Once dry, the microarray was scanned using the Agilent 2505B scanner, and image processing was performed using the Feature Extraction V9.5 software (Agilent). Data were analyzed by GeneSpring GX7.3 software (Agilent). A first set of microarrays was used for an experiment with a 4-h *H. pylori* incubation. A second set was used for a dye exchange experiment to eliminate dye bias effects

with a 4-h *H. pylori* incubation. A third set was used for an experiment with a 6-h *H. pylori* incubation.

IL-8 Assay by ELISA

The GCIY cells were cultured in a 96-well plate as described above. The indicated concentrations of BF-1 or medium were added to the cells and incubated for 3 h. Then, the sheet of cells was rinsed 3 times with fresh medium and incubated with *H. pylori* at 10^7 cfu/mL or control medium for 20 h. Clarified culture supernatants were analyzed for IL-8 protein with Immunoassay Kit Human IL-8/NAP1 (Biosource, Invitrogen) as directed by the manufacturer. Data are the means $\pm$ standard deviations of the means ($n = 3$).

Real-Time PCR

Primers for PCR were designed with Primer Express software (Applied Biosystems Inc., Foster City, CA) according to the transcription sequence data of each gene obtained from Ensembl (<http://www.ensembl.org/index.html>). The following were used as the PCR primers: IL-8 forward, 5'-CACCGGAAGGAACCA-TCTCA-3', and IL-8 reverse, 5'-AGAGCCACGGC-CAGCTT-3'; NF- κ B forward, 5'-AAATCTAATGCT-GGGATGAAGCA-3', and NF- κ B reverse, 5'-CA-CAACCTTCAGGGTCCTTTT-3'; myeloid differentiation primary response gene (88) (MyD88) forward, 5'-GAGCTGGCGGGCATCAC-3', and MyD88 reverse, 5'-TCGAAACGCTCAGGCATATG-3'; GAPDH forward, 5'-ATGGAAATCCCATCACCATCTT-3', and GAPDH reverse, 5'-CGCCCCACTTGATTTTGG-3'.

Total RNA was reverse-transcribed using the PrimeScript RT reagent Kit (Takara Bio Inc., Shiga, Japan), and 10 ng of cDNA per reaction was labeled by using Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. Quantitative analyses were performed using real-time PCR on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Results of the different RNA samples were normalized for RNA quantity using the housekeeping gene GAPDH.

Statistical Analysis and Microarray Data Deposition

Statistical differences were analyzed by paired Student's *t*-test. The entire collection of microarray data from this study were deposited in the Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/projects/geo/>; accession no.: GSE20171).

RESULTS

H. pylori Induction and BF-1 Suppression of IL-8 in Epithelial Cells

Secretion of IL-8 from GCIY cells was quantified by ELISA. Figure 1 shows that preincubation with BF-1 suppresses IL-8 induction in a dose-dependent manner. In the presence of BF-1 at 10^9 cfu/mL, IL-8 production induced by *H. pylori* strains YIT 10238, YIT 11123, and YIT 10057 was suppressed by 40, 46, and 33%, respectively ($P < 0.001$), confirming the results of previous experiments performed with YIT 10238 (Miki et al., 2007).

Microarray Analyses

Microarray analyses were performed to establish whole gene expression profiles of human gastric GCIY cells with and without BF-1 preincubation and *H. pylori* YIT 10238 infection. A slide set contains 4 independent microarrays, and the 2-color method was employed to compare the different conditions. Table 1 shows the combination of samples in each array.

In microarray 1 of the first set, the gene expression in the control cells [pretreated and incubated with medium (Cy3)] versus the infected cells [pretreated with medium (Cy5)] were compared. Of all human genes, 838 genes were shown to be upregulated more than 2.0-fold in the presence of *H. pylori* infection. We have excluded unannotated genes from the list and categorized them by function. We found that many known inflammatory genes were activated as expected, and several gene clusters of inflammatory genes could serve as signatures for *H. pylori* infection, including genes encoding chemokines, chemokine receptors, fibroblast growth factor, interleukins, tumor necrosis factor (TNF) superfamily proteins, matrix metalloproteinases, and cell adhesion molecules (Table 2). As expected, IL-8 was strongly activated.

In microarray 2, gene expression profiles from BF-1 pretreated, noninfected cells (Cy3) and BF-1 pretreated, *H. pylori*-infected cells (Cy5) were compared. The genes induced by *H. pylori* in array 1 were also upregulated in array 2; however, we found that the extent of upregulation was reduced for most of the *H. pylori*-induced genes listed in Table 2. The expression of IL-8 was suppressed drastically. These results suggest that BF-1 preincubation is responsible for the suppression of *H. pylori*-induced gene expression. These characteristic gene signatures were confirmed and selected as described in the following section.

Figure 1. Effect of *Bifidobacterium bifidum* BF-1 on IL-8 secretion (ng/mL) of human gastric carcinoma (GCIY) cells. After preincubation of BF-1 at 10^7 (E7), 10^8 (E8), and 10^9 (E9) cfu/mL or medium, *Helicobacter pylori* at 10^7 cfu/mL or medium was added. The amount of secreted IL-8 was assayed by ELISA. Data are the means $\pm$ standard deviations of the means ($n = 3$). * $P < 0.001$ and ** $P < 0.0001$ compared with the results for preincubation with medium.

To confirm and further characterize the effect of BF-1, microarray 3 compared gene expression profiles in 2 sets of *H. pylori*-infected cells, only one of which was pretreated with BF-1 (Cy5). The suppression of upregulated genes appeared as downregulated genes in this array; these results supported the results of microarray 2.

Microarray 4 demonstrated that preincubation with BF-1 does not significantly (>2 fold) affect the expression of inflammatory genes (Cy5). Expression of IL-8 was not detected in these cells.

The second slide set was used for a dye-exchange experiment after 4 h of *H. pylori* infection. The results were the mirror image of the first set, and no dye bias was observed, lending support to the validity of the results from the first set of slide. The third slide set was used to characterize samples after 6 h of *H. pylori* infection. Some differences in the degree of activation or inactivation were observed, most likely because of the difference in incubation time; however, the overall patterns of gene expression were the same (data are available from GEO).

Selection of Genes with Expression Profiles Matching IL-8

In each of the 4 microarrays, IL-8 exhibited a characteristic profile; the gene was induced by *H. pylori* and suppressed by BF-1 and was not activated in the presence of BF-1 alone. A group of genes exhibiting a profile similar to that of IL-8 throughout the 4 microarrays was selected using the GeneSpring GX7.3 software (Agilent). Typical patterns of those genes are depicted in Figure 2, which include the data of slide 2. The selected genes and their normalized intensities are listed in Table 3; these include chemokines, cytokines, TNF superfamily proteins, and adhesion molecules, all of which are consistent with those listed in Table 2. The list of these overlapping genes and the ratio of normalized expression of array 1 and array 2 are shown in Table 4. These results suggest that BF-1 can suppress many types of inflammatory processes as well as IL-8. It is also clear that most of these genes are known to be regulated by NF- κ B or are involved in the NF- κ B network. This strong positive correlation

Table 1. Sample labeling combination of microarrays

Microarray	Cyanine 3		Cyanine 5	
	Pretreatment	Treatment	Pretreatment	Treatment
1	Medium	Medium	Medium	<i>Helicobacter pylori</i>
2	<i>Bifidobacterium bifidum</i> BF-1	Medium	<i>B. bifidum</i> BF-1	<i>H. pylori</i>
3	Medium	<i>H. pylori</i>	<i>B. bifidum</i> BF-1	<i>H. pylori</i>
4	Medium	Medium	<i>B. bifidum</i> BF-1	Medium

between BF-1-suppressed genes and NF- κ B-regulated genes may suggest that BF-1 affects the regulation of NF- κ B pathways.

Real-Time PCR

The IL-8 microarray expression data were confirmed by quantitative real-time PCR. Figure 3a shows the relative expression of the gene: IL-8 was upregulated more than 49-fold at 4 h after *H. pylori* infection. With BF-1 preincubation, upregulation was reduced by 33% ($P = 0.04$).

Because the up- and downregulation of NF- κ B-related genes was not quantifiable by microarray analysis,

the expression levels of NF- κ B and MyD88 were also quantified by real-time PCR (Figure 3b). Expression of NF- κ B increased 2-fold by *H. pylori* and this increase was suppressed 87% by BF-1 preincubation (results statistically insignificant). Expression of MyD88 was not upregulated by *H. pylori* infection but was suppressed 59% ($P = 0.03$) by BF-1 preincubation.

DISCUSSION

Bifidobacterium bifidum BF-1 has been shown to be effective in improving *H. pylori*-infected gastric symptoms. To understand the mechanism by which BF-1 mediates its beneficial effects, we investigated gene

Table 2. Differentially expressed genes in *Helicobacter pylori*-infected cells in microarray 1

Gene name ¹	Normalized expression	GenBank accession no.
Adhesion molecules		
VCAM-1	7.0	NM_001078
ICAM-1	14.1	NM_000201
Laminin, α 3 (LAMA3)	5.8	NM_198129
Laminin, γ 2 (LAMC2)	2.3	NM_005562
OLR1	6.0	NM_002543
Chemokines and chemokine receptors		
CCL2	15.6	NM_002982
CCL5/RANTES	8.2	NM_002985
CCL20/LARC	26.1	NM_004591
CXCL1/Gro- α	13.9	NM_001511
CXCL2/Gro- β	6.8	NM_002089
CXCL3/Gro- γ	16.4	NM_002090
CXCL5/ENA-78	4.0	NM_002994
CXCL10	6.0	NM_001565
CX3CL1	3.4	NM_002996
CXCR4/fusin	8.0	NM_001008540
Interleukins		
IL-8	80.0	NM_000584
IL-23A	2.9	NM_016584
Growth factor and receptors		
Fibroblast growth factor (FGF)18	14.8	NM_003862
Tumor necrosis factor (TNF) superfamily and receptors		
LTB	8.3	NM_002341
TNF- α	28.6	NM_000594
TNFAIP2	7.1	NM_006291
TNFAIP3	6.3	NM_006290
TNFAIP6	2.0	NM_007115
TNFRSF9	3.3	NM_001561
TNFRSF1B	2.1	NM_001066
TNFRSF10B	2.8	NM_003842
TRAF1	5.5	NM_005658
Others		
ITGB8	2.2	BC042028
Metalloproteinase 1 (MMP1)	2.8	NM_002421
Metalloproteinase 7 (MMP7)	2.9	NM_002423
Metalloproteinase 9 (MMP9)	4.1	NM_004994
NFKBIA	6.0	NM_020529

¹VCAM-1 = vascular cell adhesion molecule-1; ICAM-1 = intercellular adhesion molecule-1; OLR1 = oxidized low-density lipoprotein receptor 1; CCL = chemokine ligands; CXCR = chemokine receptor; RANTES = regulated upon activation, normal T cell expressed and secreted; LARC = liver activation regulated chemokine; Gro = growth-regulated protein; ENA-78 = epithelial cell-derived neutrophil-activating peptide 78; LTB = leukotriene B; TNFAIP = TNF- α -induced protein; TNFRSF = TNF receptor superfamily; TRAF = TNF receptor-associated factor; ITGB8 = integrin beta-8; NFKBIA = nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha.

Table 3. Normalized intensity (log scale) of the genes in each microarray (1 to 4)

Gene name ¹	Slide 1 (green/red dye)				Slide 2 (red/green dye)			
	1	2	3	4	1	2	3	4
VCAM-1	7.016	4.589	0.765	1.187	0.114	0.17	1.279	0.891
ICAM-1	14.12	8.167	0.905	1.475	0.0609	0.0863	1.088	0.882
LAMA3	7.283	5.181	1.279	1.89	0.161	0.168	1.115	2.774
LAMC2	2.259	1.139	0.541	0.975	0.293	0.431	1.147	0.533
OLR1	6.024	4.916	0.776	1.229	0.15	0.252	1.34	1.44
CCL5	8.193	6.777	1.156	1.103	0.134	0.145	1.371	1.473
CCL20	26.12	13.7	0.852	1.026	0.025	0.027	0.719	0.451
CXCL1	13.85	11.39	0.419	0.667	0.118	0.0403	0.773	0.594
CXCL2	6.797	4.963	1.09	0.979	0.163	0.144	1.184	1.256
CXCL3	6.182	5.154	0.991	1.034	0.207	0.14	1.243	1.301
CXCL5	3.983	2.603	1.099	0.516	0.303	0.201	1.466	0.716
CXCL10	5.954	2.897	0.534	ND ²	0.211	0.395	2.19	1.588
CX3CL1	3.396	2.97	0.88	1.078	0.334	0.342	0.985	1.445
CXCR4	8.039	3.535	0.542	0.943	0.0737	0.124	1.005	0.537
IL-8	80.47	14.53	0.436	ND	0.01	0.0158	0.994	0.944
IL-23A	2.881	1.076	0.361	0.674	0.132	0.209	0.86	0.379
FGF18	14.75	8.541	0.779	0.751	0.0699	0.0611	1.43	0.631
LTB	8.286	6.627	0.98	1.097	0.141	0.157	1.323	0.833
TNF- α	28.65	11.75	0.937	1.141	0.0518	0.0593	0.983	0.935
TNFAP2	7.09	4.561	0.695	1.558	0.151	0.267	1.222	0.809

¹VCAM-1 = vascular cell adhesion molecule-1; ICAM-1 = intercellular adhesion molecule-1; LAMA3 = laminin, α 3; LAMC2 = laminin, γ 2; OLR1 = oxidized low-density lipoprotein receptor 1; CCL = chemokine ligands; CXCR = chemokine receptors; LTB = leukotriene B; TNF- α = tumor necrosis factor- α .

²ND = not detected.

Figure 2. Normalized intensities [cyanine (Cy)5/Cy3] of genes. The genes that have the same profile as IL-8 in 4 microarrays were selected using GeneSpring GX7.3 software (Agilent Technologies Inc., Santa Clara, CA). Each line represents a gene. A solid line represents IL-8 and dotted lines represent other genes. The left graph shows IL-8-like genes in slide 1 and the right graph shows IL-8-like genes in slide 2.

expression in infected cells in the presence and absence of BF-1. Infection with *H. pylori* induces gastric inflammatory responses. Comprehensive microarray analyses of human gastric cells have shown that various genes are upregulated in *H. pylori*-infected gastric cells. We confirmed these results, demonstrating by ELISA that IL-8 expression is suppressed by BF-1 pretreatment in infection models with 3 *H. pylori* strains. Microarray and real-time PCR data also support these results. The real-time PCR experiment demonstrated that IL-8 expression is activated up to 49 times after *H. pylori* infection, and preincubation of BF-1 suppresses this effect by 33%. Interleukin-8 has been specifically implicated in the pathogenesis of *H. pylori*. Although the sample combination designs of the microarrays described here are complicated, this degree of complexity was necessary to identify those genes that, like IL-8, exhibit induction profiles that are suppressed by pre-incubation with BF-1. These experiments identified many other genes (see Table 2) with profiles similar to that of IL-8, including genes of the C-C (RANTES, LARC), C-X-C (Gro- α , β , γ , ENA-78, IL-8), C-X3-C (Fractalkine) motif chemokines, chemokine receptor (CXCR4/fusin), FGF18, interleukin (IL-23A), TNF superfamily proteins (Rink and Kirchner, 1996), matrix metalloproteinases, and cell adhesion molecules (ICAM-1, VCAM-1, Laminin). Interleukin-8 and Gro- α preferentially recruit neutrophils, whereas RANTES primarily attract lymphocytes and monocytes (Baggiolini, 1998). Increased endothe-

Table 4. List of genes induced by *Helicobacter pylori* and suppressed by *Bifidobacterium bifidum* BF-1

Gene name ¹	Normalized expression	Relative fold ²	GenBank accession no.
VCAM-1	7.0	0.65	NM_001078
ICAM-1	14.1	0.58	NM_000201
LAMA3	5.8	0.46	NM_198129
LAMC2	2.3	0.50	NM_005562
OLR1	6.0	0.82	NM_002543
CCL5	8.2	0.83	NM_002985
CCL20	26.1	0.52	NM_004591
CXCL1	13.9	0.82	NM_001511
CXCL2	6.8	0.73	NM_002089
CXCL3	16.4	0.54	NM_002090
CXCL5	4.0	0.65	NM_002994
CXCL10	6.0	0.49	NM_001565
CX3CL1	5.5	0.46	NM_002996
CXCR4	8.0	0.44	NM_001008540
IL-8	80.4	0.18	NM_000584
IL-23A	2.9	0.37	NM_016584
FGF18	14.7	0.58	NM_003862
LTB	8.3	0.80	NM_002341
TNF- α	28.6	0.41	NM_000594
TNFAIP2	7.1	0.64	NM_006291
TNFAIP3	6.2	0.67	NM_006290
TNFRSF1B	2.1	0.77	NM_001066
TNFSF10B	2.8	0.66	NM_003842
TRAF1	5.5	0.51	NM_005658
MMP1	2.8	0.35	NM_002421
MMP9	4.1	0.68	NM_004994
NFKBIA	6.0	0.68	NM_020529

¹VCAM-1 = vascular cell adhesion molecule-1; ICAM-1 = intercellular adhesion molecule-1; LAMA3 = laminin, α 3; LAMC2 = laminin, γ 2; OLR1 = oxidized low-density lipoprotein receptor 1; CCL = chemokine ligands; CXCR = chemokine receptor; LTB = leukotriene B; TNF- α = tumor necrosis factor- α ; TNFAIP = TNF- α -induced protein; TNFRSF = TNF receptor superfamily; TRAF = TNF receptor-associated factor; ITGB8 = integrin beta-8; NFKBIA = nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; MMP = matrix metalloproteinases.

²Relative fold is a ratio of normalized expression of array 1 and array 2 (array 2/array 1).

lial cell expression of the adhesion molecules VCAM-1 and ICAM-1 supports the recruitment of leukocytes to the site of inflammation and perpetuates the ongoing inflammatory process (Kelly et al., 2007). These genes are known to have close interactions with each other. Although the mechanism of *H. pylori*-induced inflammation is not yet fully understood, the translation factor NF- κ B plays a key role in gene regulation during the inflammatory response. In endothelial cells, NF- κ B regulates the expression of VCAM-1 and ICAM-1, as well as that of many cytokines, including IL-8, Gro- α , RANTES, and TNF- α (Innocenti et al., 2002). It is noteworthy that the *H. pylori*-induced and BF-1-suppressed genes overlap with the genes regulated by NF- κ B. It is thus likely that suppression of gene expression by BF-1 is the result of BF-1 interference with the NF- κ B regulatory pathways.

The mechanism by which BF-1 affects gene expression is unknown. One possible explanation is that a direct competition between *H. pylori* and BF-1 occurs on the cell surface. To eliminate the effect of direct

bacteria-to-bacteria contact inhibition, the cells in our experiments were washed with medium 3 times. Bifidobacteria have strong adherent properties, so the possibility that BF-1 sticks to the surface of the cells cannot be excluded, but the effects of lactic acid, acetic acid, or pH variation produced by BF-1 were eliminated by the wash step.

Another possibility is that BF-1 inhibits the intracellular pathogenic function of *H. pylori*. *Helicobacter pylori* induces NF- κ B activation via intracellular signaling pathways in toll-like-receptor dependent and independent pathways. Toll-like receptor (TLR)2 and TLR5 have been shown to recognize *H. pylori* and to initiate signaling pathways that result in enhanced activation of NF- κ B (Smith et al., 2003; Torok et al., 2005). In our study, no variation in TLR gene expression was detected. In epithelial cells, the major roles of TLR are ambiguous (Ferrero, 2005; Hirata, et al., 2006). *Helicobacter pylori* can direct signaling in gastric epithelial cells through cytotoxin-associated gene A (CagA) to activate NF- κ B, leading to the release of chemokines

Figure 3. (a) Relative expression rate of IL-8 mRNA induced by *Helicobacter pylori* quantified by real-time PCR; (b) relative expression rate of nuclear factor- κ B (NF- κ B) and myeloid differentiation primary response gene-88 (MyD88) mRNA induced by *H. pylori*. Data are the means $\pm$ standard deviations of the means ($n = 3$). * $P < 0.04$, ** $P < 0.03$ for *H. pylori*-induced genes not preincubated versus preincubated with BF-1. M = media; B = BF-1; P = *H. pylori*. Open bar (M→P) represents the sample without preincubation. Hatched bar (B→P) represents the sample preincubated with BF-1.

such as IL-8. Although the interaction of CagA and BF-1 has not yet been studied, our preliminary data show that BF-1 affects CagA production.

The other possibility is that BF-1 directly interacts with and suppresses NF- κ B, improving gastric symptoms by suppressing the inflammatory pathway, which includes IL-8. The results of the real-time PCR experiment suggest that BF-1 does not directly suppress NF- κ B transcription; however, even a slight variation may lead to significant effects because NF- κ B is a key regulatory gene.

One final possibility is that preactivation of BF-1 may suppress the drastic activation of gene expression by *H. pylori*. No upregulation or downregulation more than 2-fold of NF- κ B or other inflammatory cytokines was detected in cells incubated with BF-1 alone, indicating that BF-1 does not induce inflammatory events in epithelial cells. It remains possible, however, that undetectable preceding stimulation by BF-1 may make

regulatory genes insensitive to the following stimulation by *H. pylori*.

Wen et al. (2004) reported the cDNA array analysis in gastric tissue obtained from biopsy specimens of *H. pylori*-infected patients. Although their results differ somewhat from those reported here, these differences likely reflect variability between cultured cells versus in situ tissues. Both reports list inflammatory genes, such as Gro- α , β , γ , ENA-78, VCAM-1, and ICAM-1, that are upregulated by *H. pylori* infection. Thus, these results allow us to speculate that the BF-1 suppression mechanism might work in the human stomach. Normalization of the numbers of infiltrating neutrophils and expression of IL-8 were achieved by eradication of *H. pylori* (Ando et al., 1998). Therefore, the suppression of IL-8 and other inflammatory genes by BF-1 may contribute to the improvement of gastric function.

The identity of the BF-1 factor responsible for its suppressing effects is not yet known. Previous studies have shown that TNF- α -induced IL-8 expression was inhibited by *B. bifidum*-conditioned medium and its DNA, but not by heat-treated bacteria in HT-29 cells (Imaoka et al., 2008). *Bifidobacterium bifidum* inhibits LPS-induced NF- κ B activation in HT-29 intestinal epithelial cells (Riedel et al., 2006). Polysaccharide fractions of *B. bifidum*, composed primarily of rhamnose, have an anti-ulcer effect in humans (Nagaoka et al., 1994). Taken together, polysaccharide fractions seem to be an important factor in cellular interactions; however, for many probiotics, it is known that the whole cell body is required to induce a strong immune response in cells (Sekine et al., 1985). The reinforcement of immunity caused by probiotics may also contribute to the suppression of *H. pylori* in vivo. The in vivo analysis of BF-1 effects on gene expression should be investigated in future studies.

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