

An immunostimulatory DNA sequence from a probiotic strain of *Bifidobacterium longum* inhibits IgE production *in vitro*

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Abstract

The immunostimulatory oligodeoxynucleotide (ODN) BL07 (5'-GCGTCGGTT-TCGGTGCTCAC-3') was identified from the genomic DNA of the probiotic strain *Bifidobacterium longum* BB536. ODN BL07 stimulated B-lymphocyte proliferation and induced interleukin-12 (IL-12) production in macrophage-like J774.1 cells. ODNs BL07 and BL07S (modified with phosphorothioate backbone) significantly inhibited immunoglobulin E (IgE) production and stimulated interferon- γ (IFN- γ) and IL-12 production, but did not affect IL-4 secretion in murine splenic cells of ovalbumin-primed BALB/c mice. These ODNs also significantly inhibited production of IgE in purified murine B cells in the presence of IL-4 and anti-CD40. The results suggest the potential of ODNs BL07 and BL07S in preventing IgE-related immune responses and the possible involvement of ODN BL07 in the antiallergic efficacy of *B. longum* BB536.

Introduction

Probiotics are defined as live commensal microorganisms that confer health benefits to the host, and have been shown by *in vitro* and *in vivo* studies to exert various health-promoting functions, such as anticarcinogenic, antimutagenic, anti-infectious and immune-modulating activities (Fuller, 1989; Naidu *et al.*, 1999; Macfarlane & Cummings, 2002). Substantial interest has been focused on the antiallergic activity of probiotics. Modulation of cytokine production and inhibition of immunoglobulin E (IgE) production have been demonstrated on different probiotic bacteria *in vitro* and *in vivo* (Matsuzaki *et al.*, 1998; Christensen *et al.*, 2002; He *et al.*, 2002; Ishida *et al.*, 2003; Fujiwara *et al.*, 2004; Kimoto *et al.*, 2004; Ohno *et al.*, 2005). Kalliomäki *et al.* (2001) demonstrated the effects of *Lactobacillus* GG (LGG) in preventing early atopic disease in high-risk children by administering LGG prenatally to mothers and postnatally to children. Effects have also been shown for oral probiotic therapy with lactic acid bacteria for improving clinical symptoms of perennial allergic rhinitis in adults and of food allergy in young children. Some bacterial cell components

such as peptidoglycans, lipoteichoic acid and secreted soluble substances reportedly play roles in these immunomodulatory responses (Matsuguchi *et al.*, 2003; Pena & Versalovic, 2003) but the primary components have yet to be identified.

Unmethylated bacterial DNA motifs comprising cytosine linked to guanine by a phosphate bond (CpG motifs), also known as immunostimulatory sequence (ISS) oligodeoxynucleotides (ODNs), are reportedly mitogenic for murine B cells (Krieg *et al.*, 1995). Recently, ISS-ODNs without CpG motifs have also been reported as displaying immunostimulatory effects (Kitazawa *et al.*, 2001; Elias *et al.*, 2003). ISS-ODNs activate innate immune cells such as macrophages and dendritic cells via Toll-like receptor 9 (TLR9), resulting in activation of the adaptive immune system by induced Th1-type cytokines (Hemmi *et al.*, 2000; Shimosato *et al.*, 2005). Improvements in allergic disease by ISS-ODNs were reported recently in animal experiments (Kline *et al.*, 1998; Sur *et al.*, 1999; Hussain & Kline, 2001), and clinical applications for hay fever improvement and vaccination in humans are expected (Simons *et al.*, 2004; Tulic *et al.*, 2004).

Rachmilewitz *et al.* (2004) revealed that genomic DNA from probiotics exerted anti-inflammatory effects in mice via

TLR9 signaling. Lammers *et al.* (2003) reported that *Bifidobacterium* genomic DNA induces secretion of anti-inflammatory cytokine interleukin-10 (IL-10) in human peripheral blood mononuclear cells (PBMCs). Iliev *et al.* (2005) recently identified a short ODN sequence with strong immunostimulatory activity from the genomic DNA of LGG strain that has shown intense potential immunomodulatory and anti-allergic effects *in vitro*, *in vivo* and clinically (Pessi *et al.*, 2000; Kalliomäki *et al.*, 2001). Such reports suggest that genomic DNA or ISS-ODNs from probiotics might possibly be involved in the immunomodulatory effects of probiotic strains, although probable roles have not yet been elucidated.

In a human trial, consumption of yogurt supplemented with *Bifidobacterium longum* BB536, a probiotic strain originating from humans, was found to alleviate subjective symptoms of Japanese cedar pollinosis and to suppress increased blood eosinophil rates and decreased interferon- γ (IFN- γ) levels (Xiao *et al.*, in press). The main objective of the present study was to identify ISS-ODNs from BB536 genomic DNA and to characterize associated immunomodulatory activities in relation to bifidobacterial antiallergic efficacy. We report herein an ISS-ODN from the genomic DNA of *B. longum* BB536 that was characterized as stimulating murine B-lymphocytes, inducing Th1 cytokines and inhibiting IgE production *in vitro*.

Materials and methods

Microorganisms

Bifidobacterium longum BB536 was originally isolated from a human fecal sample and stored at the Nutritional Science Laboratory, Morinaga Milk Industry Co., Ltd, Zama, Japan. This strain was maintained by subculturing in Brigg's liver broth with 3% (volume in volume, v/v) inoculation at 37 °C for 16 h, in accordance with the methods described by Tanaka *et al.* (1999).

Mice

Specific pathogen-free BALB/c mice (Japan SLC, Shizuoka, Japan) were housed in plastic cages at room temperature and used at 6–8 weeks old. Mice were fed a standard diet (F-2; Oriental Yeast, Tokyo, Japan) and were allowed *ad libitum* access to water throughout the experimental period. All experiments involving mice were performed according to the guidelines of Tohoku University, Japan.

Preparation of genomic DNA and ODNs

Genomic DNA from *B. longum* BB536 was prepared in accordance with the methods of Luchansky *et al.* (1991), with some modifications. Briefly, cells (20 mg) from fresh cultures were treated in 50 mM Tris/HCl buffer (pH 7.2)

with 0.5 mg *N*-acetyl muramidase SG (Seikagaku Kougyou, Tokyo, Japan), 8 mg lysozyme (Seikagaku Kougyou) and 10 μ L RNase A (Qiagen, Tokyo, Japan) for 2 h at 37 °C, followed by addition of 100 μ L protease K (Takara, Kyoto, Japan) for 16 h at 37 °C. Genomic DNA was purified using a genomic tip (Qiagen). DNA was extracted using Triton X-114 (Wako Pure Chemical, Tokyo, Japan) as described by Cotton *et al.* (1994), to remove contaminated lipopolysaccharide (LPS). After repeated ethanol precipitation, purified genomic DNA was dissolved in TE buffer (10 mM Tris/HCl buffer containing 1 mM EDTA) and stored at 4 °C until use.

Chemically synthesized ODNs CpG 1826 (Hartmann *et al.*, 2000) (5'-TCCATGACGTTCTGACGTT-3') and CpG 1826Me (5'-TCCATGAmCGTTCTGAmCGTT-3', where mC indicates 5'-methyl cytosine) were used as positive and negative controls, respectively. ODN BL07S was modified from ODN BL07 by including a phosphorothioate backbone to provide nuclease resistance. All ODNs used in this study were chemically synthesized by Sigma-Genosys (Hokkaido, Japan).

LPS contamination was confirmed as <10 ng mg⁻¹ DNA for all ODN samples using a Toxicolor system (Seikagaku Kougyou).

Proliferation assays

Splenic cells from BALB/c mice were prepared as previously described (Kitazawa *et al.*, 1992). Cells were suspended in RPMI 1640 medium (Sigma, St Louis, MO) supplemented with 2% fetal calf serum (FCS; Sigma). Next, 90 μ L of cell suspension (2.0×10^5 cells) was placed into each well of 96-well plates (Sumitomo Bakelite, Tokyo, Japan), followed by 10 μ L of DNA (500 μ g mL⁻¹) or ODN (100 μ M) solution. All cultures were incubated for 32 h at 37 °C under 5% CO₂ in air. The cultures were pulse labeled for 16 h with 9.25 kBq ³H-thymidine (Amersham Biosciences, Buckinghamshire, UK) before the end of each experimental period and harvested on the glass filter (Labo Mash, Labo Science Co., Ltd, Tokyo, Japan). The amount of ³H-thymidine incorporated into splenic cells was determined using an LSC-5100 scintillation counter (Beckman Instruments, Fullerton, CA). LPS from *Escherichia coli* 0111:B4 (Sigma) and Concanavalin A (ConA; Sigma) were used as positive controls against B and T lymphocytes, respectively. Stimulation index (SI) was calculated using the following equation:

$$SI = \frac{[(\text{counts/min})_{\text{in treated cells}} - (\text{counts/min})_{\text{background}}]}{[(\text{counts/min})_{\text{in control cells}} - (\text{counts/min})_{\text{background}}]}$$

All assays were performed in at least three separate experiments using three separate culture wells.

Isolation of mitogenic DNA clones

Genomic DNA prepared from cells of *B. longum* BB536 was digested using *Sau3AI* (Takara) for 16 h at 37 °C. DNA fragments with a molecular weight of <0.5 kb were prepared under 1.5% agarose gel electrophoresis and purified using a Quantum Prep Freeze 'N Squeeze Spin Column (Bio-Rad, Hercules, CA). DNA fragments were ligated into the *Bam*HI site of a pUC119 vector (Takara) using T4 ligase (Promega, Madison, WI). Ligates were transformed into *E. coli* JM109 cells and amplified onto a Luria–Bertani agar plate (Difco Laboratories, MI) containing X-Gal (Sigma). After incubation for 16 h at 37 °C, white colonies were selected and amplified in Terrific broth at 37 °C for 16 h. Purified plasmid DNA from *E. coli* JM109 was obtained using a Quantum Prep Plasmid Mini Prep Kit (Bio-Rad). DNA was amplified using PCR with M13 primer M4, 5'-GTTTCCAGTCACGAC-3', and M13 primer RV, 5'-CAGGAAACAGCTATGAC-3', for multicloning sites of pUC119. Reaction mixtures were heated at 95 °C for 5 min, then amplified for 30 cycles under conditions of: 95 °C for 30 s, 55 °C for 30 s and 72 °C for 5 min. Single DNA clones were obtained using a QIA-quick PCR purification kit (Qiagen). All DNA clones were cleaned using Triton X-114 (Cotton *et al.*, 1994) and tested under proliferation assay in splenic cells.

Identification of mitogenic nucleotide sequences

DNA clones were sequenced using plasmid DNA templates. Dideoxy sequencing reactions were performed with Taq DNA polymerase using a sequencing kit (Takara). Nucleotide sequences were determined using an ABI PRISM 3100-Avant Genetic Analyzer DNA sequencer (Applied Biosystems, Foster City, CA). Analysis of sequence data was performed using GENETYX-SV/RC version 6.15 software (Software Development, Tokyo, Japan). Highly homologous nucleotide sequences were analysed from among the nucleotide sequences of 14 DNA clones, and were chemically synthesized. A total of 20 ODNs were synthesized and tested by proliferation assay in splenic cells.

Determination of stimulated lymphocytes

Splenic B and T cells were separated by positive selection with anti-CD45R and anti-Thy1.2 monoclonal antibodies (mAb) (Miltenyi Biotec, Bergisch Gladbach, Germany) using a Magnetic Cell Sorting system (MACS; Miltenyi Biotec). Purity of B and T cells was confirmed by flow cytometry using a FACScalibur (Becton Dickinson, Franklin Lakes, NJ) after incubation with antimouse CD45R and anti-Thy1.2mAb (Caltag Laboratory, Burlingame, CA). Purified cells were suspended at a concentration of $2 \times$

10^6 cells mL⁻¹ in RPMI 1640 medium (Invitrogen Corp., Carlsbad, CA) supplemented with 10% FCS, 2.1 mM L-glutamine, 100 IU mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin (Invitrogen). Mitogenic activities of these separated cells were measured against synthesized ODN BL07 according to the above methods.

IL-12 induction from J774.1 by ODNs

Murine macrophage-like J774.1 cells (Riken Bioresource Center, Ibaraki, Japan) were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS (Invitrogen), 100 IU mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin (Invitrogen) at 37 °C under 5% CO₂ in air. J774.1 cells (4.0×10^5) were plated in a 96-well tissue culture plate (Sumitomo Bakelite) and incubated for 2 h. After washing, cells were stimulated using ODNs (10 µM) in RPMI 1640 medium. Culture supernatants were collected 24 h after cell stimulation and stored at -80 °C until analysis.

NF-κB reporter assays

Human primary embryonic kidney 293T cells were obtained from the TKG cell bank (Institute of Development, Aging and Cancer, Tohoku University, Miyagi, Japan) and maintained in complete Dulbecco's modified Eagle medium (DMEM; 10% FCS, 50 mg mL⁻¹ penicillin/streptomycin, 2 mM L-glutamine, 10 mM HEPES buffer, 0.11 mg mL⁻¹ sodium pyruvate, 0.5 mM 2-ME; Sigma). Next, 293T cells transfected with mouse TLR9 (mTLR9) were obtained as described previously (Shimosato *et al.*, 2005). The 293T^{mTLR9} cells (8×10^4) were precultured for 24 h and transfected for 4 h using lipofectamine reagent plus 0.5 µg pGLM-ENH-luci vector. After stimulation of ODNs (10 µM) for 24 h, a luciferase assay was performed as previously described (Shimosato *et al.*, 2005), and light output was measured for 10 s at room temperature using a Lumat LB9501 luminometer (Berthold Technologies, Bad Wildbad, Germany). SI was determined using the following equation:

$$SI = \frac{[(\text{resonance unit (RU)})_{\text{in stimulated culture}} - (\text{RU})_{\text{background}}]}{[(\text{RU})_{\text{in nonstimulation}} - (\text{RU})_{\text{background}}]}$$

Ovalbumin-induced IgE and cytokine production

The effect of ODNs on ovalbumin (OVA)-induced production of IgE and cytokines was investigated using a culture system with OVA-primed BALB/c splenic cells as described by Shida *et al.* (1998), with some modifications. Each mouse was injected intraperitoneally with 1 mg of OVA (grade V, Sigma) and 2 mg of Al(OH)₃ (Wako Pure Chemical). After 10 days, single splenic cell suspensions were prepared at 2.5×10^6 cells mL⁻¹ in RPMI 1640 medium containing 10%

FCS, $100 \mu\text{g mL}^{-1}$ penicillin and 100 U mL^{-1} streptomycin (Invitrogen). Splenic cells were incubated with ODNs in 24-well flat-bottom culture plates in the presence of $100 \mu\text{g mL}^{-1}$ OVA at 37°C in 5% CO_2 . Following incubation for 7 days, culture supernatants were harvested for total IgE and cytokine analysis.

Ig isotype switch assays

Purified B cells were isolated from splenic cells of BALB/c mice by positive selection in the MACS system. Ig isotype switch assays were performed as described by Kobayashi *et al.* (2002), with some modifications. Prepared B cells ($2.0 \times 10^6 \text{ cells mL}^{-1}$) were incubated in RPMI 1640 medium containing 10% FCS (Invitrogen), $100 \mu\text{g mL}^{-1}$ penicillin and 100 U mL^{-1} streptomycin (Invitrogen) with 10 ng mL^{-1} mouse recombinant interleukin-4 (IL-4) (R&D Systems, Minneapolis, MN) and $3 \mu\text{g mL}^{-1}$ antimouse CD40 antibody (R&D Systems) in the presence or absence of ODNs ($10 \mu\text{M}$) at 37°C in 5% CO_2 . After 10 days, supernatants were collected for analysis of total IgE, IgG1 and IgG2a levels.

ELISA

Cytokines examined in this study comprised IFN- γ , IL-4 and IL-12. Cytokine levels in RPMI 1640 culture medium were quantified using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) according manufacturer's recommendations. Total IgE, IgG1 and IgG2a levels were assessed using an ELISA kit (Bethyl Laboratory Inc., Montgomery, TX).

Statistics

Experimental data are expressed as mean $\pm$ standard deviation. Statistical analyses were conducted using Student's *t*-test compared with controls. Multiple groups were statistically compared with ANOVA, followed by Dunnett's test. $P < 0.05$ was considered statistically significant.

Results

Mitogenic activity of DNA from *Bifidobacterium longum* BB536

Figure 1 shows the mitogenic activity of DNA from *Bifidobacterium longum* BB536 and DNA digested by *Sau3AI* in murine splenic cells. DNA from *B. longum* BB536 displayed significant activities. SI was 4.0–15.0 and was dose-dependent for concentrations of 1– $50 \mu\text{g mL}^{-1}$. Digested DNA showed higher activity (SI 14.8) than natural DNA (SI 5.4) at a concentration of $10 \mu\text{g mL}^{-1}$. In the present experimental system, SIs for the positive controls were 45 for LPS and 80 for ConA.

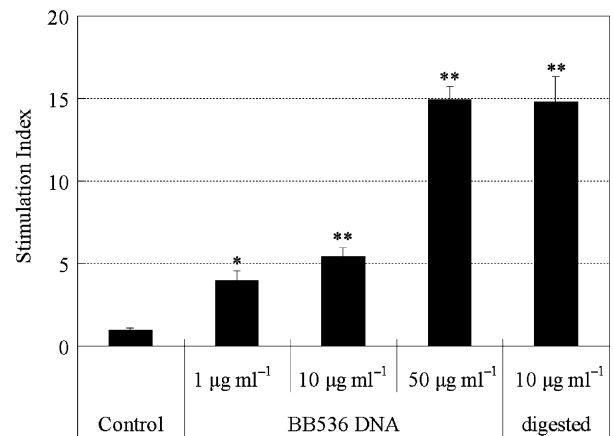


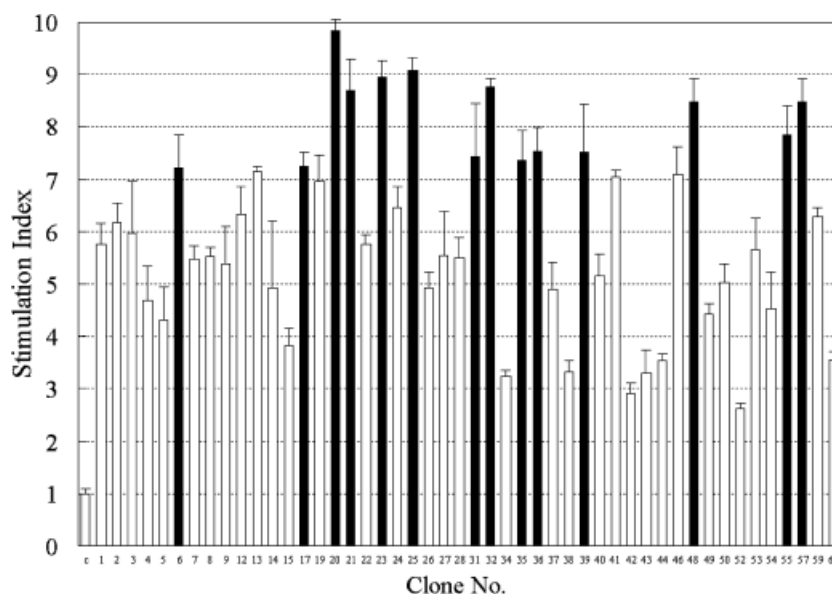
Fig. 1. Mitogenic activity of genomic DNA and digests from *Bifidobacterium longum* BB536. Splenic cells from BALB/c mice were cultured in 96-well microplates at a concentration of 2.0×10^5 cells in the presence of DNA or *Sau3AI*-digested DNA for 32 h at 37°C . After pulse labeling with ^3H -thymidine for 16 h, the amount of ^3H -thymidine incorporated into splenic cells was determined. Data represent mean ($\pm$ SD) stimulation index (SI) of triplicate cultures. * $P < 0.05$; ** $P < 0.01$ compared to control.

Identification of ISS-ODN

During color selection from colonies of *E. coli* JM109, 62 white colonies were selected as positive colonies with inserted DNA. The inserted DNA in each plasmid was amplified by PCR and the molecular weights of each single DNA fragment (DNA clone) were analysed by 2.5% agarose electrophoresis. Among the 48 clones studied, 14 exhibited strong mitogenic activity in murine splenic cells at a concentration of $50 \mu\text{g mL}^{-1}$, with SI ranging from 7 to 10 (Fig. 2). Homologous sequences ($n = 20$) were selected from sequences of the 14 clones, and representative 20-mer ODNs were designed, as ODNs BL01–20 (Fig. 3a). The 20 ODNs were chemically synthesized and tested for mitogenic activity. Only ODN BL07 (5'-GCGTCGGTTTCGGTGCTCAC-3') induced significant proliferation of splenic cells, with an SI of 5.0 (Fig. 3b). When B or T lymphocytes were stimulated using ODN BL07, significant proliferation was observed in B cells but not in T cells (Fig. 3c). In this experiment, the SIs of CpG 1826 and CpG 1826Me as positive and negative controls were 15 and 1.1, respectively.

To determine the effects of ODN BL07 on the innate immune system, the 20 selected ODNs were also tested for IL-12 induction in macrophage-like J774.1 cells (Fig. 3d). ODN BL07 was found to be one of the sequences with high IL-12 induction. ISS-ODNs such as CpG ODN were recognized by TLR9 on innate immune cells, such as macrophages and dendritic cells. To investigate whether ODN BL07 was recognized by mTLR9, luciferase activities dependent on NF- κB in 293T^{mTLR9} cells were measured. ODN BL07

Fig. 2. Mitogenic activity of genomic DNA clones. DNA clones were amplified by PCR and amplified DNA fragments were extracted. DNA was tested for mitogenic activity in splenic cells from BALB/c mice. Cell proliferation was assayed as described in Fig. 1. Data represent mean stimulation index (SI) of triplicate cultures compared with controls stimulated using amplified DNA from pUC119 without inserted DNA. For the 14 mitogenic DNA clones (closed bars), nucleotide sequences displaying high homology were analysed.



showed a significantly increased SI value (2.4) compared with control (Fig. 3e). The SI value of CpG 1826 and CpG 1826Me used as positive and negative controls were 4.8 and 1.0, respectively, in this experiment.

Given the above results, ODN BL07 was selected from among the 20 ODNs as an ISS-ODN and used for further functional studies.

Effects of ODN BL07 on IgE production and cytokine production

To evaluate the function of ODN BL07 against allergic diseases, effects on IgE and cytokine production were tested in murine splenic cells. ODNs BL07 and BL07S significantly inhibited IgE synthesis in a dose-dependent manner (Fig. 4). Levels of Th1-type cytokines (IFN- γ and IL-12) after stimulation by ODN BL07 (10 μ M) and BL07S (1 μ M) were significantly higher compared with control levels (Figs 5a and b). By contrast, no marked differences in IL-4 levels were noted between ODN treatments and control (Fig. 5c).

Regulation of Ig production in purified B cells by ODN BL07

Purified B cells (>98%) were cultured with ODNs in a medium containing recombinant mouse IL-4 and anti-CD40 antibody to examine effects on Ig class switching. Secretion of IgE was significantly inhibited and secretion of IgG2a was markedly increased in the presence of ODNs BL07 and BL07S, whereas IgG1 production was not significantly affected (Table 1). No significant IFN- γ was detected with either treatments or control (data not shown).

Discussion

Immunostimulatory CpG and non-CpG ODNs have already been identified from the genomic DNA of probiotics (Kitazawa *et al.*, 2001, 2003; Iliev *et al.*, 2005). In the present study, ODN BL07 with three CpG bonds was identified as an ISS-ODN from the genome of *Bifidobacterium longum* BB536, with a sequence of 5'-GCGTCGGTTTCGGTG CTCAC-3'. This ODN stimulated murine B lymphocytes, induced Th1 cytokines and inhibited IgE production *in vitro*. The present study is the first to identify an ISS-ODN from bifidobacteria. Immunostimulatory activity of ODN BL07 was confirmed as independent of LPS contamination. As no homology with reported ISS-ODNs was found, ODN BL07 might be considered as a novel ISS-ODN. A survey of public genome databases identified ODN BL07 in the genome of *B. longum* NCC2705, but not in the genomes of other bacteria such as lactobacilli or streptococci. We have also confirmed the existence of ODN BL07 in the genomes of *B. longum* BB536, *Bifidobacterium breve* M-16V and *Bifidobacterium infantis* M-63, for which genome sequence information has recently been obtained (N. Takahashi, unpublished data). ODN BL07 might thus represent a sequence unique to bifidobacteria.

Certain CpG ODNs (A-class) are considered to be particularly potent for activating natural killer (NK) cells and inducing IFN- α production by plasmacytoid dendritic cells, whereas other CpG ODNs (B-class) represent potent B-cell activators (Klinman, 2004). C-class ODNs display the characteristics of both A- and B-class ODNs. These ISS-ODNs were modified with a phosphorothioate backbone,

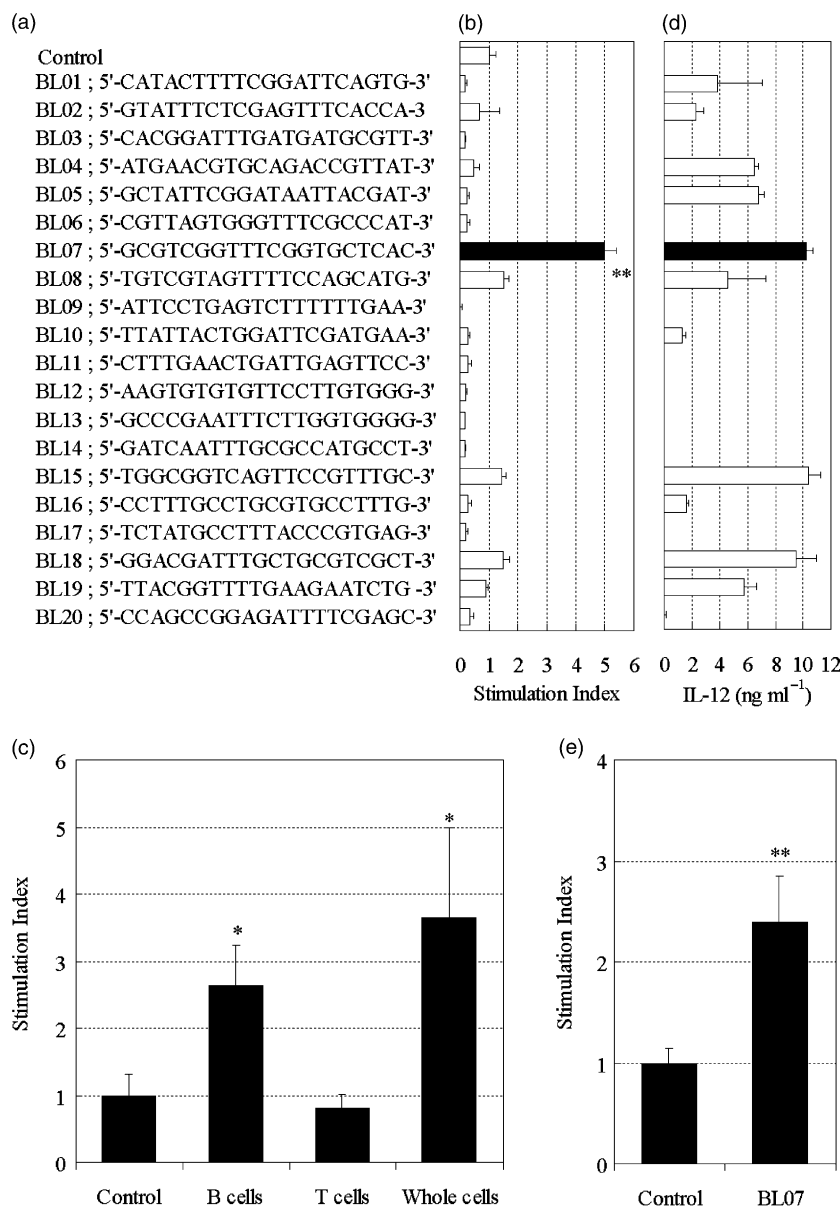


Fig. 3. Identification of an immunostimulatory sequence oligodeoxynucleotide (ISS-ODN) from genomic DNA of *Bifidobacterium longum* BB536. (a) Sequences of synthesized ODN sequences from mitogenic DNA clones. Mitogenic DNA was sequenced using a DNA sequencer, and homologies among nucleotide sequences were analysed. A total of 20 oligodeoxynucleotides (20 mers) displaying high homology were obtained and designated BL01 to BL20. (b) Mitogenic activity of homologous ODN at concentrations of 10 μ M. Cell proliferation and presentation of data are as in Fig. 1. *** P < 0.01 compared with control. (c) Mitogenic activity in murine B and T lymphocytes by synthesized ODN BL07. Splenic whole, B and T lymphocytes from BALB/c mice were cultured in 96-well microplates at 2.0×10^5 cells in the presence of ODN BL07 (10 μ M). Cell proliferation and presentation of data are as in Fig. 1. * P < 0.05 compared with control. (d) IL-12 levels in supernatants were determined using an ELISA kit. J774.1 cells (4.0×10^5 cells mL⁻¹) were stimulated with the selected 20 ODNs (10 μ M) for 16 h. Data represent mean ($\pm$ SD) of three independent experiments. ND, not detected. (e) Luciferase activities of mouse TLR9-transfected cells at 24 h after treatment with ODN BL07 (10 μ M). Data shown represent mean ($\pm$ SD) of three independent experiments. ** P < 0.01 compared with control.

conferring nuclease resistance. However, ODN BL07 could not be classified among these three ODN classes, owing to the presence of natural phosphodiester bonds only. ODN BL07S modified to contain a phosphorothioate backbone strongly activated B cells (data not shown), suggesting that this might be considered as a B-class ODN. A CpG motif comprising GACGTT is known to represent the optimal CpG motif for murine cells (Krieg *et al.*, 1998; Yi *et al.*, 1998), but does not exist in ODN BL07. Furthermore, activity of the CpG motif was recently reported to be related to secondary structure (Kandimalla *et al.*, 2003). The mechanisms underlying the immunomodulatory activity of ODN BL07 remain to be resolved.

Type I allergy is caused by a characteristic immune response to particular antigens, primarily mediated by Th2 cells (Romagnani, 1994). Th2 cells synthesize IL-4, IL-5 and IL-13, subsequently leading to the production of allergen-specific IgE, which triggers allergic inflammatory reactions such as the release of histamine and leukotriene from mast cells. By contrast, Th1 inhibits IgE synthesis by secreting IFN- γ . Evaluation of IgE levels thus provides a cue for evaluating antiallergic effects. The prophylactic and therapeutic effects of ISS-ODNs in allergy have been demonstrated by selective down-regulation of IgE and Th2 cytokine production in mouse models of asthma and in human PBMCs from atopic patients (Kline *et al.*, 1998;

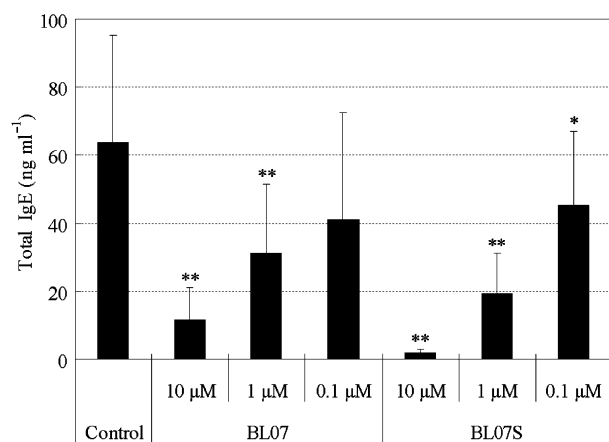


Fig. 4. Oligodeoxynucleotide (ODN) BL07 inhibits IgE production from splenic cells of ovalbumin (OVA)-sensitized BALB/c mice. Splenic cells of BALB/c mice sensitized with OVA were cultured at 2.5×10^6 cells mL⁻¹ using $100 \mu\text{g mL}^{-1}$ OVA in the presence of ODNs for 7 days. Total IgE levels in supernatants were measured using ELISA. Data are shown as mean ($\pm$ SD) of three independent experiments. * $P < 0.05$; ** $P < 0.01$ compared with control.

Bohle *et al.*, 1999; Parronchi *et al.*, 1999; Sur *et al.*, 1999). In general, these ISS-ODNs were artificially designed, synthesized and modified with a phosphorothioate backbone to provide nuclease resistance. Few studies have examined the activity of natural ODNs without modification for nuclease resistance. ISS-ODNs with natural sequences identified from probiotic lactobacilli have been described as exerting significant B-cell mitogenic activity (Kitazawa *et al.*, 2001, 2003; Iliev *et al.*, 2005), but antiallergic activity has not been clearly demonstrated. Whether ISS-ODNs from probiotics play any role in the antiallergic effects of lactobacilli remains unclear. In the present study, in addition to their B-cell mitogenic activity, both ODNs BL07 and BL07S with the phosphorothioate backbone were found to inhibit IgE production in Th2-polarized model systems *in vitro*. The finding that ODN BL07 derived from probiotics with a natural sequence exhibits such antiallergic activity is extremely interesting.

Table 1. Immunoglobulin levels produced by purified B cells

Group	IgE (ng mL ⁻¹)	IgG1 (ng mL ⁻¹)	IgG2a (ng mL ⁻¹)
Control	19.8 $\pm$ 5.3	71.6 $\pm$ 15.9	ND (< 3.9)
BL07	10.9 $\pm$ 4.9**	76.6 $\pm$ 13.1	14.4 $\pm$ 4.9
BL07S	4.3 $\pm$ 1.6**	94.8 $\pm$ 36.1	11.6 $\pm$ 12.5

Purified splenic B cells of BALB/c mice were cultured at 2.0×10^6 cells mL⁻¹ with 10 ng mL^{-1} IL-4 and $3 \mu\text{g mL}^{-1}$ anti-CD40 antibody in the presence of oligodeoxynucleotides ($10 \mu\text{M}$) for 7 days. Total IgE, IgG1 and IgG2a levels in supernatants were measured using ELISA. Data are shown as mean ($\pm$ SD) of three independent experiments.

** $P < 0.01$ compared with control. ND, not detected.

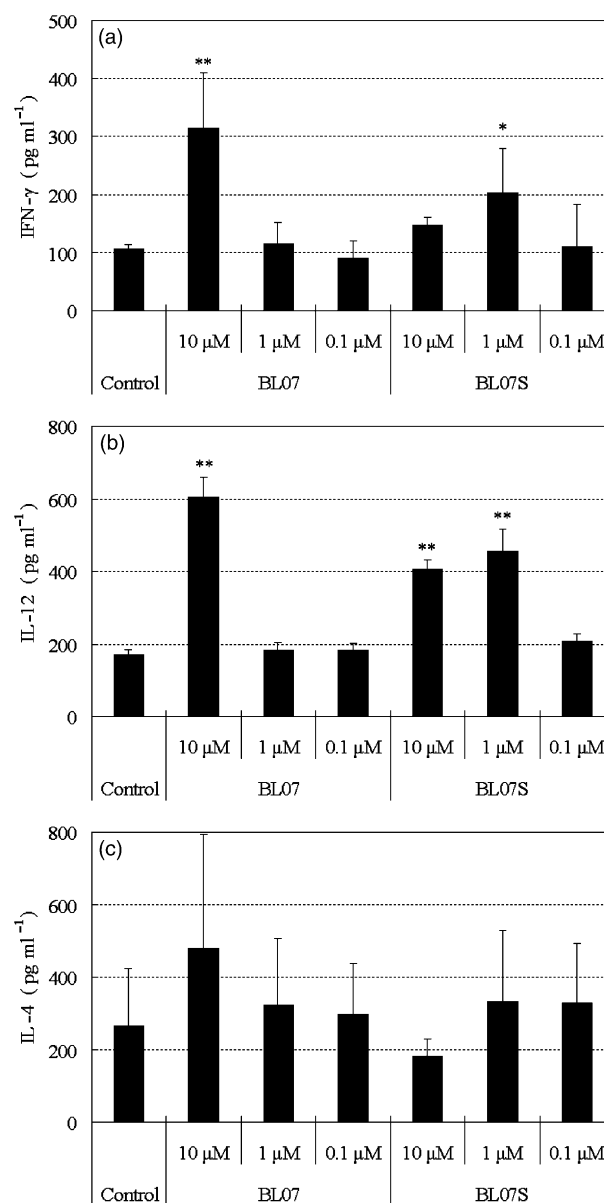


Fig. 5. Cytokine levels in splenic cell cultures stimulated by oligodeoxynucleotides (ODNs). Splenic cells of BALB/c mice sensitized with ovalbumin (OVA) were cultured at 2.5×10^6 cells mL⁻¹ with $100 \mu\text{g mL}^{-1}$ OVA in the presence of ODNs for 7 days. Cytokine levels in supernatants were measured using ELISA. Data are shown as mean ($\pm$ SD) of three independent experiments. (a) IFN-γ; (b) IL-12; (c) IL-4. * $P < 0.05$; ** $P < 0.01$ compared with control.

Liu *et al.* (2003) reported that CpG ODN directly inhibits IgG1 and IgE production in B cells *in vitro*, suggesting that CpG ODN might trigger antiallergic immune responses by directly regulating T-bet expression via signaling pathways in B cells. Similarly, ODN BL07 significantly inhibited secretion of IgE in B cells in the presence of IL-4 and anti-CD40. These results indicate that ODN BL07 could also

directly inhibit IgE synthesis in B cells without the modulation of Th1/Th2 balance. However, Peng *et al.* (2003) reported that T-bet is dispensable for inhibiting IgE using B cells from T-bet-deficient mice. Further studies are thus needed to reveal the mechanisms of IgE inhibition by ISS-ODNs in B cells.

In conclusion, the present study has demonstrated that ODN BL07 might represent a novel candidate as a therapeutic agent against allergic diseases and has identified the possible involvement of ODN BL07 in the antiallergic efficacy of *B. longum* BB536. Further study is in progress to evaluate the *in vivo* antiallergic effects of ODN BL07 in BALB/c mice.

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