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RESEARCH ARTICLE

Genetically engineered *Bifidobacterium animalis* expressing the *Salmonella flagellin* gene for the mucosal immunization in a mouse model

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Abstract

Background A critical component of the host defense against enteric infections is the immunological response of the mucosal membrane, a major starting point of infectious disease, such as typhoid fever. The mucosal immune system consists of an integrated network of lymphoid tissues, mucous membrane-associated cells, and effector molecules. In the present study, we developed a recombinant *Bifidobacterium animalis* (*B. animalis*) genetically modified with the *Salmonella flagellin* gene for mucosal immunization as an oral typhoid vaccine.

Methods We constructed an oral vaccine against *Salmonella typhimurium*, consisting of recombinant *B. animalis* containing the *flagellin* gene of *Salmonella*. The recombinant *B. animalis* was administered orally to mice every other day for 6 weeks. Anti-flagellin antibodies in the serum and stools were measured by enzyme-linked immunosorbent assay (ELISA).

Results We detected significantly higher levels of flagellin-specific IgA in the serum and stools of the mice treated with the recombinant *B. animalis* containing the *flagellin* gene than was seen in those treated with parental *B. animalis*.

Conclusions Our findings suggest that an oral vaccination using recombinant *B. animalis* genetically modified with the *flagellin* gene of *Salmonella* may be effective against *Salmonella* infections. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords *Bifidobacterium animalis*; mucosal vaccine; *Salmonella typhimurium*; flagellin

Introduction

Typhoid fever caused by the enteric pathogen *Salmonella enterica serovar* Typhi (*S. typhi*) remains an important public health problem in many parts of the world, especially in south-east Asia, Africa, and South America. According to WHO estimates, 16 million people are affected with this disease which causes 600 000 deaths each year worldwide [1]. The infection is spread by the fecal-oral route and is closely associated with poor food hygiene and inadequate sanitation. The control of typhoid fever by vaccination of high-risk populations is the most cost-effective means of containing this disease.

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As the current typhoid vaccines, the Vi capsular polysaccharide of *S*. Typhi was identified over 30 years ago [2], and the attenuated *S*. Typhi strain Ty21a was developed by chemical mutagenesis about 30 years ago [3]. However, according to a recent meta-analysis of studies of vaccines against typhoid, the 3-year cumulative efficacy was 30% to 71% for the Vi vaccine and 35% to 63% for the Ty21a vaccine [4–7]. The Vi capsular polysaccharide vaccine is usually administered subcutaneously to minimally induce the mucomembrane antibody. The Ty21a vaccine is administered orally, and associated with a 2.1% incidence of vomiting and a 5.1% incidence of diarrhea, and cannot be used for an immunocompromised host and below 5 years old [7]. Thus, there is a need for a novel genetically modified live oral vaccine that is both more safe and efficacious.

The use of intestinal indigenous live bacteria as an antigen delivery vehicle for mucosal immunization may be an alternative choice for vaccination against enteric infectious diseases. Bifidobacteria are predominant members of the intestinal microflora, and their health-promoting benefits have long been recognized [8]. They play an important role in protecting against intestinal infections by balancing the natural microflora of the gut [9], and are considered to have both immunostimulating and anticarcinogenic effects [10,11]. Although several efforts have been made to construct bifidobacteria vectors [12,13], the bifidobacteria DNA technology has remained relatively undeveloped considering its commercial potential.

Previously, we developed a *Escherichia coli-Bifidobacterium* shuttle vector, pBLES100, which was constructed by cloning with a *Bifidobacterium longum* plasmid and an *E. coli* vector pBR322 [13]. In the present study, we constructed a recombinant *Bifidobacterium animalis* (*B. animalis*) genetically modified with the *Salmonella flagellin* gene using the pBLES100 shuttle vector system. *B. animals* is normally used in probiotics and receives much attention in the food industry and in medical science [14]. Here, we examine whether this genetically engineered *B. animalis* could elicit the mucosal immune response against *Salmonella* in a mouse experimental model.

Material and methods

Bacteria

B. animalis and *Salmonella typhimurium* (*S. typhimurium*) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The ATCC numbers were 27536 for *B. animalis* and 14028 for *S. typhimurium*. *B. animalis* was grown in MRS broth (Becton, Dickinson and Co., USA) at 37 °C anaerobically. *S. typhimurium* was grown in LB broth (Invitrogen Life Technologies, Japan) at 37 °C. For mouse challenge experiments, these bacteria were suspended in phosphate-buffered saline (PBS).

Construction of the recombinant *B. animalis* expressing the *flagellin* gene

The open reading frame of the *flagellin* gene of *S. typhimurium* has been sequenced previously [15]. The *flagellin* gene was cloned out from genomic DNA of *S. typhimurium* by polymerase chain reaction (PCR) using the forward primer 5′-CATGCCATGGATGGCACAGTCATTAATACA (including an NcoI site), and the reverse primer 5′-CGCGGATCCTTAACGCAGTAAAGAGAGGAC (including a BamHI site). An *Escherichia coli-Bifidobacterium* shuttle vector, pBLES100, which was constructed by cloning with a *Bifidobacterium longum* plasmid, pTB6 [16], and an *E. coli* vector, pBR322 [13], was used to construct the plasmid, pBLES-FliC. The PCR mixture was performed using 10 ng of *S. typhimurium* gene in a 25 mL reaction, 0.5 units of Ampli Taq DNA polymerase and a reaction kit (Applied Biosystems, NJ, USA). The PCR profile was as follows: 94 °C for 10 min followed by 30 cycles (each cycle: 94 °C for 45 s, 55 °C for 45 s, 72 °C for 90 s) and a final extension at 72 °C for 10 min. The 1507 base pair of the amplified *flagellin* gene fragment was inserted into the pBLES100 vector with the *hup* (a histone-like protein HU of *B. longum*) promoter [17] to obtain the pBLES-FliC (Figure 1). Previously, Nakamura and colleagues [18] demonstrated the gene expression of the cytosine deaminase gene of *E. coli* in *B. longumin* using this *hup* promoter and pBLES100 shuttle vector. The pBLES-FliC was transformed into competent cells of *B. animalis* by an electroporation method (5 kV, 10 μs). For this competent cell line, *B. animalis* was suspended in 10% of glycerol after washing by PBS. Transfected *B. animalis* was grown under anaerobic conditions at 37 °C in BL agar (Nissui, Japan) containing 50 μg/mL of spectinomycin for selection.

Western blotting

Western blotting was employed to examine the expression of the flagellin protein in the recombinant *B. animalis*. Primary antibody, which was specific for the flagellum protein present in most *Salmonella* species, was obtained from Viro Stat Inc. (Portland, Maine, USA). As a positive control, we used 10 ng of flagellin derived from *S. typhimurium* (Alexis Biochemicals, San Diego, CA, USA).

For these determinations, *B. animalis* was lysed by lysation buffer containing 1% Tween 100. The lysates were diluted with an equal volume of loading buffer (126 mM Tris·HCl, 20% glycerol, 4% SDS, 1.0% 2-mercaptoethanol, 0.05% bromophenol blue, pH 6.8) and 5 μg of lysates was loaded onto Tris glycine polyacrylamide gels. The proteins were separated by electrophoresis. Reference protein standards with known molecular weights were run in conjunction with the experimental samples. The proteins were then transferred to nitrocellulose membranes. After electroblotting, they were blocked for 1.0 h at room

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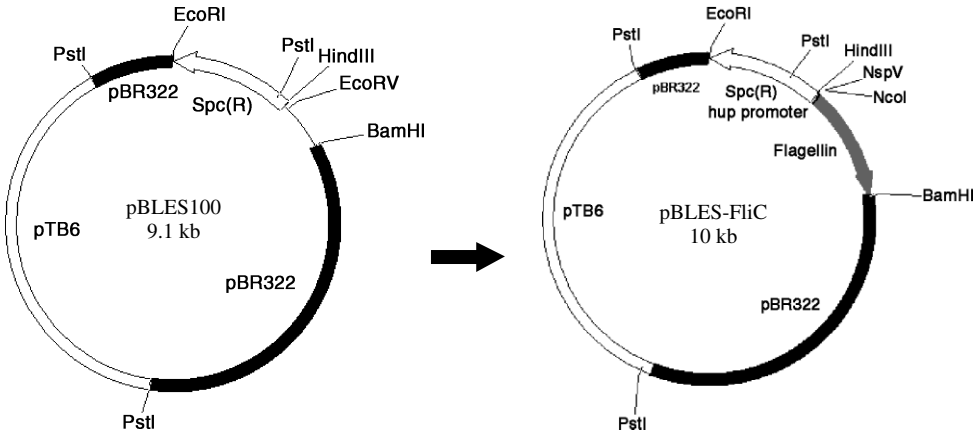

Figure 1. Construction of pBLES-FliC. The 1507 base pair of the amplified *flagellin* gene fragment was inserted into the *Escherichia coli-Bifidobacterium* shuttle vector, pBLES100, with the *hup* (a histone-like protein HU of *B. longum*) promoter to obtain the pBLES-FliC. The *Escherichia coli-Bifidobacterium* shuttle vector, pBLES100, was constructed by cloning with a *Bifidobacterium longum* plasmid pTB6 and an *E. coli* vector pBR322

temperature in PBS containing 0.5% skimmed milk and 0.05% Tween 20. After washing in PBS Tween, the membranes were incubated overnight at 4 °C with primary antibody (Viro Stat-Inc., Portland, OR, USA) to targeted proteins, 1 : 200. The membranes were then washed extensively and subsequently incubated with anti-mouse horseradish peroxidase-conjugated IgG (Santa Cruz Biotechnology, USA) 1 : 500 for 3 h at room temperature. Antibody binding to proteins was detected by enhanced chemiluminescence (Perkin Elmer Sciences, Boston, MA, USA).

Animal experiments

Female BALB/C mice, 8–12 weeks of age, were purchased from Charles River Japan (Yokohama, Japan). Twenty-one mice were randomly assigned to three experimental groups as follows: group 1, recombinant *B. animalis* immunization (7 mice); group 2, parental *B. animalis* control (7 mice); group 3, PBS control (7 mice). The fresh recombinant *B. animalis* or parental *B. animalis* (2.5×10^7 colony-forming units/50 μL of PBS) was orally administered in bacterial PBS suspension directly into the stomach using a feeding needle every other day for 6 weeks. Blood samples were collected from the tail vein on days 0, 14, 28, and 42 and were centrifuged to obtain the serum. Fecal samples were collected on days 4, 7, 11, 14, 18, 21, 25, 28, 32, 35, 39, and 42. The feces were vortexed in PBS containing 5% non-fat dry milk, 0.1 mg of soybean trypsin inhibitor/mL, and 2 mM phenylmethylsulfonyl fluoride (WAKO, Japan) along with 20 μL/mg dry feces, and the mixture was centrifuged at 15 000 *g* for 20 min at 4 °C. The clear supernatants were then frozen until analysis [19]. All aspects of the experimental design and procedure were reviewed and approved by the institutional ethics and animal welfare committees of the Kobe University School of Medicine.

Enzyme-linked immunosorbent assay (ELISA)

Nunc Immunoplate Maxisorb F96 plates (Nalge Nunc. Rochester, NY, USA) were coated with flagellin (10 g/L) in PBS and incubated overnight at 4 °C. The plates were blocked with 1% bovine serum albumin (BSA) in PBS for 2 h at room temperature and were washed three times with PBS. The antisera or extracts of feces were diluted to appropriate concentrations in PBS and added to the plates. The plates were then incubated for 3 h and washed. The secondary antibodies, goat anti-mouse IgG (Santa Cruz Biotechnology, USA) (1 : 500), goat anti-mouse IgA (Santa Cruz Biotechnology, USA) (1 : 500) and goat anti-mouse IgM (Santa Cruz Biotechnology, USA) (1 : 500), were added and incubated for 3 h at room temperature. The tertiary antibody, FITC-conjugated rabbit anti-goat IgG (1 : 500), was added and incubated for 3 h at room temperature. The absorbance was measured at 405 nm in a Fluoroskan II (Dainippon Pharmaceutical, Co., Japan).

Statistical analysis

Statistical significance was determined by Student's *t* test, with $p < 0.05$ considered to be statistically significant.

Results

Generation of the recombinant *B. animalis*

To obtain the recombinant *B. animalis*, the pBLES-FliC, which was a flagellin-expressing *Escherichia coli-Bifidobacterium* shuttle vector, was transformed into competent cells of *B. animalis* by an electroporation method. The expression of flagellin protein in this recombinant *B. animalis* was confirmed by Western blotting, and the

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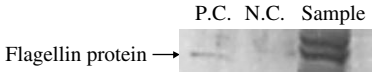

Figure 2. Western blot analysis. Bacterial lysates of *B. animalis* expressing flagellin. P.C., positive control (10 ng of flagellin derived from *S. typhimurium*). N.C., negative control (5 μg protein lysates of parental *B. animalis*). Sample: 5 μg protein lysates of the recombinant *B. animalis*

induction of antibodies against *S. typhimurium* flagellin was confirmed by the mouse experimental model.

Expression of the flagellin protein in the recombinant *B. animalis*

To determine whether the recombinant *B. animalis* could express the flagellin protein, whole-cell lysates of the recombinant *B. animalis* were analyzed by Western blotting after sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE), using anti-*Salmonella* antibody for the detection of flagellin. An immunoreactive band was detected in the recombinant *B. animalis*, whereas there was no band in the control lanes of the parental *B. animalis*. Furthermore, the electrophoretic mobility of the band from the recombinant *B. animalis* was similar to that of the purified flagellin from *S. typhimurium* (Figure 2). There was also an upper band of flagellin which we considered to be a complex between flagellin and other proteins.

The recombinant *B. animalis* induced the antibody against *S. typhimurium* flagellin in the mouse model

We assessed the anti-flagellin antibody responses of three different experimental groups by ELISA. In group 1, the oral administration of the recombinant *B. animalis* every other day for 6 weeks induced significantly ($p < 0.001$) higher levels of anti-flagellin IgA antibody in feces from day 14 as compared with the other groups (i.e., the parental *B. animalis* and PBS groups) (Figure 3). Also, a significant ($p = 0.0024$) anti-flagellin serum-antibody response was observed in group 1 at day 48 as compared with the other groups (Figure 4A). However, no significant response of anti-flagellin IgG or IgM serum-antibody was detected in group 1 (Figure 4).

Discussion

Pathogens that enter an organism initially may elicit a mucosal or systemic response depending on the route of entry. The initial infection with *Salmonella* occurs at the mucosa of the intestines and, after penetrating the mucosal epithelium, the infection becomes systemic. Although T-cell-mediated immunity is important for acquired resistance to systemic *Salmonella* infection, the

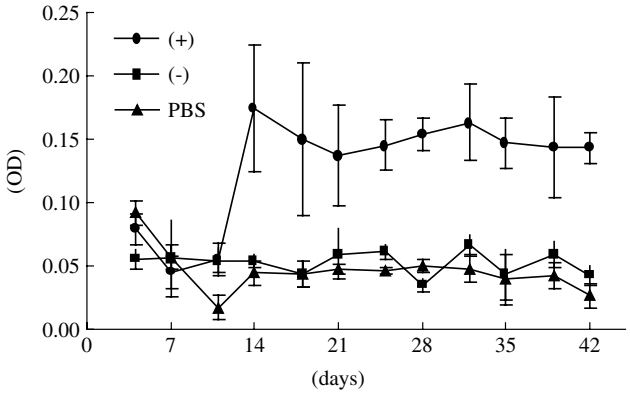

Figure 3. Anti-flagellin IgA antibody levels in stools following oral vaccination (n = 7 in each group). (+), recombinant *B. animalis* group; (−), parental *B. animalis* group; PBS, the PBS group. Blood was collected on days 4, 7, 11, 14, 18, 21, 25, 28, 32, 35, 39, and 42. Antibodies were determined by ELISA for each mouse sample (dilution of 1 : 10). Each point represents the average of each groups; bars, ±SD. A significantly higher level of anti-flagellin IgA antibody was observed in the recombinant *B. animalis* group from day 14 as compared with the other groups ($p < 0.001$)

secretory intestinal antibody response for preventing mucosal invasion may also play a significant protective role against *Salmonella* infection. The humoral response at the mucosa is mediated predominantly by secretory immunoglobulin A (IgA) antibody [20]. To date, several oral vaccine delivery systems have been explored in an attempt to develop mucosal vaccines [20]. In the present study, we describe a novel mucosal vaccine delivery system using *B. animalis*, and demonstrated that the oral administration of recombinant *B. animalis* genetically modified with the *Salmonella flagellin* gene could induce the anti-flagellin IgA antibody as evident in stools and serum, using a mouse model. In our animal experiments, anti-flagellin IgA antibody responses were detected in the stools from day 14 and in the blood serum at day 42, whereas we detected neither IgG nor IgM antibody responses in the blood serum. These results suggested that our oral vaccine delivery system using the recombinant *B. animalis* was suited to the mucosal immunization by inducing a humoral immune response.

Recently, significant progress has been made in the development of attenuated *S. enterica* strains not only as candidate typhoid fever vaccines, but also as heterologous antigen carriers such as the tetanus toxin fragment C [21]. However, a major concern is that the attenuated pathogen is not suited for use in immunocompromised or weak hosts, for whom it can potentially be pathogenic [20]. In the present study, we selected the *B. animalis*, which is a non-pathogenic intestinal microflora, as an oral vaccine of specific antigen carrier. In many previous studies, lactobacilli have been used as candidates for oral live vaccine delivery vectors [20]. Bifidobacteria and lactobacilli are similar in many ways, and both have been widely used in food preparation, being consumed without causing health problems for thousands of years. Although there are many studies on oral vaccines carrying

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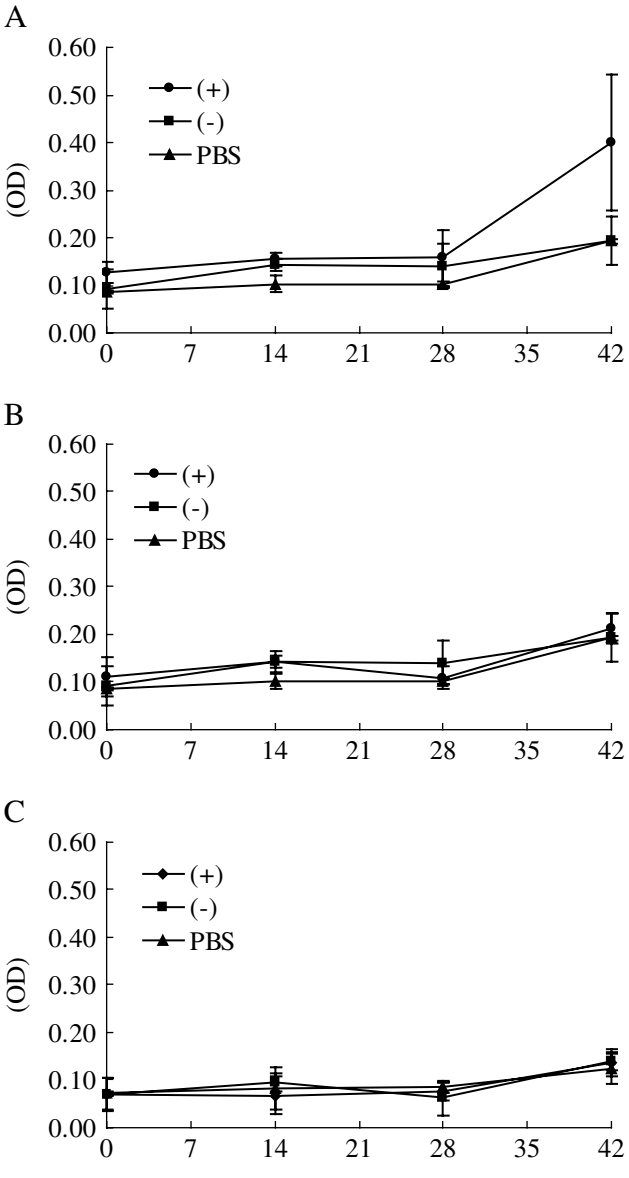

Figure 4. Anti-flagellin antibody levels in serum following oral vaccination (n = 7 in each group). (A) IgA in the serum; (B) IgG in the serum; and (C) IgM in the serum. Blood was collected on days 0, 14, 28, and 42. (+), recombinant *B. animalis* group; (−), parental *B. animalis* group; PBS, the PBS group. Antibodies were determined by ELISA for each mouse sample (dilution of 1 : 100). Each point represents the average of each group; bars, ±SD. A significantly higher level of anti-flagellin serum-IgA antibody was observed in the recombinant *B. animalis* group at day 48 as compared with the other groups ($p = 0.0024$)

a specific antigen using the live lactobacilli, few studies have focused on a bifidobacteria vaccine [20].

Many previous studies have suggested that these probiotic bacteria in the gut can elicit a multiplicity of inhibitory effects against pathogens [22]. There are several potential mechanisms by which probiotic bacteria may inhibit intestinal infections: by strengthening the mucosal barrier, by competitive exclusion to inhibit the adhesion of pathogens, by suppressing intestinal inflammation, and by affecting the immune system [23]. With regard to stimulating the immune system, different bacterial strains appear to differentially induce cytokine responses. Lammers and colleagues suggested that bifidobacteria might play an important role in such immunological responses as secretion of the cytokines, interleukin 10 and interleukin 1 [24]. In addition, bifidobacteria contain DNA with a high GC content and hence a high fraction of unmethylated CpG motifs that affect the immune system by interacting with Toll-like receptor (TLR) 9, triggering the production of proinflammatory cytokines and promoting the Th-1 response [25]. Another report also suggested that CpG motifs could effect mucosal immunity by enhancing mucosal cytokine production and the influx of immune cells to mucosa [26]. In the present study, we succeeded in inducing bifidobacteria to express flagellin as a *Salmonella* antigen using a novel *Escherichia coli-Bifidobacterium* shuttle vector system [13]. We confirmed the expression of the flagellin protein in the recombinant *B. animalis* by Western blotting.

Our recombinant *B. animalis* carries the specific antigen of *Salmonella*, the flagellin gene. Cookson *et al*. demonstrated that a flagellin-specific $CD4^+$ T cell response was strongly induced by oral immunization with attenuated *Salmonella* in a mouse model [27]. In addition, flagellin also binds to TLR5 and activates proinflammatory cytokines (e.g., tumor necrosis factor-α, interleukin-1β) [28]. These observations indicated to us the great potential of a genetically engineered *B. animalis*, that could effectively carry flagellin, a strong antigen, to the intestinal mucosa and thereby elicit a humoral and cellular immune response to the pathogen. To further develop this novel oral vaccine delivery system for typhoid fever, additional mouse experiments including challenges with *S. typhimurium* would be required to establish the efficacy of this vaccination against *Salmonella.* Further, a modification of the formula of this live vaccine, which may include freeze-drying or other preparations, should be investigated with the ultimate goal of creating a more practical clinical application.

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