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Development of Recombinant Vaccines in Lactobacilli for Elimination of *Salmonella*

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Many *Lactobacillus* and *Lactococcus* strains are generally regarded as safe for consumption because they are utilized for food fermentation or inhabit the intestinal mucosa as commensals. Recently, vaccine delivery systems using lactic acid bacteria (LAB) have been under development. Our research group has been investigating the development of oral mucosal vaccines against *Salmonella enterica* serovar Enteritidis (SE) using *Lactobacillus casei* IGM393 as an antigen delivery vehicle. Recombinant lactobacilli expressing SE antigens, FliC, SipC, and OmpC, have been constructed and orally administered to mice. Antigen specific immune responses and protective immunity were elicited after the immunization. For adjuvant-delivery, IL-1β-secreting *L. casei* was also engineered and its effects evaluated *in vitro* and *in vivo*. This article reviews a novel approach to the elimination of *Salmonella* via the development of a vaccine in lactobacilli.

Key words: *Lactobacillus*; *Salmonella*; vaccine; flagellin; recombinant

BACKGROUND

Salmonellosis is caused by consumption of eggs contaminated with *Salmonella* and constitutes a major public health concern not only for developing countries but also industrialized countries. In Japan, 123 outbreaks of food poisoning involving 2,025 patients (1 death) caused by non-typhoidal *Salmonella* were reported in 2006 (annual report of the Ministry of Health, Labour and Welfare). Although the number of cases has been decreasing since 2002 (465 cases, 5,833 patients, 2 deaths), salmonellosis remains a major food-borne disease. In 2010, the United States of America experienced a nationwide outbreak of *Salmonella* Enteritidis (SE) infection which was likely caused by contaminated eggs (CDC, http://www.cdc.gov/salmonella/enteritidis/index. html). A survey by the Food Standards Agency of the United Kingdom found that 0.3% of UK-produced eggs on retail sale during 5 months in 2003 were contaminated with *Salmonella* of which 78% was SE. In another report, *Salmonella* was detected in 13.3% and 0.6% of eggs sampled that were produced in Spain and France, respectively *(1)*.

Transmission of SE caused by external fecal contamination of egg shells can be avoided via cleaning and inspecting eggs; however, SE can latently infect the ovaries of hens contaminating the inside of the eggs before their shells are formed. In this occurs, there is no means of removing SE from the eggs. Hence, SE infection of hens has to be prevented in order to control egg-borne salmonellosis. Vaccination of hens is an effective option for avoiding SE infection and several laboratory or field trials have demonstrated the efficacy of SE vaccines *(2–6)*. The present anti-SE vaccines for hens are administered by injection and induce systemic immune responses; however, a totally effective vaccine has not yet been developed. Mucosal vaccines are considered more effective vaccine candidates because they can induce not only systemic immunity but also local immune responses at the mucosal site, which is the first line of defense against the pathogens.

Vaccine delivery systems using lactic acid bacteria (LAB) for mucosal immunization are under development. Many *Lactobacillus* and *Lactococcus* strains are generally regarded as safe (GRAS) because they have been consumed for centuries in fermented foods, or as probiotics originating as commensals of the human intestinal tract. It is now well documented that LAB can provide immune modulating/stimulating activities and contribute to health maintenance *(7, 8)*. Recent studies have revealed that several cell surface components of probiotic bacteria are recognized by immune cells via pattern recognition receptors (PRRs) *(9)*. In particular, lipoteichoic acid (LTA), peptidoglycan (PG), and muramyl dipeptide (MDP), a subcomponent of PG, are

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Table 1. Immune responses induced by FliC-displaying *L. casei* and purified flagellin

Dose (i.g.)	Dosage (per mouse)	α-FliC sIgA	α-FliC IgG	Specific IFN-γ	Protection
LCF	1×10^{10} cfu	–	–	+++	+
LCF	1×10^{9} cfu	–	–	++	ND
LCN + Flagellin	1×10^{10} cfu + 5 μg	–	–	+	ND
LCN	1×10^{10} cfu	–	–	–	–
Flagellin	50 μg	+	+	++	+
Flagellin	5 μg	–	–	+	ND
Flagellin	0.5 μg	–	–	–	ND
PBS		–	–	–	–

known to be major immune stimulators recognized by Toll-like receptor 2 (TLR2) and nucleotide-binding oligomerization domain 2 (NOD2) *(10–12)*. The combination of safety and immunogenicity of LAB satisfies the key requirements of vaccine-delivery vehicles. Several studies have demonstrated the potential of *Lactococcus lactis* and *Lactobacillus* strains for immunization as described in previous reviews *(13–15)*. Recently, recombinant lactobacilli expressing *Salmonella* antigens have been constructed and their potential as mucosal vaccines has been reported. In the present review, a series of studies on the development of vaccines against SE using *Lactobacillus casei* as a delivery vehicle are outlined.

PROTECTIVE IMMUNITY AGAINST SE INDUCED BY FLIC-DISPLAYING *L. CASEI*

Flagellin is a predominant subunit of flagella and includes highly conserved regions among Gram-negative and Gram-positive bacteria *(16)*. In the bacterial flagellum, thousands of copies of flagellin are polymerized to form a helical tube structure *(17)*. A flagellin of *Salmonella*, FliC, has been reported to act as a protective antigen against SE infection in mice *(18)*. Thus, this antigen in combination with lactic acid bacteria would appear to be a suitable vaccine candidate. For expression of the heterologous antigen on the bacterial cell surface, plasmid vector, pLP401, was employed. A plasmid with an inserted *fliC* gene was introduced into *L. casei* IGM393. Since FliC fused to a signal peptide and LPXTG-included anchor motif, SE antigen was displayed on the cell surface. Mice were immunized with this FliC-producing *L. casei* (LCF) via the intragastric (i.g.) route. After immunization, the mice were challenged with SE, then sacrificed and the number of SE in spleens was counted. Compared to the non-immunized or the control strain of *L. casei* (LCN)-immunized mice, roughly 10-fold less SE cfu were recovered from the LCF-immunized mice. This result indicates that FliC-producing lactobacilli can confer protective immunity against SE. Because neither FliC-specific serum IgG nor fecal IgA were detected in this immunization, other immunity factors likely contributed to the protection. *Ex vivo* re-stimulation of spleen and mesenteric lymph node (MLN) cells with SE flagellin showed that relatively high levels of IFN-γ were produced from the cells of LCF-immunized mice compared to spleen/MLN cells from the other groups. Oral immunization with purified SE flagellin also provided protection against SE, albeit the immune responses were dissimilar to those of LCF-immunization. In particular, FliC-specific serum IgG and fecal IgA were induced but the level of IFN-γ produced by spleen/MLN cells was moderate. Even when the amount of FliC was normalized across the samples, FliC-displaying *L. casei* induced higher IFN-γ production than purified FliC or LCN mixed with purified FliC. This result suggests that the physical state of FliC is important for the adjuvanticity. Furthermore, a previous study reported that flagellin conjugated to polyacryl starch microparticles was less immunogenic than the free form of flagellin *(18)*. Taken together, both the physical location and components of the bacterial cells would appear to be important for the adjuvant effect. The immune responses determined in this study are summarized in Table 1. Detailed information is shown in the original paper *(19)*.

IMMUNOLOGICAL PROPERTIES OF RECOMBINANT *L. CASEI* DISPLAYING OTHER SALMONELLA ANTIGENS WITH OR WITHOUT FLIC FUSION

Although FliC-displaying *L. casei* provides protection against SE, its efficacy is in need of improvement. Since FliC is not the only protective antigen which can be used, additional antigens might increase the efficacy of immunization. Two other *Salmonella* antigens, SipC and OmpC, were employed to investigate whether either of those proteins in combination with FliC improves protection against SE infection. SipC is a member of the proteins involved in type III secretion systems (TTSSs)

Table 2. Immunological properties of recombinant *L. casei* displaying *Salmonella* antigens.

Recombinant *L. casei*	*In vitro* assays	Route of immunization	Immune responses	Protection against SE	References
FliC	IL-8 from Caco-2 cells	i.g.	IFN-γ	Yes	19
cSipC	ND	i.p.	α-cSipC IgG	ND	28
FliC-cSipC	IL-8 from Caco-2 cells	i.p./i.g.	α-cSipC/FliC IgG IFN-γ	Yes	28, Kajikawa *et al.* unpublished data
cSipC-FliC	IL-8 from Caco-2 cells	i.p.	α-cSipC/FliC IgG	ND	28
OmpC	Decrease in TNF-α from RAW264.7 cells	ND	ND	ND	27
FliC-OmpC	ND	i.g.	IFN-γ	Yes	Kajikawa *et al.* unpublished data

and possesses dual functions, including translocation of effectors and actin modulation *(20, 21)*. A specific immune response to SipC is induced during infection by *Salmonella*, and the $CD4^+$ T cell epitope I-A^d/SipC 381-94 has been defined *(22)*. The C-terminal region of SipC (cSipC), including the T cell epitope, was used for this study. OmpC is a major outer membrane porin of *Salmonella*, the function of which is the formation of a channel for the diffusion of nutrients and low molecular weight compounds across the outer membrane *(23)*. OmpC is also known as a protective antigen for vaccination against *Salmonella* because OmpC-specific antibodies exert a bactericidal effect *(24–26)*. The genes encoding either cSipC or OmpC were introduced into *L. casei* IGM393 via the pLP401 vector and expressed on the cell surface. Interestingly, the recombinant *L. casei* producing OmpC demonstrated decreased cell viability and elicited a relatively low TNF-α response in RAW264.7 cells. This unexpected characteristic of OmpC-producing strains was concluded to be due to weakened cell wall integrity *(27)*. In order to determine the combinational effect with FliC, recombinant lactobacilli displaying FliC-fusion antigens, cSipC fused to either the N- or C-terminus of FliC, and OmpC conjugated to the C-terminus of FliC, were also constructed. The immunological properties of these strains were evaluated both *in vitro* and *in vivo*, and the results are summarized in Table 2. In order to investigate whether these recombinant lactobacilli have TLR5-stimulating activity, IL-8 release from stimulated Caco-2 cells was determined. Remarkable amounts of IL-8 were detected from each culture stimulated with recombinant *L. casei* producing either FliC or FliC-fusion antigens, suggesting that these recombinant lactobacilli retain TLR5-stimulating activity. Parenteral immunization of mice with *L. casei* displaying FliC or FliC-fusion proteins evoked specific immune responses to the cell surface antigens. Interestingly, the α-FliC:α-cSipC IgG ratios were different between the groups immunized with either the recombinant lactobacilli expressing FliC-cSipC or cSipC-FliC, implying that physical structure affects the immunogenicity of cell surface-displayed antigens. In this study, an antigen located on the far side from a cell wall anchor tended to induce antibody production more efficiently. Compared to antibodies induced by soluble antigens, the IgG2a ratio was relatively high in antigen-specific IgG elicited by the recombinant *L. casei*. Cytokine profiling showed that soluble antigens promoted IL-4 secretion from stimulated spleen cells while the antigen-displaying lactobacilli accelerated IFN-γ production. These results suggest that antigens in combination with *L. casei* are likely to skew Th1 polarization. Detailed information of this study is provided in the original paper *(28)*. Oral immunization of mice with *L. casei* displaying either FliC-cSipC or FliC-OmpC provided protection against SE, albeit their efficacies were no better than that of *L. casei* expressing FliC alone. Analysis of antigen-specific immune responses indicated that only FliC but not the fusion partners were recognized by T cells (Kajikawa *et al*., unpublished data). In the case of oral immunization, unfortunately, the fusion of additional antigens to FliC is unlikely to sufficiently improve vaccines against SE using *L. casei* as a delivery vehicle.

ADJUVANT EFFECTS OF RECOMBINANT *L. CASEI* SECRETING BIOLOGICALLY ACTIVE IL-1B

Oral administration is one of the most desirable means of immunization because it is needleless and can elicit both mucosal and systemic immune responses. However, development of oral vaccines is challenging due to the highly tolerant nature of gastrointestinal mucosal immunity. Lactic acid bacteria are considered safe but unlikely to be very immunogenic in oral immunization. In previous studies, a large amount of cells ($>10^9$ cfu) were repeatedly dosed in order to elicit decent immune

Table 3. Adjuvant molecules provided by recombinant lactic acid bacteria

Host strain	Adjuvant molecules	Antigens	Effects	References
L. lactis	Mouse IL-2/IL-6	TTFC co-expression	Increase in Ab production (nasal)	32
L. lactis	Mouse single chain IL-12	E7 (HPV) Provided *in trans*	Protection improved	33
L. casei	Mouse IL-1β	Heat killed SE Provided *in trans*	Increase in Ab production (oral, nasal)	35, Kajikawa *et al.* unpublished data
L. acidophilus	DC peptide	PA fusion	Protection improved	34
L. acidophilus	FliC	Gag (HIV-1) co-expression	Increase in IgA-producing cells	Kajikawa *et al.* unpublished data

responses *(29–31)*. For improvement of immunogenicity, adjuvants have been used in combination with antigen-producing lactic acid bacteria. As listed in Table 3, cytokines and other molecules are provided by recombinant lactic acid bacteria and improve vaccine efficacy. Steidler *et al*. carried out a pioneering study, which demonstrated the adjuvant effect provided by *L. lactis* intracellularly expressing TTFC and also secreting either murine interleukin-2 (IL-2) or IL-6 *(32)*. In another report, a single chain murine IL-12 was produced by *L. lactis* and enhanced antigen-specific Th1 cytokine production *(33)*. Recently, a unique strategy to accelerate bacterial uptake by dendritic cells (DCs) was developed by Mohamadzadeh *et al*. *(34)*. They achieved effective oral vaccination using recombinant *Lactobacillus acidophilus* secreting a protective antigen of *Bacillus anthracis* in combination with a DC-targeted peptide. In our previous study, recombinant *L. casei* secreting mouse IL-1β was constructed for adjuvant delivery *(35)*. IL-1β is produced by activated monocytes, macrophages, dendritic cells, and other cells as a precursor, which is proteolytically processed into a mature form by IL-1β-converting enzyme (ICE), also known as caspase-1 *(36)*. Cleavage and activation of caspase-1 is triggered via recognition of pathogen-associated molecular patterns (PAMPs) by NOD-like receptors (NLRs) and formation of macromolecular complexes known as the inflammasomes *(37)*. Since NLRs are cytoplasmic proteins, viable pathogens, but not dead or commensal bacteria, can activate this cascade. We hypothesized that IL-1β provided by recombinant lactobacilli might mimic the "danger signal" of pathogens and complement the efficacy of vaccine candidates. IL-1β and other IL-1 family cytokines play an important role in modulating the adaptive immune response *(38)*. In fact, previous studies have demonstrated the adjuvant effect of IL-1β in both mucosal and systemic immunization *(39)*. In a previous study, a matured mouse IL-1β-encoding gene was combined with a bacterial signal sequence and introduced into *L. casei*. The resulting recombinant *L. casei* secreted IL-1β into its culture supernatant. The secreted proinflammatory cytokine elicited IL-8 production from Caco-2 cells, suggesting that the IL-1β was biologically active. In order to determine cytokine responses at the intestinal mucosa, a ligated intestinal loop assay followed by RT-PCR was performed. Among the detected cytokines, expression of IL-6 was specifically up-regulated in response to the IL-1β-secreting lactobacilli. IL-6 production by isolated Peyer's patch cells was also confirmed *in vitro*. For evaluation of adjuvant effect, the IL-1β secreting *L. casei* mixed with heat-killed SE was orally administered to mice. Relatively high *Salmonella*-specific IgG in serum and IgA in feces was detected after the immunization. This result suggests that IL-1β-secreting *L. casei* provides an adjuvant effect which delivers a promising improvement to vaccine efficacy.

CONCLUDING REMARKS

A series of studies on the development of vaccines against SE using *L. casei* as a delivery vehicle has been pursued. Recombinant lactobacilli displaying FliC or FliC-fused antigens can induce both innate and adoptive immune responses. Moreover, intragastric immunization of mice with these *L. casei* strains can confer protective immunity against SE infection. Biologically active matured murine IL-1β secreted by recombinant *L. casei* accelerated SE-specific antibody production. This adjuvant molecule may deliver a promising improvement to the efficacy of vaccines based on lactic acid bacteria.

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