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# Studies on Mucosal Immunity Induced by Transmissible Gastroenteritis Virus Nucleocapsid Protein Recombinant *Lactobacillus casei* in Mice and Sow

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## Abstract

Mucosal immunity plays an important role in protecting pigs against transmissible gastroenteritis virus (TGEV) infection. To elicit mucosal immune response against TGEV, we developed a surface antigen display system using the poly- $\gamma$ -glutamate synthetase A (pgsA) protein of *Bacillus subtilis* as an anchoring matrix to express recombinant fusion proteins of pgsA and nucleocapsid protein of TGEV in *Lactobacillus casei*. Surface location of fusion protein was verified by ELISA and indirect immunofluorescence test. Oral and intranasal inoculations of pregnant sow and mice with recombinant *L. casei* resulted in high levels of serum immunoglobulin G (IgG) and secretory immunoglobulin A (sIgA) against recombinant N protein as demonstrated by ELISA. More importantly, the level of specific sIgA in colostrum significantly increased compared with that of IgG. The serum IgG levels of the piglets increased after suckling colostrum produced by sows was previously inoculated with recombinant *L. casei*. These results indicate that immunization with recombinant *L. casei* expressing TGEV N protein on its surface elicited high levels of specific sIgA and circulating IgG against TGEV N protein.

**Key words:** transmissible gastroenteritis viral, lactic acid bacteria, N protein

## INTRODUCTION

Live vaccine vehicles offer a powerful approach for inducing protective immunity against pathogenic microorganisms. A variety of viruses, bacteria, and protozoans have been used successfully as vaccine delivery systems in several experimental models. The delivery of vaccine subunits to the mucosal surface by a suitable live microbial vector is a rational approach to get around the obstacles encountered by parenteral vaccines. Oral antigen delivery systems that potentiate mucosal immune responses against adherent pathogens have received considerable attention because parenteral immunization usually elicits little or no mucosal immunity. However, potential safety and environmen-

tal considerations, particularly the immune status of the vaccine recipients in developing countries, still raise much concern over the use of the mucosally delivered vector candidates such as *Escherichia coli*, *Salmonella*, and vaccinia virus. Therefore, nonpathogenic, food grade, or commensal bacterial vectors have begun to receive attention for their vaccine potential (Lee *et al.* 2006).

Transmissible gastroenteritis virus (TGEV) is a coronavirus that causes acute diarrhea in piglets, characterized by up to 100% mortality among neonatal pigs (Sestak *et al.* 1996, 2002). Maternal antibodies, passed to piglets in colostrums and milk, provide effective protection against infection. Against the TGEV infection, a variety of viral vaccines (virulent, attenuated, inactivated, and subunit) and routes of administration

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(oral, intranasal, intramuscular, subcutaneous, and intramammary) have been tested for induction of neonatal immunity (Kaji and Shimizu 1978; Moxley and Olson 1989; Saif *et al.* 1994). However, only oral administration of live virulent virus to pregnant sows generally gave the highest level of immunity, resulting in protective immunity for the sow and consistently producing high level of persisting IgA TGEV antibodies in milk associated with protective neonatal immunity for sucking piglets. During this decade, emphasis had been laid on the development of new vaccines for active and passive immunity to TGEV. Since stimulation of intestinal immunity is important to induce protection against TGEV, investigators have attempted to develop genetically engineered vaccines using live recombinant vectors such as attenuated strains of *Salmonella typhimurium* (Smerdou *et al.* 1996). However, no pig protection studies have been reported. Studies are in progress using a live eukaryotic vector, human adenovirus 5, to express TGEV S glycoprotein that induced neutralizing antibodies in the serum of swine inoculated orally, nasally, and intraperitoneally (Torres *et al.* 1996). No results were reported on the direct TGEV challenge to the recombinant TGEV adenovirus-inoculated pigs. In recent years, major technique advances in a great deal to generate antibodies including hybridoma, bacteria, and phage systems made it possible that a new recombinant vaccine might be a promising candidate vaccine for the control of swine disease (Maynard and Gerorgiou 2000; Humphreys and Glover 2001).

In this study, we developed a novel expression vector using *pgsA* gene product as an anchoring matrix. Intranasal and oral vaccination of mice and sow with the live and inactivated recombinant *Lactobacillus casei* 525 elicited high levels of circulating antibodies and local mucosal immunity against TGEV N antigen, suggesting a potential use for the control of TGEV infection.

## MATERIALS AND METHODS

### Construction of minimal surface display vector

The minimal surface display plasmid, pLA-TGEV N, was constructed by PCR amplification using pGEM-T-TGEV N as templates as follows: PCR-amplified 1 124

bp DNA fragment with 5'-CGCGGATCCGCC AACCAGGG ACAA-3' and 5'-CGCGGATCC TTAGTTCGTT ACCTCATCAAT-3' encoding the nucleocapsid protein of TGEV was digested by *Bam*H I and inserted into pLA. Restriction fragment analysis was performed to select right-oriented clones, creating pLA-TGEV N.

*L. casei* 525, isolated from Korean food, was transformed by electroporation (Pulse Controller Plus, Bio-RAD, USA) with pLA-TGEV N. The lactobacilli were cultured in MRS medium (Difco, USA) supplemented with 16 µg mL<sup>-1</sup> of erythromycin at 30°C. The cells were harvested, and the existence of the fusion proteins on their surface was examined by ELISA and indirect immunofluorescence test.

### ELISA and indirect immunofluorescence test

Detection of the surface display of the recombinant N proteins on *L. casei* cells was performed using the method of Laitinen *et al.* (2002). Briefly, the cells were harvested after expression and resuspended in phosphate-buffered saline (PBS) to OD<sub>600</sub> of 1.0. Aliquots of 1 mL from this resuspension were centrifuged at 3 000 × g for 30 min and washed twice with PBS before resuspended in 200 µL of PBS. Four microliters of rabbit polyclonal antibodies against TGEV N prepared previously were added into the cell suspension and incubated for 1 h. After washing, the cells were resuspended in 200 µL of PBS. Two microliters of anti-rabbit IgG conjugated with horseradish peroxidase (Sigma, USA) were added into the cell suspension and incubated for 1 h. The cells were washed twice with PBS and once with H<sub>2</sub>O<sub>2</sub> substrate buffer containing OPD chromogen and then resuspended in 1 mL of the same buffer to make the OD<sub>600</sub> value of 0.02 to 0.20. The cell suspension (100 µL) was added into the wells of the microtitration plates and incubated 15 min in the dark at room temperature. In the same manner, *pgsA* on the cell surface was detected to examine the fusion protein using the anti-*pgsA* antibody.

For immunofluorescence microscopy, cells labeled with anti-TGEV N polyclonal antibody and fluorescence isothiocyanate-conjugated anti-rabbit antibody were examined using a Carl Zeiss Axioskop 2 fluorescence microscopy (Olympus, Japan). Photographs were taken

with an AxioCam high-resolution camera (Olympus, Japan).

### Western blotting

The pgsA-TGEV N protein was examined by SDS-PAGE and immunoblotting according to the method of Burnette (Burnette 1981). Briefly, protein samples were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Roche Diagnostic Corp., USA), which was blocking with 5 mL of 1 M Tris-HCl, pH 8.0 solution containing 5% skim milk (Difco, USA) for 1 h and probed with rabbit anti-TGEV N and anti-pgsA polyclonal antibodies (1:1 000 dilution). Following overnight incubation, the membrane was extensively washed with washing buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween-20). Biotinylated anti-rabbit antibody (Sigma, USA) (1:3 000 dilution) was added to the membrane and incubated for 3 h. After washing the membranes with washing buffer, the membranes were treated with avidin and biotin complex (Vectastain ABC Kit, Vector Lab, USA) following the manufacturer's instructions. Color was developed with diaminobenzidine (DAB) solution (Vector Lab, USA).

### Immunization of mice and sows with TGEV N proteins and serum sample collection

240 C57BL/6 mice were randomly divided into 12 groups with 20 mice in each group. Each group of mice was immunized orally or intranasally with live *L. casei* or live *L. casei* bearing TGEV N protein. The recombinant live *L. casei* cells displaying TGEV N on their surface were resuspended in 100  $\mu$ L sterile PBS at the concentration of  $5 \times 10^9$  for the oral route. The suspension was administered daily via intragastric lavage on day 0 to 4, 7 to 11, 21 to 25, and 49 to 53. For the intranasal route,  $2 \times 10^9$  *L. casei* cells in 20  $\mu$ L suspension were administered into nostrils of lightly anesthetized mice on day 0 to 2, 7 to 9, 21, and 49. Blood samples were collected from the tail vein on day 0 (preimmune), 14, 28, 42, 56, and 70. Sera were prepared from the blood and stored at  $-20^\circ\text{C}$  until use. Mice were sacrificed at day 56 and 70 for collection of bronchoalveolar and intestinal lavage fluids. Samples were centrifuged at  $2\,500 \times g$  for 20 min at  $4^\circ\text{C}$ , and the

supernatants were stored at  $-20^\circ\text{C}$  until use.

Ten Yorkshire pregnant sows one month before farrowing, seronegative to the TGEV, were assigned to two different experiment groups. The sows were fed with a commercial diet for their age. Each group was kept in isolated facilities to prevent virus circulation among them. The inactivated recombinant *L. casei* cells were added to the feed at the final cell concentration of  $10^6$  cells  $\text{kg}^{-1}$  feed. The sows in the treatment group were fed with this feed throughout the experiment. The sows in the untreated group were fed with the plain feed throughout the experiment. The colostrum was collected at the farrowing day. The piglets' serum was collected on day 10 after sucking colostrums.

### Antibody detection

The ELISA of TGEV N protein was developed for detection of antigen-specific IgG and sIgA in sera and colostrum as previously described (Yu and Hou 2005).

## RESULTS

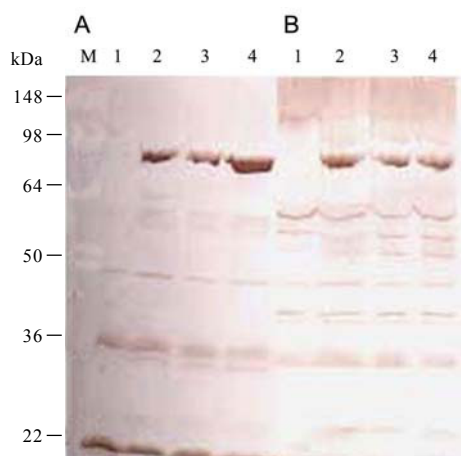
### Construction of surface display vector and expression of TGEV N viral-antigen hybrids on the surface of *L. casei*

To express fusion protein on the surface of *L. casei*, pgsA was used as the anchor protein. We cloned TGEV N gene directed downstream to pgsA gene in a shuttle vector pLA. *E. coli* carrying the plasmid was grown overnight at  $37^\circ\text{C}$ . The cells were harvested, and expression of the expected chimeric protein was confirmed by immunoblotting using anti-pgsA and anti-TGEV N polyclonal antibodies (data not shown). *L. casei* cells were then transformed with the plasmid (pLA-TGEV N) and cultured at  $30^\circ\text{C}$ . Expression of the pgsA-TGEV N fusion protein was detected by immunoblotting (Fig. 1). The pgsA-TGEV N was stably expressed through serial passages and maintained its predicted molecular mass (84 kDa). When reacted with anti-TGEV N and anti-pgsA polyclonal antibodies, the same bands corresponding to the interested protein size 84 kDa were exhibited strongly on the membranes.

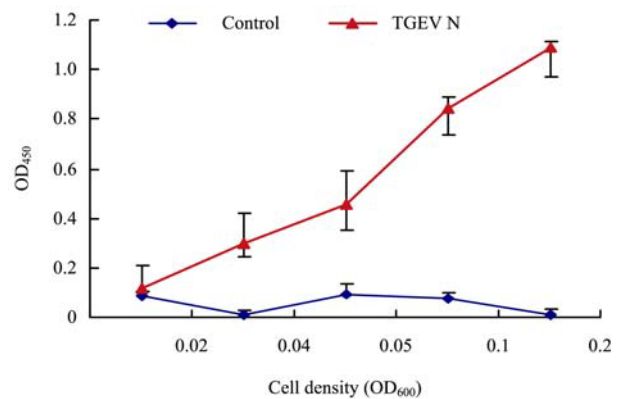
ELISA and fluorescence microscopy were used to determine the cellular localization of the recombinant protein on the surface of *L. casei* (Figs. 2 and 3, respectively). The ELISA color intensity increased with increasing cell density of recombinant *L. casei*, whereas the color intensity for *L. casei* host cells remained at a very low level (Fig. 2). Analyses by immunofluorescence microscope also revealed fluorescence only in the recombinant bacteria harboring pLA-TGEV N but not in the control cells (Fig. 3). These results showed that TGEV N protein was properly and efficiently displayed on the cell surface of *L. casei* using pgsA as a membrane-anchored protein display motif.

### Immunogenicity of fusion proteins expressed on *L. casei*

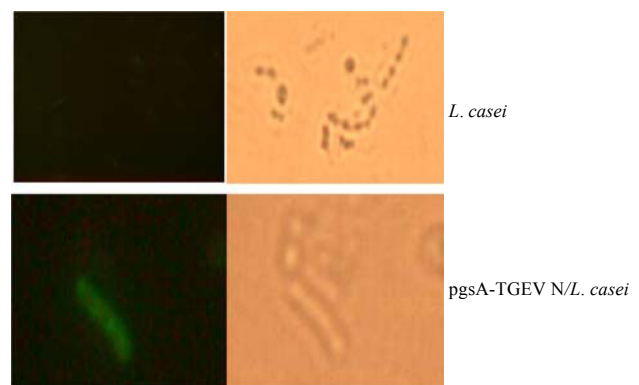
To characterize the immunogenicity of TGEV N surface displayed on *L. casei*, the N proteins were administered into C57BL/6 mice and pregnant sows by mucosal administration. C57BL/6 mice (20 per group in 12 groups) were orally and intranasally administered with  $5 \times 10^9$  cells/mouse and  $2 \times 10^9$  cells/mouse of the recombinant live *L. casei* and control group with *L. casei* host cells. Levels of specific antibody in the serum were evaluated by ELISA (Fig. 4). During the first two series of immunization, very low levels of IgG antibody were detected (Fig. 4, day 14). The higher IgG



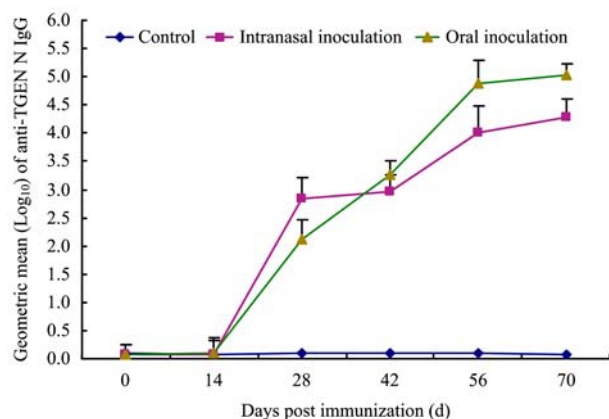
**Fig. 1** Detection of pLA-TGEV N expressed on *L. casei* by Western blotting with anti-TGEN (A) and anti-pgsA (B) polyclonal antibodies. Lane 1 shows control *L. casei*; lanes 2-4 show the recombinant *L. casei*. Protein bands of about 84 kDa corresponding to the expected size of pgsA-TGEV N were detected.



**Fig. 2** Confirmation of surface-displayed TGEV N on *L. casei* by ELISA.



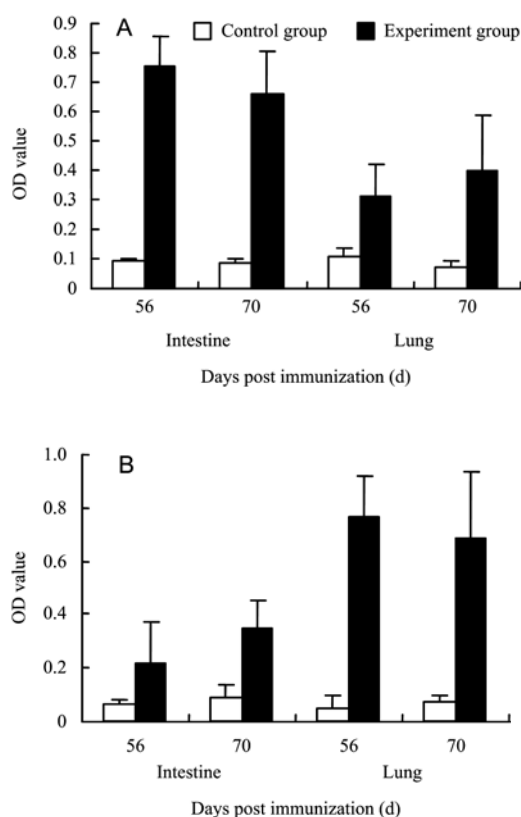
**Fig. 3** Representative immunofluorescence images of control cells harboring pLA and recombinant cells expressing pgsA-TGEV N. Bright-field images are shown in the right.



**Fig. 4** Antigenicity of surface-displayed TGEV N antigen in lactic acid bacteria display. Anti-TGEV N mouse serum IgG absorbances following intranasal and oral immunization with recombinant *L. casei*. There were no statistically significant differences between the two groups as determined by Mann-Whitney *U*-test.

levels were detected shortly after the third immunization (day 28,  $P < 0.01$ ). After the fourth immunization, further increase in IgG titer was observed (day 56,  $P < 0.01$ ) by oral inoculation. The results also showed that there were no significant differences between oral and intranasal routes.

To assess mucosal immune response, N protein-specific sIgA levels in intestinal and bronchoalveolar lavage fluids were determined by ELISA. Fluids collected on days 56 and 70 after immunization were examined using the purified recombinant N protein expressed in *E. coli* as coating antigens. Both intranasal and oral immunization elicited N protein-specific IgA responses at the site of inoculation, as well as the remote mucosal sites (Fig. 5). The sIgA level was higher in experiment group than the control group for all sets of experiments that anti-TGEV N sIgA antibody responses ( $P < 0.01$ ).

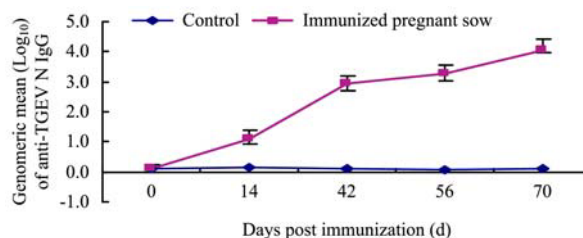


**Fig. 5** Anti-TGEV N sIgA antibody responses. Intestinal and bronchoalveolar lavage fluids, harvested from mice sacrificed 56 or 70 days postimmunization, were analyzed by ELISA. Concentrations of samples from animals immunized orally (A) or intranasally (B) are shown. Fluids from control animals are shown as black bars. The error bars represent standard deviations. The differences between the two groups are statistically significant as determined by Mann-Whitney *U*-test.

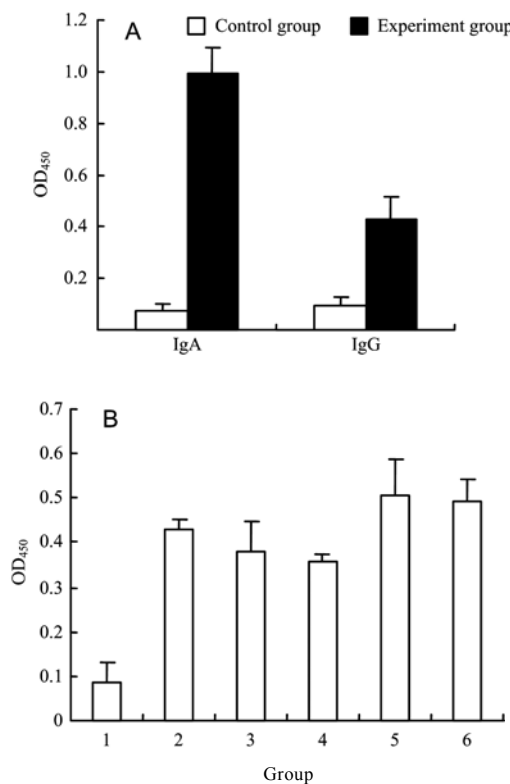
The results also showed that the sIgA level in intestine (Fig. 5-A, left) was higher than that in lung (Fig. 5-A, right) in oral route for both day 56 and day 70 ( $P < 0.01$ ); however, the opposite results were found in intranasal routes ( $P < 0.01$ ). The sIgA was barely detected in control mice. To better characterize antibody responses against TGEV N protein, 5 TGEV-free pregnant sows (1 month before farrowing the piglets) were immunized with heat-killed recombinant TGEV N *L. casei* by oral administration and four as controls. Specific IgG antibodies were detected on days 0, 14, 42, 56, and 70 after immunization. The concentration of IgG was increased in a time-dependant manner (Fig. 6, day 42,  $P < 0.01$ ). IgG and sIgA colostrum antibodies following oral immunization with recombinant *L. casei* were also detected by rELISA. The concentration of sIgA in colostrum (Fig. 7-A, left) was increased significantly while IgG slightly (Fig. 7-A, right,  $P < 0.01$ ), and they are all higher than their corresponding control ( $P < 0.01$ ). After suckling colostrum secreted from sows previously inoculated recombinant *L. casei*, IgG levels of the piglets were increased slightly (Fig. 7-B).

## DISCUSSION

The recombinant live oral vaccines expressing pathogen-derived antigens on the surface of bacteria can be an alternative method for providing protection. Mucosal immunization offers a number of advantages over other routes of antigen delivery, including convenience, cost effectiveness, and induction of both local and systemic immune responses (Lee *et al.* 2006; Mannam *et al.* 2004; Seegers 2002; Xin *et al.* 2003). Our goal is



**Fig. 6** Anti-TGEV N pregnant sow's serum IgG concentration following oral immunization with recombinant *L. casei*. Coating antigen, 100 ng/well; serum, 1:100; and conjugation, 1:3000. The differences between the two groups are statistically significant as determined by Mann-Whitney *U*-test.



**Fig. 7** Detection of TGEV N sIgA and IgG levels in colostrum secreted from sow previously inoculated recombinant *L. casei* (A). Coating Ag, 100 ng/well for TGEV N; colostrums, 1:100; conjugation, 1:3 000. Colostrum from control animals is shown as black bars. The error bars represent standard deviations. TGEV N IgG antibody in piglets' serum on day 10 after sucking colostrums (B). Coating Ag, 100 ng/well; serum, 1:100; conjugation, 1:3 000; lane 1, negative control; and lanes 2-6, the piglet sera randomly selected from offerings of the five immunized pregnant sows. The differences between the two groups are statistically significant as determined by Mann-Whitney *U*-test.

to provide an effective method that can eliminate pathogens at the mucosal surface. But there is growing evidence that surface display of large antigens on the bacterial surface can perturb membrane topology. True surface exposure of antigens requires a transmembrane anchor that is long enough to cross the cell wall (Sestak *et al.* 1996). At least 100 amino acids are needed to properly cross the cell wall (Leenhouts *et al.* 1999).

In this article, we have developed a surface display system using the pgsA protein as the transmembrane anchor to present heterologous proteins on the surface of *L. casei*. To investigate the feasibility of using lactic acid bacteria as a carrier of immunogenic peptides to mucosal immune system, we immunized mice and pregnant sow orally and/or intranasally with *L. casei* bearing TGEV. Although the level of fusion TGEV N pro-

teins expressed on the surface of *L. casei* was not very higher than TGEV N expressed in *E. coli* (data not shown), oral and intranasal immunizations of swine and mice with these recombinant *L. casei* resulted in systemic and local immune responses. Mucosal delivery of vaccines induces mucosal immunity more efficiently than parenteral immunization (Mutwiri *et al.* 1999). To investigate the feasibility of using lactic acid bacteria as a carrier of immunogenic peptides to mucosal immune system, we immunized mice intranasally and orally with *L. casei* anchoring nucleocapsid proteins of TGEV. Our data showed that there was no significant difference in both routes of administration.

Protection against neonatal infectious agents such as TGEV and porcine epidemic diarrhea viral (PEDV) is currently best obtained by passive immunization of piglets after induction of colostrum antibodies in the sow (Moon and Bunn 1993; Saif 1996). Colostrum antibodies can be induced by the oral delivery of protective antigens activating the gut-associated lymphoid tissues of sows. For this purpose, pregnant sows were orally administrated with recombinant *L. casei* before farrowing. The higher sIgA levels in these sow colostrums were detected by ELISA, although their IgG levels were relatively lower. Furthermore, the piglets that sucked these colostrums showed a certain extent IgG level. Since swine are born without immunoglobulins, immunoprotection for newborn piglets mainly consists of passive immunity through colostrum immunoglobulins from immunized dam (Kweon *et al.* 2000). Thus, passive immunity from colostrum is of primary importance in piglets for protection against infectious enteric diseases.

In conclusion, we have demonstrated that nucleocapsid protein of coronavirus TGEV exposed on the surface of nonpathogenic strain *L. casei*, resisting gastric acidity, delivered orally to animals, elicited both systemic and mucosal immune responses. The immunogenicity of N antigens expressed by such strains may present significant opportunities for TGEV vaccine development.

Despite the rapid advancement of knowledge and concurrent development of techniques, a number of problems have to be solved before the first laboratory vaccine can be marketed. Although it is possible to determine accurately the levels of antigen expression in

the test tube, we cannot measure the gene expression in the gut. Therefore, methods will have to be developed to analyze expression of the antigen encoding genes *in situ* so as to control the efficiency and duration of expression. In addition, how long colonizing bacteria are present in the gut and what the effects are of continuous expression of a LAB-associated antigen remain to be determined.

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### References

- Humphreys D P, Glover D J. 2001. Therapeutic antibody production technology: molecules, applications, expression and purification. *Current Opinion in Drug Discovery & Development*, **4**, 172-185.
- Kaji T, Shimizu Y. 1978. Passive immunization against transmissible gastroenteritis virus in piglets of ingestion of milk of sows inoculated with attenuated virus. *National Institute of Animal Health Quarterly* (Tokyo), **18**, 43-52.
- Kweon C H, Kwon B J, Woo S R, Kim J M, Woo G H, Son D H, Hur W, Lee Y S. 2000. Immunoprophylactic effect of chicken egg yolk immunoglobulin (IgY) against porcine epidemic diarrhea virus in piglets. *The Journal of Veterinary Medical Science*, **62**, 961-964.
- Laitinen R, Malinen E, Palva A. 2002. PCR-ELISA I: application to simultaneous analysis of mixed bacterial samples composed of intestinal species. *Systematic and Applied Microbiology*, **25**, 241-248.
- Lee J S, Poo H Y, Han D P, Hong S P, Kim K, Cho M W, Kim E, Sung M H, Kim C J. 2006. Mucosal immunization with surface-displayed severe acute respiratory syndrome coronavirus spike protein on *Lactobacillus casei* induces neutralizing antibodies in mice. *Journal of Virology*, **80**, 4079-4087.
- Leenhouts K, Buist G, Kok J. 1999. Anchoring of proteins to lactic acid bacteria. *Antonie Van Leeuwenhoek*, **76**, 367-376.
- Mannam P, Jones K F, Geller B L. 2004. Mucosal vaccine made from live, recombinant *Lactococcus lactis* protects mice against pharyngeal infection with *Streptococcus pyogenes*. *Infection and Immunity*, **72**, 3444-3450.
- Maynard J, Gerorgiou G. 2000. Antibody engineering. *Annual Review of Biomedical Engineering*, **2**, 339-376.
- Moon H W, Bunn T O. 1993. Vaccines for preventing enterotoxigenic *Escherichia coli* infections in farm animals. *Vaccine*, **11**, 200-213.
- Moxley R A, Olson L D. 1989. Clinical evaluation of transmissible gastroenteritis virus vaccination procedures for inducing lactogenic immunity in sows. *American Journal of Veterinary Research*, **50**, 111-118.
- Mutwiri G, Watts T, Lew L, Beskorwayne T, Papp Z, Baca-Estrada M E, Griebel P. 1999. Ileal and jejunal Peyer's patches play distinct roles in mucosal immunity of sheep. *Immunology*, **97**, 455-461.
- Saif L J. 1996. Mucosal immunity: an overview and studies of enteric and respiratory coronavirus infections in a swine model of enteric disease. *Veterinary Immunology and Immunopathology*, **54**, 163-169.
- Saif L J, van Cott J L, Brim T A. 1994. Immunity to transmissible gastroenteritis virus and porcine respiratory coronavirus infections in swine. *Veterinary Immunology and Immunopathology*, **43**, 89-97.
- Seegers J F. 2002. Lactobacilli as live vaccine delivery vectors: progress and prospects. *Trends in Biotechnology*, **20**, 508-515.
- Sestak K, Lanza I, Park S K, Weilnau P A, Saif L J. 1996. Contribution of passive immunity to porcine respiratory coronavirus to protection against Transmissible gastroenteritis virus challenge exposure in suckling pigs. *American Journal of Veterinary Research*, **57**, 664-671.
- Sestak K, Saif L J, Morilla A, Yoon K J, Zimmerman J J. 2002. Trends in emerging viral infections of swine. Iowa State Press, Ames, Iowa.
- Smerdou C, Urniza A, Cuitis III R, Enjuanes L. 1996. Characterization of transmissible gastroenteritis coronavirus S protein expression products in avirulent *S. typhimurium*  $\Delta$ cyamit  $\Delta$ crp: persistence, stability and immune response in swine. *Veterinary Microbiology*, **48**, 87-100.
- Torres J M, Alonso C, Ortega A, Mittal S, Graham F, Enjuanes L. 1996. Tropism of human adenovirus type 5-based vectors in swine and their ability to protect against transmissible gastroenteritis coronavirus. *Journal of Virology*, **73**, 3770-3780.
- Xin K Q, Hoshino Y, Toda Y, Igimi S, Kojima Y, Jounai N, Ohba K, Kushiro A, Kiwaki M, Hamajima K, Klinman D, Okuda K. 2003. Immunogenicity and protective efficacy of orally administered recombinant *Lactococcus lactis* expressing surface-bound HIV Env. *Blood*, **102**, 223-228.
- Yu L Y, Hou X L. 2005. Detection of TGEV antibody by enzyme-linked immunosorbent assay using recombinant nucleocapsid proteins. *Agricultural Sciences in China*, **4**, 469-474.

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