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Recombinant S-Layer Proteins of *Lactobacillus brevis* Mediating Antibody Adhesion to Calf Intestine Alleviated Neonatal Diarrhea Syndrome

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A chimeric gene encoding enhanced green fluorescent protein (EGFP) and a S-layer protein from *Lactobacillus brevis* KCTC3102, and/or two copies of the Fc-binding Z-domain, a synthetic analog of the B-domain of protein A, was constructed and expressed in *Escherichia coli* BL21(DE3). The S-layer fusion proteins produced in a 500-l fermentor were likely to be stable in the range of pH 5 to 8 and 0°C to 40°C. Their adhesive property enabled an easy and rapid immobilization of enzymes or antibodies on solid materials such as plastics, glass, sol-gel films, and intestinal epithelial cells. Owing to their affinity towards intestinal cells and immunoglobulin G, the S-layer fusion proteins enabled the adhesion of antibodies to human epithelial cells. In addition, feeding a mixture of the S-layer fusion proteins and antibodies against neonatal calf diarrhea (coronavirus, rotavirus, *Escherichia coli*, and *Salmonella typhimurium*) to Hanwoo calves resulted in 100% prevention of neonatal calf diarrhea syndrome ($p<0.01$), whereas feeding antibodies only resulted in 56% prevention.

Keywords: Antibody adhesion, enzyme immobilization, Hanwoo calf, *Lactobacillus brevis*, neonatal diarrhea, recombinant S-layer

Lactobacillus species have been used as probiotics owing to their beneficial effects on human and animal health by lowering the pH and preventing the attachment of many pathogenic bacteria [21]. The surface layer (S-layer) proteins of *Lactobacillus acidophilus*, *Lactobacillus brevis*, and *Lactobacillus crispatus* were reported to mediate the organisms' binding to intestinal epithelial cells or to collagen [13, 28, 32]. S-layer functions are generally believed to be cell-protective coats, molecular sieves, and cell adhesion mediators [30]. S-layer proteins can also contribute to virulence when they are present as a structural component of the cell envelope of pathogens [27]. The S-layer lattice has oblique, square, or hexagonal symmetries. S-layer proteins isolated from the cell wall can maintain the ability to self-assemble in suspension or to recrystallize on solid supports [27]. This specific feature of S-layers has opened a broad potential for application in nanobiotechnology by making fusion proteins with enzymes, antibodies, allergens, or ligands [29, 31].

Infectious diarrhea is an important cause of neonatal calf morbidity and mortality that results in significant economic losses in the beef and dairy industries. Most virulent pathogens are present within the gastrointestinal tract of many healthy cattle, with the exception of *Salmonella* spp. [2]. Newborn calves growing in a limited space nearby mature cattle in Korean cattle (Hanwoo) farm-houses have a high risk of the occurrence of neonatal diarrhea syndrome by multiple infections. Although numerous pathogens have been implicated in the occurrence of neonatal calf diarrhea, only relatively limited viruses and bacteria are commonly involved: of these, bovine coronavirus and rotavirus that replicate within small intestinal epithelial cells are frequently associated with the neonatal calf diarrhea syndrome. Most of the diarrhea viral infections occur in calves less than 3 week of age [25]. Passive protection in calves against coronavirus, rotavirus, enterotoxigenic *Escherichia coli*, or *Salmonella* species was possibly obtained by use of specific immunoglobulins obtained from chicken egg yolk [14, 15, 19]. It was reported that the N-terminal part of the S-layer protein of *Lactobacillus brevis* ATCC 8287 played an important role in its adhesion to human intestinal cell lines and fibronectin [13]. On the basis of this knowledge, it is assumed that *Lactobacillus* S-layers might also enhance passive immunization in calves by mediating antibody adhesion to calf intestine. In this report, we describe the construction of S-layer fusion proteins consisting of enhanced green fluorescent protein),

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(EGFP) S-layer proteins of *Lactobacillus brevis*, and two copies of Z-domain, a synthetic analog of the immunoglobulin G (IgG)-binding B-domain of staphylococcal protein A, and a successful field application of the S-layer fusion protein and specific antibodies against coronavirus, rotavirus, *Escherichia coli*, and *Salmonella typhimurium* obtained from chicken egg yolks to treat neonatal calf diarrhea syndrome.

Materials and Methods

Bacterial Strains, Plasmids, DNA Manipulation, and Culture Conditions

For PCR amplification of the S-layer (SlpA) gene, *Lactobacillus brevis* KCTC3102 was cultured in MRS broth (Difco, U.S.A.) and its chromosomal DNA prepared by using the QIA DNA minikit (Qiagen, Germany). The plasmid pEGFP-N1 (Clontech, Palo Alto, CA, U.S.A.) was used for PCR amplification of the EGFP gene. For PCR amplification of the ZZ domain gene only, the plasmid pEZZ18 (GE Healthcare) was used. For cloning PCR fragments into a vector, pGEM-T easy (Promega, U.S.A.) was used. Recombinant plasmids of pGEM/EGFP, pGEM/SlpA, pGEM/ZZ, and pGEM/EGFP/SlpA were used to transform *E. coli* DH5α. For expression, *E. coli* BL21(DE3) was used as a host strain for the constructs pET30b/EGFP/SlpA and pET30b/EGFP/SlpA/ZZ. Recombinant *E. coli* was grown on Luria–Bertani medium (Invitrogen, Carlsbad, CA, U.S.A.) for cloning experiments at 37°C and for expression experiments at 28°C. For selection of transformants harboring the pET30b derivative, kanamycin was added to the medium to a final concentration of 30 μg/ml. DNA fragments were recovered from agarose gels by using the QIAEX II Gel Extraction Kit (Qiagen). Digestion of DNA with all restriction endonucleases (Takara, Japan), separation of DNA fragments, and transformation procedures were performed as described previously [26].

Construction of Chimeric Genes of EGFP/SlpA and EGFP/SlpA/ZZ

The oligonucleotide primers of GF1K and GF2B (Table 1), which introduced the restriction sites KpnI at the 5' end and BamHI at the 3' end of the coding sequence, respectively, were used for PCR amplification of the EGFP gene from plasmid pEGFP-N1. The gel-purified PCR fragment was inserted into the pGEM-T easy vector, and the derivative pGEM/EGFP was established in *E. coli* DH5α.

The oligonucleotide primers of S1B and S2E (Table 1), which introduced the restriction sites BamHI at the 5' end and EcoRI at the 3' end of the coding sequence, respectively, were used for PCR amplification of the SlpA structural gene from chromosomal DNA of *L. brevis* KCTC3102. The gel-purified PCR fragment was inserted into the pGEM-T easy vector, and the derivative pGEM/SlpA was established in *E. coli* DH5α.

The oligonucleotide primers of FZ1H and RZ2H (Table 1), which introduced the restriction site HindIII both at the 5' end and at the 3' end of the coding sequence, respectively, were used for PCR amplification of the ZZ domain gene from plasmid pEZZ18. The gel-purified PCR fragment was inserted into the pGEM-T easy vector, and the derivative pGEM/ZZ was established in *E. coli* DH5α.

The S-layer gene was isolated by the digestion of plasmid pGEM/SlpA with BamHI and EcoRI, whereas the EGFP gene was isolated by the digestion of plasmid pGEM/EGFP with KpnI and BamHI as shown in Fig. 1.

To make a fusion gene of EGFP::S-layer, DNA fragments of the entire EGFP and S-layer genes were ligated at the BamHI site and then the ligated DNA fragment was reamplified with primers of GF1K and S2E. The gel-purified PCR fragment was inserted again into the pGEM-T easy vector, and the derivative pGEM/EGFP/SlpA was established in *E. coli* DH5α.

Since the pGEM-T easy vector was designed to release a cloned gene by a single digestion of EcoRI, the digestion of plasmid pGEM/EGFP/SlpA with EcoRI resulted in a single DNA fragment containing a fusion gene of EGFP::SlpA. The fusion gene was inserted into the EcoRI site of an expression vector, pET30b (Novagen, Germany), and the derivative pET30b/EGFP/SlpA (pES) was established in *E. coli* BL21(DE3). To make certain of an open reading frame translated from the fused gene, the cloned gene in pET30b was verified by DNA sequencing (ABI 480 System; Applied Biosystems, U.S.A.).

The digestion of plasmid pGEM/ZZ with HindIII resulted in a single DNA fragment containing a ZZ gene. The ZZ gene was inserted into the HindIII site of the pET30b/EGFP/SlpA and the derivative pET30b/EGFP/SlpA/ZZ (pESZ) was established in *E. coli* BL21(DE3) (Fig. 1).

Expression of the Fusion Proteins EGFP/SlpA and EGFP/SlpA/ZZ

For expression, recombinant plasmids pET30b/EGFP/SlpA (pES) and pET30b/EGFP/SlpA/ZZ (pESZ) were transformed into *E. coli* BL21(DE3), which were cultured in Luria-Bertani medium at 37°C. Expression of each gene in a small scale (10-l fermentor) was induced by the addition of isopropyl β-D-thiogalactoside (IPTG; Sigma) to a final concentration of 1 mM at an attenuance ($A_{600\,nm}$) of 0.8. For large-scale expression in a pilot plant (500-l fermentor) of KRIBB (Korea Research Institute of Bioscience and Biotechnology), IPTG was replaced with lactose to a final concentration of 20 g/l at

Table 1. Oligonucleotides of the primers used in this experiment.

Primer name	Oligonucleotides	Introduced restriction site
GF1K	5'-GGGGTACCATGGTGAGCAA-3'	KpnI
GF2B	5'-CGGGATCCCTTGTACAGCT-3'	BamHI
S1B	5'-CGGGATCCAAGTCATACGCTA-3'	BamH1
S2E	5'-CCGGAATTCTTAGTTGAACCAAGT-3'	EcoRI
FZ1H	5'-CGCGCGAAGCTTGACAACAAATTCAACAAAGA-3'	HindIII
RZ2H	5'-ATATAAGCTTCCGCCAGCCATTGCAACGGAATCG-3'	HindIII

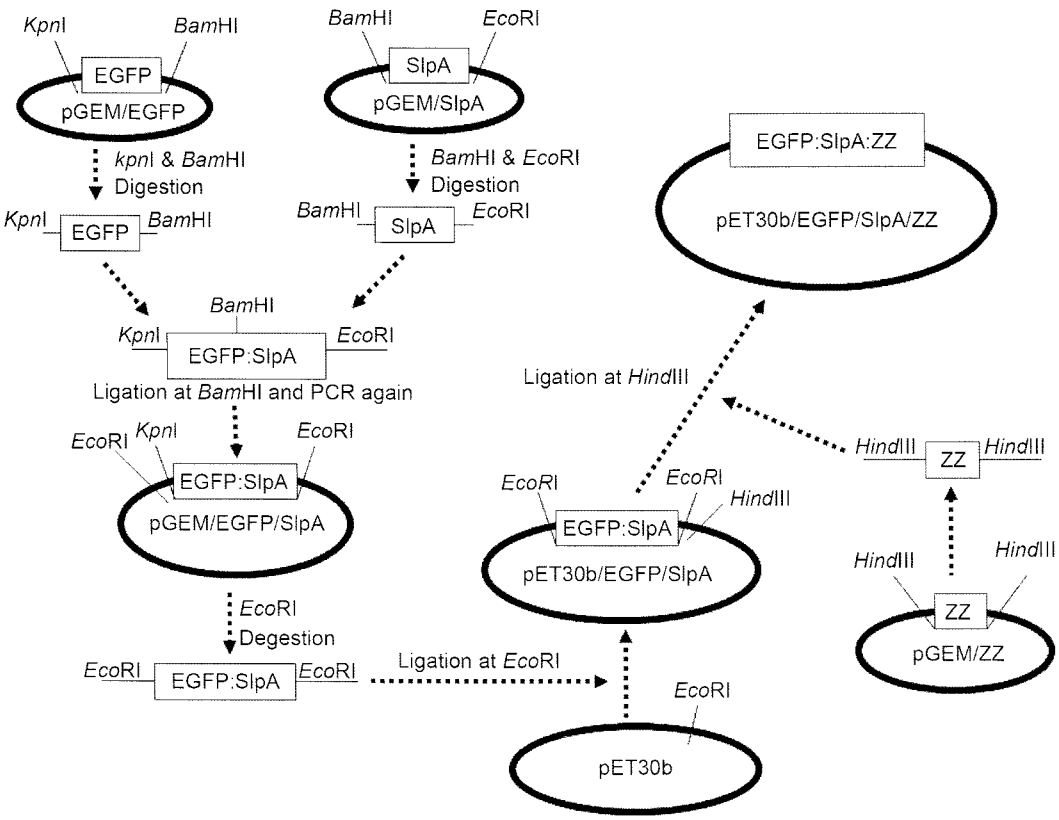

Fig. 1. Schematic diagram of the construction of pET30b/EGFP/SlpA/ZZ.

an attenuance ($A_{600\,nm}$) of 0.8. Protein expression was carried out at 28°C for another 18 h after induction.

Purification of Fusion Proteins EGFP/SlpA and EGFP/SlpA/ZZ

After 18 h of expression, the recombinant *E. coli* BL21(DE3) cells were harvested by centrifugation (10,000 ×*g*, 20 min, 4°C). Subsequently, the pellet was resuspended with a phosphate buffer (50 mM, pH 7.0). Cell disruption was performed by using a French press (100 psig). The cell extract was centrifuged (20,000 ×*g*, 20 min, 4°C) and the obtained pellet was resuspended in 5 M LiCl solution to extract the fusion proteins from the disrupted cytoplasmic membranes for 18 h at 4°C. After centrifugation (20,000 ×*g*, 20 min, 4°C), the supernatant containing LiCl was dialyzed against phosphate buffer (50 mM, pH 7.0) for another 18 h at 4°C. The dialyzed solution was concentrated by use of Amicon ultramembrane (YM-30) and the filtrate was subjected to a gel chromatography (Sephadex G-100). Fractions containing green color due to the expression of EGFP of the fusion protein were stored in ice for 18 h. The S-layer fusion proteins were slowly precipitated by aggregation from their self-assembly and then collected by centrifugation (20,000 ×*g*, 20 min, 4°C). For electron-microscopic investigation, self-assembled S-layer fusion proteins were dried, coated with argon gas, and viewed with a scanning electronic microscope (HITACHI: S-4100; Japan).

S-Layer-Mediated Enzyme Immobilization on Solid Materials

A portion of the fusion protein of EGFP/SlpA was mixed with cellulase (0.2 uint) – a model enzyme – in sodium acetic acid buffer (50 mM, pH 5.0), and then they were placed on slide glass plates at 0°C for 38 h. After the unbound proteins were removed from the slide glass by washing twice with sodium acetic acid buffer (50 mM, pH 5.0), the glass plates were dried at 20°C. For enzyme reaction, immobilized cellulases on the slide glass were reacted with 0.1% (w/v) carboxy methyl cellulose (CMC) at 37°C for 10 min. The activity of immobilized cellulases was measured by a DNS method [18]. Sol-gel technique [20] was also applied for the immobilization of cellulase.

***In Vitro* Assay of Antibody Adhered to Human Intestinal Epithelial Cells**

Human intestinal epithelial cells (HT29) were seeded to a microplate (1.5×10^5 cells/cm^2) and cultured with RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum at 37°C for 24 h. Then, the cells were washed twice with PBS (3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl, pH 7.4) and incubated with the fusion proteins of EGFP/SlpA/ZZ at 37°C for 2 h in serum-free media. Unbound proteins were removed by washing twice with PBS, and fluorescence of the fusion proteins bound to the cells was observed with an epifluorescent microscope (Olympus BX61; Japan). To measure the amount of the immunoglobulin G (IgG) adhered to the epithelial cells, IgGs conjugated with a peroxidase (Sigma) were mixed with the proteins of EGFP/SlpA/ZZ and then exposed to the human epithelial HT29 cells. After 2 h incubation in serum-free medium, the HT29 cells were washed twice with PBS and reacted with substrate H_2O_2 (8 mM) in the presence of 1 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid,

(ABTS Sigma) for 30 min at 25°C. The activity of peroxidase-IgG adhered to the cells was measured at 415 nm (Molecular Devices SpectraMax Plus384; U.S.A.).

***In Vivo* Assay of Antibodies Adhered to Mouse Intestine**

The specific polyclonal antibodies against neonatal calf diarrhea pathogens (coronavirus, rotavirus, *Escherichia coli* GB96000170, and *Salmonella typhimurium* GB96000180) from chicken egg yolks were kindly provided by Gyeongsangbuk-do Veterinary Service Laboratory in Korea.

About 30 mg of proteins of EGFP/SlpA/ZZ were mixed together with 1 mg protein of the antibodies. A portion of the antibody solution (1 ml) only was fed to 4 mice as a control, and the same portion of the mixture (1 ml) was fed to 4 mice, respectively. The antibodies adhered to the intestine were titered by an ELISA method at 492 nm [36]. To obtain relative retention rates of the antibodies in the intestine, each mouse was sacrificed in an hour and its small intestine (20 cm) was cut out and washed twice with distilled water. The initial amount of antibodies fed to a mouse as a control yielded an average titer value of 1.05 from four samples obtained in the mouse intestine. The mean relative retention rate was calculated by the residual amount of antibodies in an hour divided by the initial amount of antibodies in the mouse intestine. The attached S-layer fusion proteins in the intestine were observed with an epifluorescent microscope.

Field Test of the S-Layer Fusion Proteins to Hanwoo Calves

Passive immunization was performed by feeding sample solutions to 29 Hanwoo calves having neonatal diarrhea syndrome in farm-houses located at 7 different cities in Gyeongsangbuk-do, Korea. Sample A solution contained only specific antibodies against neonatal calf diarrhea pathogens (coronavirus, rotavirus, *E. coli* GB96000170, and *S. typhimurium* GB96000180) and was used as a control. Sample B solution was prepared by mixing the antibody solution (density 1.00 g/l) with the solution of S-layer proteins (density 1.05 g/l). The ratio of the S-layer solution to the antibody solution was 0.6 (v/v). A portion of each solution (35 ml) was fed to a newborn Hanwoo calf twice within 12 hours.

RESULTS

Construction of Expression Vectors for S-Layer Fusion Proteins

To establish a reporter system with which to study S-layer protein, EGFP was fused to the N-terminal and C-terminal parts of the S-layer of *Lactobacillus brevis*. The expression of the S-layer fusion proteins under T7 promoter was assessed by the fluorescence of EGFP under UV light. The fusion protein of EGFP connected to the N-terminal part of the S-layer (EGFP/SlpA) yielded a strong green fluorescence, but not the fusion protein of EGFP connected to the C-terminal part of the S-layer (SlpA/EGFP), indicating inappropriate folding of the EGFP structure when fused with the C-terminal part of the S-layer.

To give the EGFP/SlpA fusion protein an affinity toward the Fc domain of immunoglobulins, two copies of Z-domain, a synthetic analog of the IgG-binding B-domain of protein A of *Staphylococcus aureus*, were connected to the EGFP/SlpA. Both the chimeric proteins of ZZ/EGFP/SlpA and EGFP/SlpA/ZZ managed to produce a green fluorescence. For the sake of EGFP stability, a fusion protein of EGFP/SlpA/ZZ, in which ZZ peptides were linked to the C-terminal part of the SlpA protein rather than to the N-terminal part of EGFP, was chosen for further experiment.

Characterization of the EGFP/SlpA Fusion Proteins

Now that EGFP was used as a bioreporter, it was easy to study the physical properties of *L. brevis* S-layer proteins. The recombinant S-layer proteins were mostly adhered to *E. coli* cell cytoplasmic membranes even after cell disruption by a French press. Accordingly, they were extracted using a high concentration of LiCl (5 M). Upon removal of LiCl molecules by dialysis, the S-layer fusion proteins were allowed to be precipitated by self-assembling. The self-assembled S-layer products were aggregated in solution consisting of the net-like structures in nanometer scale (Fig. 2).

The S-layer proteins aggregated in microtubes disappeared in either pH 4 or pH 9 solutions (Fig. 3A), indicating that the self-assembled S-layer proteins seemed to be stable in the range of pH 5 to pH 8. The lost self-assembling ability of S-layer proteins in either pH 4 or pH 9 solutions could be recovered when its buffer solution was adjusted to pH 7.0 (data not shown). The aggregated S-layer proteins in microtubes began to be swollen at temperatures of higher than 50°C, indicating that the self-assembled S-layer proteins seemed to be stable at lower than 50°C (Fig. 3B).

S-layers of *L. brevis* were found to be well adhered on solid surfaces in this study (Fig. 4). Thus, it is suggested that this adhesive feature of *L. brevis* S-layers may mediate an easy and rapid immobilization of enzymes or antibodies to chief materials such as glass, plastics, sol-gels, *etc.* As a model case of enzyme immobilization mediated by S-

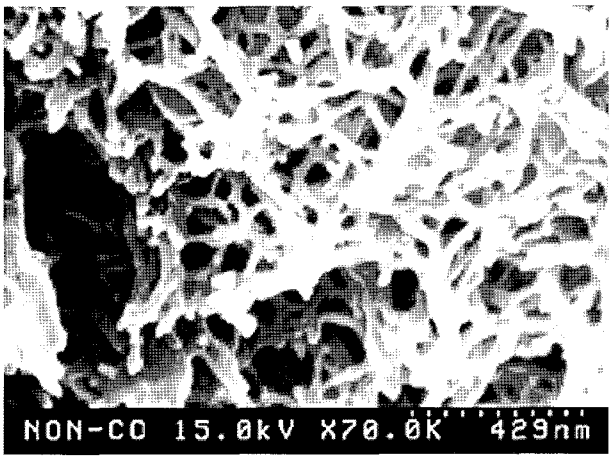

Fig. 2. Electron micrograph of the net-like structures of EGFP/SlpA fusion protein.

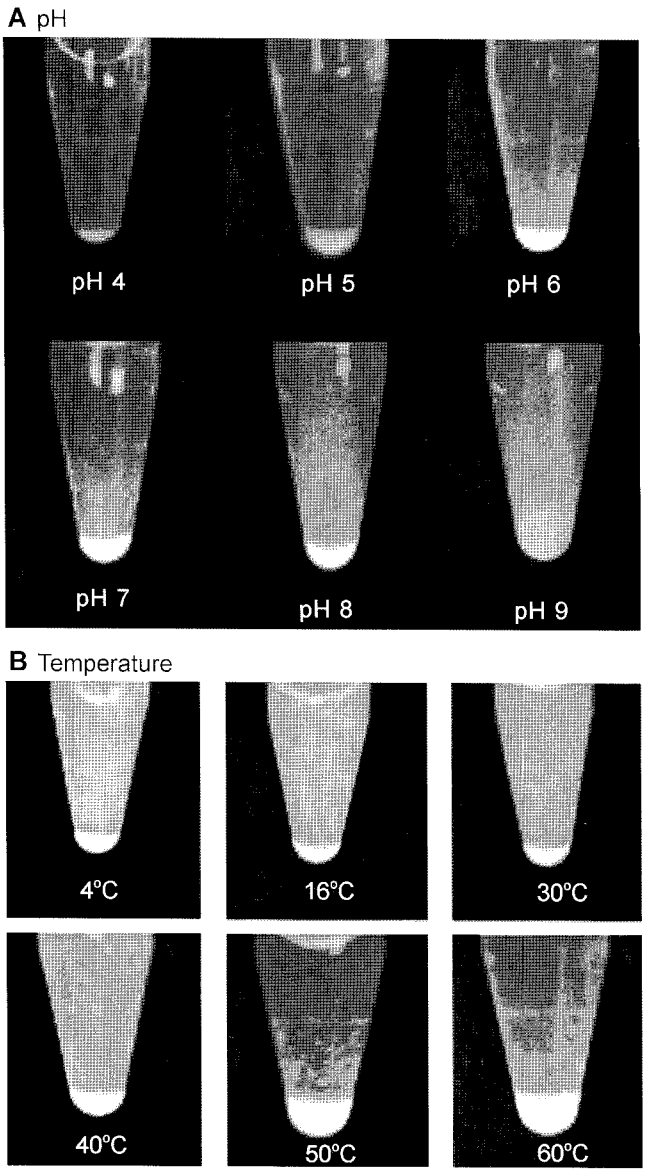

Fig. 3. Effect of pH and temperature on the stability of EGFP/SlpA fusion protein.
The aggregated EGFP/SlpA proteins in microtubes disappeared at either pH 4 or pH 9 solutions and were swollen at higher than 50°C, indicating that they were unstable in these physical conditions.

layers, cellulase was chosen to be immobilized onto slide glass. Cellulase alone was hardly attached onto the surface of glass plates, but the addition of S-layers enabled the attachment of cellulase to the glass plates. Sol-gel techniques have been developed to give a good mechanical, thermal, and photochemical stability to immobilized bioactive compounds by embedding them in the form of thin layers on inorganic matrices [20]. S-layer mediating cellulase also adhered well to the surface of sol-gel films, but under the sol-gel films, the activity of cellulase significantly decreased because of the mass transfer resistance of its substrate, carboxymethyl cellulose (Fig. 5).

Fig. 4. Adhesive property of EGFP/SlpA fusion protein on non-living materials.
The EGFP/SlpA fusion proteins adhered to (**A**) pipet tips, (**B**) dialysis membrane, and (**C**) vinyl wrap.

Adhesion of EGFP/SlpA Fusion Proteins to Intestinal Cells Both *In Vitro* and *In Vivo*

As the adherence of S-layers of *L. brevis* ATCC 8287 to human intestinal epithelial cells was reported [1], we also found that the EGFP/S-layer fusion proteins adhered well to human epithelial cells (Fig. 6).

In the condition where they are directly fed to mice, however, the adhesion of S-layer fusion proteins to the intestine may not be expected, possibly because the S-layer proteins passing through the stomach must meet extreme environmental conditions of low pH of the gastric acid and various proteases, under which the S-layer proteins may be completely disintegrated before getting to the intestine. In the present study, as the EGFP/SlpA fusion proteins were directly fed to mice, strong fluorescence emitted by EGFP was observed in the intestinal cells, as shown in Fig.7, indicating that they could reach successfully to the intestine despite there were extremely unfavorable conditions *in vivo*.

Adhesion of EGFP/SlpA/ZZ Mediating Antibodies to Intestinal Cells Both *In Vitro* and *In Vivo*

A recent study describes an S-layer fusion protein with two Z-binding domains to remove IgG from human plasma from patients suffering from an autoimmune disease [34]. Based on this knowledge, the S-layer fusion protein containing the two Z-binding domains, EGFP/SlpA/ZZ, was constructed to make antibodies bind to the S-layer fusion proteins. To prove that the EGFP/SlpA/ZZ fusion protein has a specific affinity to antibody, EGFP/SlpA/ZZ fusion proteins were mixed with rabbit anti-mouse immunoglobulin G conjugated with peroxidase. Then, the mixture was added to the human epithelial cells (HT29) cultured in a microplate. After washing out unbound S-layer fusion proteins, the activity of peroxidase-IgG bound to the HT29 cells was measured. IgGs themselves hardly adhered to the HT29 cells, but they adhered well to the HT29 cells along with EGFP/SlpA/ZZ proteins *in vitro* (Fig. 8), indicating that the IgGs were well bound to EGFP/SlpA/ZZ proteins.

To obtain more definitive evidence *in vivo* that the chimeric S-layer protein mediates the binding of antibodies

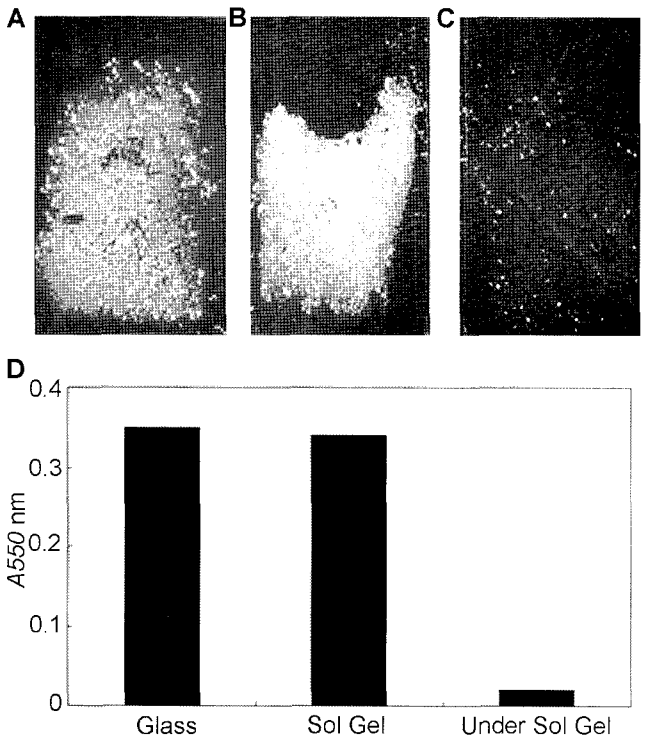

Fig. 5. Immobilization of cellulase with the S-layer fusion proteins on glass plates.
Cellulase (0.2 units) was immobilized (**A**) on the surface of glass (Glass), (**B**) on the surface of sol-gel film (Sol Gel), and (**C**) on the surface of glass and then coated with sol-gel film (Under Sol Gel). **D.** The activity of the immobilized cellulase was measured by use of 0.1% (w/v) CMC for 10 min at 550 nm, as described in Materials and Methods.

to mouse intestinal guts, the EGFP/SlpA/ZZ proteins were mixed with polyclonal antibodies raised against pathogens that caused neonatal calf diarrhea, such as coronavirus, rotavirus, *E. coli*, and *S. typhimurium*, and then they were

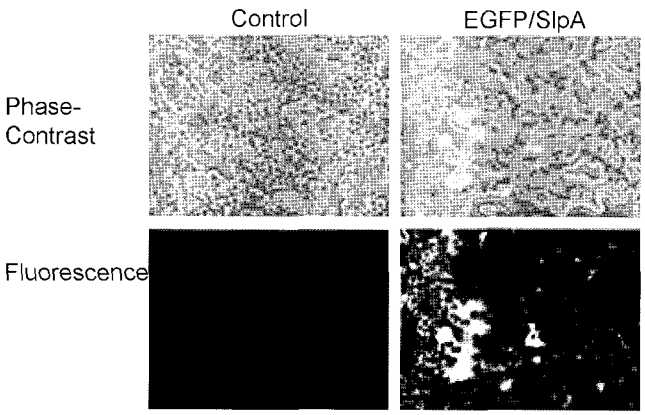

Fig. 6. Micrograph of the S-layer fusion proteins adhered to human epithelial cells.
Human intestinal epithelial cells (HT29) (1.5×10^5 cells/cm^2) were incubated without (a control) and with the EGFP/SlpA/ZZ fusion proteins at 37°C for 2 h in serum-free media before taking a fluorescent micrograph.

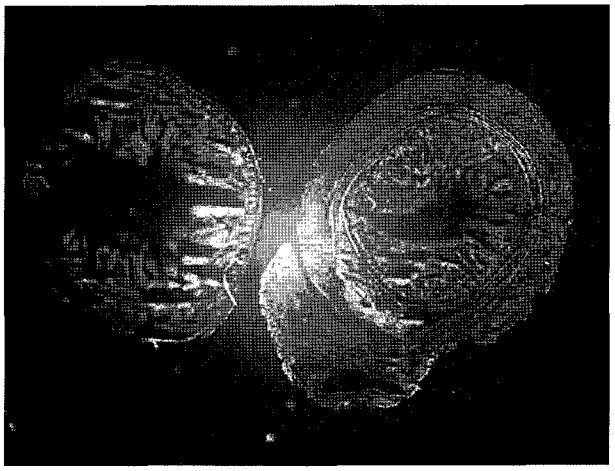

Fig. 7. Micrograph of the S-layer fusion protein adhered to mouse intestine.
Dissected mouse intestines were observed with a fluorescent micrograph at 2 h after feeding the EGFP/SlpA fusion proteins directly to a mouse.

directly fed to mice. After washing out the small intestines obtained from mice, antibodies adhered to the mouse intestines were titered with each pathogen by an ELISA method. Feeding antibodies alone to mice resulted in about 50% loss of antibodies in an hour, except for the coronavirus antibody, but antibodies mixed with the S-layer chimeric proteins resulted in only 10–20% loss of antibodies in an hour, which was statistically significant ($p<0.01$), suggesting that the S-layer proteins mediated adhesion of the specific antibodies to the mouse intestine (Fig. 9).

Feeding the EGFP/SlpA/ZZ Fusion Proteins to Hanwoo Calves

To obtain evidence that the chimeric EGFP/SlpA/ZZ protein plays an important role in reducing neonatal calf

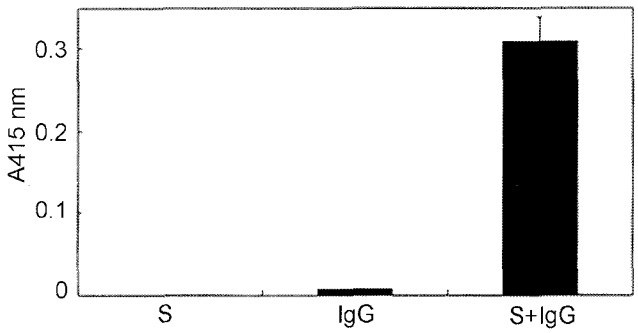

Fig. 8. S-Layer-mediated adhesion of IgG to human epithelial cells.
The EGFP/SlpA/ZZ fusion proteins (S), IgG conjugated with a peroxidase (IgG), and the mixture of the EGFP/SlpA/ZZ fusion proteins and peroxidase-IgGs (S+IgG) were loaded to the human epithelial HT29 cells as described in Materials and Methods. The activity of peroxidase-IgG adhered to the HT29 cells was measured at 415 nm after incubating with substrate H_2O_2 (8 mM) for 30 min.

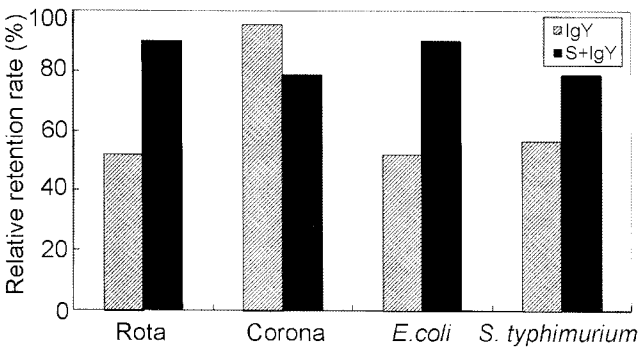

Fig. 9. S-Layer-mediated adhesion of specific antibodies to mouse intestine.

The specific polyclonal antibodies against neonatal calf diarrhea pathogens (coronavirus, rotavirus, *Escherichia coli*, and *Salmonella typhimurium*) from chicken egg yolks (IgY) and the mixture of the EGFP/SlpA/ZZ fusion proteins and the antibodies (S+IgY) were directly fed to mice. Antibodies adhered to the mouse small intestine (20 cm) were titered by use of an ELISA method. Relative retention rate was calculated by the residual amount of antibodies per hour divided by the initial amount of antibodies in the mouse intestine.

diarrhea, the mixtures of the polyclonal antibodies and the chimeric S-layer proteins were applied to diarrheic Korean calves (Hanwoo) in farm-houses before ten days of age. Feeding sample A solution containing antibodies only as a control resulted in stopping the neonatal diarrhea syndrome in 9 calves out of 16 calf herds (56%). In contrast, feeding sample B solution containing the mixture of antibodies and the chimeric S-layer proteins caused all of 13 calves (100%) to recover from the neonatal diarrhea syndrome. This difference was statistically significant ($p<0.01$), strongly indicating that the chimeric S-layer proteins played a significant role in preventing neonatal calf diarrhea syndrome possibly because of mediating the antibody adhesion to calf intestine.

DISCUSSION

Lactobacillus species have been known to prevent gastrointestinal infections not only by lowering the pH but also by adhering to the gastrointestinal tract, hence creating fewer opportunities for spoilage microorganisms to grow [10]. Their adhesion to the intestine was related with S-layer proteins [1, 4, 7, 33]. In this study, the recombinant S-layer proteins of *L. brevis* were used to make antibodies adhere to calf intestines for preventing neonatal calf diarrhea, due to the reported adhesive property of the S-layer protein of *Lactobacillus brevis* to human intestinal cell lines [13].

A number of recombinant S-layer proteins of *L. brevis* were produced in a pilot plant, and then the S-layer physical properties, such as protein stabilities at different pHs and temperatures, the abilities of self-assembly and adhesion to solid materials, were investigated with EGFP as a bioreporter. The self-assembly properties of the *L. brevis* S-layer fusion proteins allowed their simple purification by precipitation. Enzyme immobilization in the nanostructured sol-gel matrix has been used for the stabilization of a number of proteins against aggressive chemical and thermal environments [20]. The adhesion properties of the S-layer part enabled a simple and rapid immobilization of an industrial enzyme to various inorganic surfaces such as glass or sol-gel films.

A C-terminally truncated form of the S-layer protein of *Bacillus sphaericus* CCM 2177 was fused with the EGFP and expressed in *Escherichia coli* to study the uptake of liposomes coated with the S-layer proteins into HeLa cells by endocytosis [17]. The mature S-layer protein (aa 31–1,099) of *Geobacillus stearothermophilus* ATCC 12980 was also fused with the EGFP and expressed in the yeast *Saccharomyces cerevisiae* and human HeLa cells [3].

Instead of EGFP, other functional peptide sequences were used to make a fusion protein with S-layer protein for introducing them on regularly structured lattices in a nanometer range. For example, the major birch pollen allergen was fused to the truncated S-layer form (aa 31–920) of *B. stearothermophilus* ATCC 12980 comprising amino acids 31–1,099 [5, 16]. Streptavidin was fused with the S-layer protein of *G. stearothermophilus* PV72/p2 for building blocks and patterning elements for nanobiotechnology [22].

It was reported that the B-domain of staphylococcal protein A had two contact sites that interact with the Fc part of IgG [11], which led to constructing the 58 amino acids of the synthetic Z-domain [6, 23]. To obtain an S-layer fusion protein capable of binding IgG, the two Z-binding domains were fused with the C-terminally truncated form of the S-layer protein of *Bacillus sphaericus* CCM [34]. Another IgG-binding domain of streptococcal protein G was connected with the S-layer protein of *Caulobacter crescentus* [24]. We also connected the two Z-binding domains to the S-layer fusion protein of *L. brevis*, hence creating an affinity toward the antibodies that were raised from hens against neonatal calf diarrhea pathogens.

Neonatal diarrhea is a major cause of morbidity and mortality in calves during the first three weeks of life, resulting in considerable economic loss. Etiological agents include coronavirus, rotavirus, enterotoxigenic *Escherichia coli*, *Salmonella* spp., and *Cryptosporidium parvum* [9, 12]. Feeding newborn calves with colostrum of their dams, previously vaccinated with an inactivated adjuvant rotavirus vaccine, could prevent the neonatal diarrhea possibly by the activities of their maternal antibodies [8].

Most of these viral infections occur in calves less than 3 week of age. All diarrheal viruses replicate within small intestinal epithelial cells [25]. Immunity against these viral infections, therefore, must be directed toward protection of the susceptible intestinal epithelial cells. We found in the

field test that feeding the mixture of the *Lactobacillus* S-layer fusion proteins and the specific antibodies against coronavirus, rotavirus, *E. coli*, and *S. typhimurium* to newborn Hanwoo calves twice a day resulted in successful protection from neonatal calf diarrhea syndrome for about three weeks. To our knowledge, this is the first report to treat neonatal calf diarrhea syndrome effectively using the recombinant *Lactobacillus* S-layer proteins. Thus, the results open promising applications of the bacterial S-layer proteins in the animal industry as well as in the bioindustry for protein immobilization.

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