Immunogenicity of Recombinant Classic Swine Fever Virus CD8+ T Lymphocyte Epitope and Porcine Parvovirus VP2 Antigen Coexpressed by Lactobacillus casei in Swine via Oral Vaccination

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Classical swine fever virus (CSFV) and porcine parvovirus (PPV) are highly contagious pathogens, resulting in enormous economic losses in pig industries worldwide. Because vaccines play an important role in disease control, researchers are seeking improved vaccines that could induce antiviral immune responses against CSFV and PPV at the mucosal and systemic levels simultaneously. In this study, a genetically engineered Lactobacillus strain coexpressing the CSFV-specific cytotoxic T lymphocyte (CTL) epitope 290 and the VP2 antigen of PPV was developed, and its immunopotentiating capacity as an oral vaccine in pigs was analyzed. The data demonstrated that in the absence of any adjuvant, the recombinant Lactobacillus strain can efficiently stimulate mucosal and systemic CSFV-specific CD8+ CTL responses to protect pigs against CSFV challenge. Moreover, anti-PPV-VP2 serum IgG and mucosal IgA were induced in pigs immunized orally with the recombinant Lactobacillus strain, showing a neutralizing effect on PPV infection. The results suggest that the recombinant Lactobacillus microecological agent may be a valuable component of a strategy for development of a vaccine against CSFV and PPV.

Classical swine fever virus (CSFV), a member of the genus Pestivirus of the family Flaviviridae, is a small, enveloped, single-stranded RNA virus. Under natural conditions, the pig is the only susceptible species, and the virus can cause acute, subacute, or chronic disease (10, 26, 29). Porcine parvovirus (PPV), characterized as a member of the autonomous parvoviruses, is a major cause of reproductive failure in swine, resulting in early embryonic death, fetal death, stillbirths, and delayed return to estrus (5, 24, 28). Enormous economic losses to pig industries have been caused by these two pathogens. Therefore, the development of an efficient vaccine against CSFV and PPV simultaneously is of practical significance.

For vaccines against CSFV, the important role of humoral immune responses has been investigated, particularly in terms of neutralizing antibodies. For instance, pigs that were immunized with recombinant virus expressing CSFV E0 or E2 protein were protected against CSFV challenge (47). In some cases, immunization of animals with recombinant virus expressing other CSFV proteins failed to induce detectable neutralizing antibodies, whereas the animals were protected against lethal CSFV infection, which indicated that virus-specific T lymphocytes participated in a protective immune response against CSFV (38, 44, 48). Currently, cellular immune responses, especially production of virus-specific cytotoxic T lymphocytes (CTL), are receiving more attention for their potential roles in developing efficient epitope vaccines against CSFV (18, 35). Several CSFV-specific T-cell epitopes have been identified (3, 8, 32). Of them, the epitope peptide 290 (KHKVRNEVMVHWFDD), located at amino acid residues 1446 to 1460 of the CSFV nonstructural protein NS2-3, could be advantageous, as it harbors a CSFV-specific helper T-cell epitope and a CTL epitope, which could elicit both CD4+ and CD8+ T-cell responses (3). Therefore, peptide 290 is a promising candidate for an epitope vaccine for the control of CSF. Among vaccines against PPV, the inactivated vaccine is the one most often used to prevent and control infection, and humoral immune responses, in particular, neutralizing antibodies, play an important role. Therefore, the development of efficient vaccines that induce antibodies which neutralize PPV infection is desirable. The VP2 protein of PPV encompasses major antigenic domains and is therefore regarded as a promising candidate immunogen with the capacity to induce neutralizing antibodies (27, 41).

Moreover, CSFV and PPV initiate their infectious cycle at the mucosal surfaces. Although parenteral vaccination is usually efficient in eliciting a protective immune response, the parenteral routes generally fail to stimulate mucosal immune responses and cannot efficiently prevent the pathogens from entering the body via the mucosae. Therefore, efficient protection against mucosal invasion requires the development of new vaccines to induce protective mucosal immune responses at the infection point (17, 21). In this respect, mucosal immunization has been proven to be an effective approach (9, 22). Thus, it is necessary to develop efficient and safe antigen vectors that could trigger mucosal and systemic immune responses. One
promising approach relies on the use of live vehicles (2). Lactobacillus strains possess many properties that make them attractive candidates as antigens carriers for the presentation to the mucosae of compounds with pharmaceutical interest, in particular, immunomodulators and vaccines. Lactobacilli are well known for having beneficial effects on the health of humans and animals. In addition, lactobacilli can survive in and colonize the intestinal tract (1, 50) and, furthermore, induce a nonspecific immunoadjuvant effect (30). The potential of live recombinant Lactobacillus to deliver heterologous antigens to the immune system has been investigated (14, 31, 33, 34, 36, 40, 51), suggesting the feasibility of using lactobacilli as safe oral vaccines.

In the present study, a recombinant Lactobacillus strain co-producing a CSFV-specific CTL epitope and PPV VP2 protein was developed using the plasmid pPG612.1 as an expression vector, and its immunogenicity as an oral vaccine used to elicit antiviral mucosal and systemic immune responses in pigs was analyzed. Our data showed that oral immunization with the recombinant strain was able to induce CSFV-specific CTL responses against CSFV challenge and neutralizing antibodies against PPV infection in pigs, which indicate a new strategy for the development of CSFV and PPV vaccines.

MATERIALS AND METHODS

Bacteria, plasmas, and viruses. Lactobacillus casei ATCC 393 and plasmid pPG612.1 were kindly gifted by J. Seegers (NIZO, Netherlands). CSFV strain Shimen and PPV NADL-2 strain were kindly supplied by the China Institute of Veterinary Drug Control. PPV strain LJL12 was preserved in the Veterinary Department, Northeast Agricultural University, Harbin, People’s Republic of China.

Construction of the recombinant Lactobacillus strain. All DNA manipulations were performed according to standard procedures (39). The genomic DNA of PPV strain LJL12 propagated on swine testicular (ST) cells was extracted by the sodium dodecyl sulfate (SDS)-protease K (Sigma) method, and then the VP2 gene of PPV was obtained by PCR with the primers 5'-GCAGAGCAGATCTGAGTATGG TCTACTGTTGTCAGACGACGG-3'(upper) and 5'-GCTCTGGAGCATGCTGCTA CCTGATTTACCCAGA-3'(lower), containing a SalI site and an XhoI site (underlined), respectively. The VP2 gene was cloned as a SalI/XhoI fragment into the vector pET-20b(+) (Amersham), generating a plasmid pET-VP2. The gene encoding CSFV peptide 290 was synthesized by PCR assay with the primers 5'-CGACGATCTGATGAA ACACAAGTAGAAGATGAACTG-3'(upper) and 5'-TCTGGTCG ACGTTCTCAAAGCAGGACATCTACTGTCT-3'(lower), containing a BamHI site and a SalI site, respectively, which overlapped each other by 18 bases. The PCR product was cloned as a BamHI/SalI fragment into pET-VP2, giving rise to pET-E290-VP2. Then the BamHI/XhoI fragment of pET-E290- VP2 was subcloned into pPG612.1, generating pPG612.1-E290-VP2 (Fig. 1). Subsequently, the recombinant Lactobacillus was constructed by electroporation according to the method described previously (50). The resulting transformant, Lc393-E290-VP2, was identified by enzyme digestion and sequencing.

Colonization capacity of the recombinant Lactobacillus in intestinal tracts. The recombinant strain Lc393-E290-VP2 was labeled with the fluorescent dye 5'-6-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) (Sigma). In brief, a group of three pigs (2 weeks old) were fed approximately 1010 CFU/ml of the CFDA-SE-labeled recombinant strain. As controls, two other groups of three pigs were fed either CFDA-SE-labeled L. casei ATCC 393 or sterile PBS. On the 1st, 3rd, 5th, and 7th days after oral administration, the duodenum, jejunum, ileum, and colon of the pigs were extracted in compliance with ethical guidelines. Individual sections were cut longitudinally, and any visible residual food particles or fecal materials were removed prior to centrifugation. CFDA-SE-labeled lactobacilli in different intestine sections were enumerated on an Epics Elite flow cytometer (Beckman Coulter). The excitation wavelength was 488 nm, and the emission was measured through a 75-nm sort sense flow cell at 827 kPa of pressure. Upon excitation at 488 nm in the flow cytometer (BD), CFDA-SE gives a maximal emission signal in the green fluorescence at 518 nm. Data were recorded in the FCSExpress 4.0 file format by using Coulter Epics Elite software and then were analyzed using WinMDI 2.9. The fully cFDA-SE-labeled lactobacilli were used as positive controls, and the unlabeled lactobacilli were used as negative controls.

Protein expression and Western blotting. Lc393-E290-VP2 and L. casei ATCC 393 harboring pPG612.1 were cultivated overnight in MRS medium supplemented with 2% xylose. The overnight cultures were centrifuged at 5,000 × g for 10 min, and the supernatant was collected, concentrated 10 times using a Ultrafree-CL PBCC centrifugal filter unit (Millipore), and analyzed by SDS-10% polyacrylamide gel electrophoresis (PAGE). Then, proteins were transferred onto a nitrocellulose membrane (Invitrogen). After blotting, nonspecific protein-binding sites were blocked overnight with blocking solution (Tiangen, Beijing, China) at 4°C, and the immunoblots were developed using mouse anti-PPV-VP2 serum and anti-CSFV peptide 290 mouse serum. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma) was used, and immunolabeled bands were then visualized with a chemiluminescent substrate reagent (Pierce) according to the manufacturer’s instructions.

Immunization. Lc393-E290-VP2 was cultured and centrifuged. Cell pellets were resuspended in sterile suspension buffer to a concentration of ~1 × 1010 CFU/ml. A vaccination group of 15 pigs (2 to 3 months old; no maternal antibodies to CSFV or PPV) obtained from the Harbin TianXiang pig farm, Harbin, People’s Republic of China, and five miniature pigs of the dd/haplo type obtained from HuaiBei, Beijing, People’s Republic of China, were immunized on an oral immunization strain per kg and control groups were immunized with equivalent doses of Lc393-pPG612.1 and PBS (15 pigs per group). The immunization protocol was administered on two consecutive days (days 0 and 1). A booster immunization was given at days 14 and 15, and a second booster was given at days 28 and 29.

Cytotoxic-T-cell assays. Five vaccinated miniature pigs of the dd/haplo type kept under specific-pathogen-free (SPF) conditions were bled on the 35th day after the first immunization, and then peripheral blood mononuclear cells (PBMC) were separated using density gradient centrifugation as described previously (42). At the same time, intestinal tissue was obtained by laparotomy, and lamina propria lymphocytes were prepared as previously described (4). The immune cells were seeded in 96-well round-bottom microplates to a concentration of 1 × 104/ml RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Sigma) and restimulated with 1 × 106 50% tissue culture infective doses (TCID50) of CSFV Shimen per well for 5 days and were used as effector cells. Max cells, a kind gift from G. H. Zhang (Northeast Agricultural University, Harbin, China) were infected with CSFV Shimen for 48 h and were used as target cells in cytotoxicity assays (3). In brief, 1 × 104/ml target cells were labeled with 100 μCi of Na251CrO4 (Amersham) for 1 h at 37°C in 5% CO2 and then washed and resuspended in culture medium. For the CTL with peptides, 1 × 104/ml noninfected chromium-labeled MAX cells were incubated with 25 μg of CSFV peptide 290 at 37°C in a 5% CO2 incubator for 2 h. CSFV-infected or peptide-loaded target cells (1 × 105 cells per well) were added to various concentrations of effector cells (ranging from 1.25 × 104 to 5.0 × 104 cells per well). All experiments were performed in triplicate cultures. The cells were centrifuged at 100 × g for 5 min and incubated at 37°C in 5% CO2 for 4 h. The chromium levels in the supernatants were measured using a COBRA Auto-Counter model 9503 gamma counter (Packard). The percentage of specific cytolytic activity was calculated as follows: [[[cpm experimental release] – [cpm spontaneous release]]/[[cpm total release] – [cpm spontaneous release]]] × 100. Mask-infected or negative peptide-loaded target cells served as the controls. Furthermore, an enzyme-linked immunoassay (ELISPOT) assay was used to detect peptide-specific gamma interferon (IFN-γ)-secreting cells as described previously (3), with minor modifications. PBMCs incubated with an irrelevant peptide served as the negative control.

CSFV challenge experiment for pigs immunized with Lc393-E290-VP2. CSFV Shimen was propagated in porcine kidney 15 (PK-15) cells at 37°C in a 5% CO2 incubator for 3 to 5 days. Virus titers were determined by endpoint dilution titrations on PK-15 cells according to the method described previously (19, 35). All groups of pigs (those that were orally fed with recombinant Lc393-E290- VP2 and the Lc393-pPG612.1 and PBS controls) were challenged orally with a lethal dose of CSFV strain Shimen (100 TCID50/animal). The pigs were examined daily for disease symptoms and fever, defined by the rectal temperature of the pig (25). Survival rates were recorded during 15 days after challenge. Serum samples were collected at various time points before and after CSFV challenge, and virus titers of serum samples were determined by endpoint dilution titrations (18).

ELISA analysis for anti-PPV-VP2 antibody levels in pigs. Sera were prepared on days 0, 7, 14, 21, 28, 35, and 42 after the first immunization and stored at −70°C until required. At the same time, intestinal lavage fluids of pigs were collected and prepared for enzyme-linked immunosorbent assay (ELISA) as previously described (11, 37). Polystyrene microtiter plates were coated over-
night at 4°C with PPV strain LJL12 propagated on ST cells. The ELISA plates were washed three times with PBS containing 1% Tween 20 and then saturated with PBS–5% skim milk at 37°C for 2 h. Serum or intestinal lavage samples were diluted with PBS–1% bovine serum albumin (BSA) and used as primary antibodies. After incubation at 37°C for 1 h, the plates were washed another three times. Bound antibodies were detected using HRP-conjugated goat anti-pig IgA or IgG diluted 1:2,000 (Sigma), followed by color development using tetramethylbenzidine (TMB) as the substrate, and then absorbance was measured at 490 nm.

Neutralizing ability of antibodies to PPV obtained from immunized pigs. Serum IgG and mucosal IgA samples obtained from the pigs immunized orally with Lc393-rE290-VP2 on days 0, 7, 14, 21, 28, 35, and 42 after the first immunization were evaluated using a plaque reduction assay to determine their ability to neutralize PPV infection. The samples obtained from pigs fed Lc393, pPG612.1 or PBS were used as controls. In brief, nondiluted 50-μl samples were prepared in a 96-cell plate. PPV adjusted to 200 TCID50 in 50 μl of virus diluent (10% concentrated Hanks balanced salt solution, 0.1% bovine serum albumin [pH 7.4]) was added to the cell plate, mixed, and incubated at 37°C for 1 h. Then 100 μl of ST cells (used for virus infection) was added to the antibody-virus mixture and incubated in a 5% CO2 incubator at 37°C for 5 days. The overlay medium was then discarded, after which the wells were washed three times with sterile PBS, pH 7.4, and stained with 1% crystal violet solution. Differences in the numbers of plaques formed on the cells receiving the different treatments were examined for the level of significance by Student’s t test after analysis of variance.

RESULTS

Colonization by recombinant Lc393-rE290-VP2. Pigs were orally given cFDA-SE-labeled Lc393-rE290-VP2, and then the different intestine sections were isolated on the 1st, 3rd, 5th, and 7th days after oral immunization. Following the collection of cFDA-SE-labeled Lc393-rE290-VP2, the ability of recombinant Lc393-rE290-VP2 to colonize swine intestine was determined by flow-cytometric analysis. Data showed that the
responses against CSFV was analyzed rE290-VP2 to stimulate mucosal and systemic cytotoxic re-
cytolytic response against peptide 290-loaded MAX cells was negative—peptide-loaded target cells served as the controls. A target MAX cells labeled with chromium. Mock-infected or activity was tested by evaluating the lysis of peptide-loaded obtained on the 35th day after the first immunization and in vivo strain with the control strain Lc393:pPG612.1 (lanes 3 and 4). lane 1) and anti-CSFV peptide 290 mouse antiserum (lane 2) in a position similar to that observed with SDS-PAGE using Lc393-rE290-VP2 using mouse anti-PPV-VP2 serum (Fig. 2B, immunoreactive band was detected in the supernatants of Lc393-rE290-VP2 but not Lc393:pPG612.1, as shown in Fig. 2A. A 70-kDa expressed and secreted into the supernatants of Lc393-rE290-VP2 (lanes 2 and 3, arrow) but not in Lc393:pPG612.1 (lane 4). Results of SDS-PAGE showed that a 70-kDa fusion protein was then analyzed by SDS-PAGE and Western blotting. The re-
xpression of the fusion protein. The supernatants of the recombinant strain Lc393-rE290-VP2 cultured overnight in MRS containing 2% xylose were concentrated 10 times and then analyzed by SDS-PAGE and Western blotting. The results of SDS-PAGE showed that a 70-kDa fusion protein was expressed and secreted into the supernatants of Lc393-rE290-VP2 but not Lc393:pPG612.1, as shown in Fig. 2A. A 70-kDa immunoreactive band was detected in the supernatants of Lc393-rE290-VP2 using mouse anti-PPV-VP2 serum (Fig. 2B, lane 1) and anti-CSFV peptide 290 mouse antiserum (lane 2) as primary antibodies, whereas there was no band of interest with the control strain Lc393:pPG612.1 (lanes 3 and 4).

Induction of specific cytotoxic responses by the recombinant strain in vivo. The capacity of the recombinant strain Lc393-rE290-VP2 to stimulate mucosal and systemic cytotoxic responses against CSFV was analyzed in vivo. The PBMC and lamina propria lymphocytes of vaccinated miniature pigs were obtained on the 35th day after the first immunization and restimulated in vitro with CSFV Shimen, and then the cytotoxic activity was tested by evaluating the lysis of peptide-loaded target MAX cells labeled with chromium. Mock-infected or negative-peptide-loaded target cells served as the controls. A cytolytic response against peptide 290-loaded MAX cells was observed not only in mucosal tissue (Fig. 3d) but also at the systemic level (Fig. 3b), which indicated that Lc393-rE290-VP2 was able to stimulate a CSFV-specific CD8⁺ CTL response in vivo.

In order to confirm the reactivity of CSFV-specific T cells against CSFV antigens, PBMCs obtained after vaccination and challenge infection were tested for their ability to produce IFN-γ upon stimulation with virus peptide in ELISPOT assays. Data showed that high numbers of IFN-γ-secreting cells were observed when PBMCs from infected pigs were stimulated with peptide 290, while none of the unvaccinated animals had detectable circulating virus-specific IFN-γ-producing cells (Fig. 4). The release of IFN-γ by peptide 290 was sequence specific, because only a few spots were observed after incubation of PBMCs with an irrelevant peptide.

### TABLE 1. Colonization efficacy of recombinant Lc393-rE290-VP2 compared with L. casei ATCC 393 in intestinal tracts of pigs

<table>
<thead>
<tr>
<th>L. casei strain</th>
<th>Day</th>
<th>Duodenum</th>
<th>Jejunum</th>
<th>Ileum</th>
<th>Colon</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 393</td>
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<td>7.92E08</td>
<td>8.97E08</td>
<td>1.53E09</td>
<td>9.85E08</td>
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<td>5.99E08</td>
<td>7.85E08</td>
<td>9.76E08</td>
<td>8.79E08</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3.68E08</td>
<td>4.97E08</td>
<td>8.37E08</td>
<td>7.66E08</td>
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<td>3.22E08</td>
<td>6.96E08</td>
<td>6.43E08</td>
</tr>
<tr>
<td>Lc393-rE290-VP2</td>
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<td>7.86E08</td>
<td>8.36E08</td>
<td>1.47E09</td>
<td>9.72E08</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5.85E08</td>
<td>7.71E08</td>
<td>9.58E08</td>
<td>8.58E08</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3.49E08</td>
<td>4.55E08</td>
<td>7.95E08</td>
<td>7.53E08</td>
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<td></td>
<td>7</td>
<td>1.57E08</td>
<td>2.82E08</td>
<td>6.37E08</td>
<td>5.85E08</td>
</tr>
</tbody>
</table>

### FIG. 2. Secretion expression of fusion protein from Lc393-rE290-VP2. The supernatants were analyzed by SDS-PAGE and Western blotting. (A) SDS-PAGE analysis. Lane 1, molecular mass marker. A 70-kDa fusion protein was observed in the supernatants of Lc393-rE290-VP2 (lanes 2 and 3, arrow) but not in Lc393:pPG612.1 (lane 4). (B) Western blotting. An immunoreactive band was observed (lanes 1 and 2) in a position similar to that observed with SDS-PAGE using anti-PPV-VP2 monoclonal antibody 3C9 and anti-CSFV peptide 290 mouse antiserum as primary antibodies, respectively, but no immunoblots were observed with the supernatants of Lc393:pPG612.1 (lanes 3 and 4).

### FIG. 3. Specific cytotoxicity of CSFV CTL to target cells sensitized by CSFV Shimen (a and c) or peptide 290 (b and d). PBMCs (a and b) and lamina propria lymphocytes (c and d) of pigs vaccinated with Lc393-rE290-VP2 and restimulated with CSFV in vitro were used as effector cells. The cytotoxicity of the T cells was determined in a chromium release assay by lysis of virus-sensitized (a and c, ●) or peptide-sensitized (b and d, ○) MAX cells as targets. MAX cells incubated with supernatants of mock-infected cells (a and c, ○) or with irrelevant peptide (b and d, ○) were used as the controls. Data are means from triplicate samples ± standard errors of the means.
Recombinant Lc393-rE290-VP2 protects pigs against CSFV challenge. In order to analyze the capacity of the recombinant strain Lc393-rE290-VP2 to protect pigs against CSFV infection, the vaccinated pigs were challenged orally with a lethal dose (10⁵ TCID₅₀/animal) of CSFV strain Shimen on the 35th day after the first immunization, and the pigs immunized orally with Lc393:pPG612.1 or PBS were used as controls. Results demonstrated effective protection (86.7%) against CSFV challenge performed on day 35 in vaccinated pigs, while control groups of pigs developed severe clinical symptoms of CSF after viral infection (Fig. 5). Little virus was detected in vaccinated groups of surviving pigs on day 15 after challenge, indicating efficient viral clearance (Table 2).

Anti-PPV-VP2 immune responses induced in pigs. The local mucosal and systemic immune responses were investigated by measuring the anti-VP2 IgA level in intestinal lavage samples and the anti-VP2 IgG level in serum of pigs after oral immunization, respectively. The levels of IgA and IgG against VP2 were determined via ELISA using PPV strain LJL12 as a coated antigen. As shown in Fig. 6a, there was no substantial difference (P > 0.05) in mucosal IgA level between the vaccinated group and the control groups prior to immunization, while oral immunization of Lc393-rE290-VP2 elicited an antigen-specific mucosal IgA response. After the second booster, high levels of anti-VP2 IgA were obtained in intestinal lavage fluids of the vaccinated pigs, showing a substantial difference (P < 0.01) relative to the control groups. Moreover, after the first booster, the pigs orally immunized with Lc393-rE290-VP2 exhibited a prompter and stronger anti-VP2 serum IgG response. No significant levels of anti-VP2 antibodies were observed in the control groups of pigs (P > 0.05) (Fig. 6b).

PPV-neutralizing ability of antibodies. Plaque reduction assays were performed to test the PPV-neutralizing ability of antibodies collected from the pigs orally immunized with Lc393-rE290-VP2. The samples of serum antibody IgG and mucosal antibody IgA were obtained from the vaccinated pigs on days 0, 7, 14, 21, 28, 35, and 42 and after the first immunization. Neutralizing ability was expressed as the maximum
inhibition rate. The results demonstrated that the presence of anti-VP2 IgA or IgG in the culture medium conferred a statistically significant (P < 0.05) ability to neutralize PPV infection. Maximum inhibition rates were nearly 96.7 ± 1.29% for anti-VP2 IgG (Fig. 7a) and 70.6 ± 1.05% for anti-VP2 IgA (Fig. 7b), which indicated that Lc393-rE290-VP2 could induce neutralizing antibodies to PPV.

**DISCUSSION**

In the development of virus vaccines, antigen delivery vehicles play a critical role in the effectiveness of vaccination against pathogens. Several strategies have been developed to deliver exogenous antigens into the cytosol. Protein or peptide antigens delivered in association with appropriate adjuvants (e.g., incomplete Freund’s adjuvant, liposomes [52], ISCOMs [43], or complete Freund’s adjuvant [16]) or in particulate form linked to latex microspheres efficiently stimulate cellular or humoral immune responses (20). Recombinant live vectors, including attenuated viruses (such as vaccinia virus [15], Mongo virus [2], or pseudorabies virus [48]) or bacteria (such as bacillus Calmette-Guérin [49], Lactobacillus [14, 31, 36, 50], Salmonella [13], or Listeria [12]), have also been shown to induce a protective immune response in vivo. In contrast, among the available approaches to stimulate efficient mucosal responses, using lactobacilli as carriers to deliver vaccine antigens constitutes one of the most successful strategies (23).

At present, CSF is controlled by stamping out-methods and vaccination, or a combination of the two, but the eradication strategy has inevitably resulted in enormous economic losses. Though traditional CSFV avirulent vaccines, in particular the lapinized C strain of CSFV, have been successfully used in several countries to prevent and control CSF, the use of traditional CSFV avirulent vaccines has been banned since 1990 by the European Union, mainly because infections with vaccine and field viruses cannot be distinguished serologically (32). Therefore, the design of new efficacious vaccines to protect animals against CSFV is being pursued. Previous studies have shown that a cellular immune response, especially production of CSFV-specific CD8+ CTLs, represents an important defense mechanism in the elimination of cells infected by

**TABLE 2. Serum virus titers**

<table>
<thead>
<tr>
<th>Vaccination</th>
<th>Postchallenge day</th>
<th>Titer (TCID50/ml serum) in pig</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Lc393-rE290-VP2</td>
<td>3</td>
<td>10^3.3</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>10^1.2</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>10^1.0</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>ND</td>
</tr>
<tr>
<td>Lc393:pPG612.1</td>
<td>3</td>
<td>10^3.5</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>10^3.2</td>
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<td></td>
<td>18</td>
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*ND, not determined; —, animal was euthanized when moribund, in compliance with ethical guidelines.

**FIG. 6.** Anti-VP2 IgA levels in intestinal lavage fluid samples (a) and anti-VP2 IgG levels in serum (b). Pigs were immunized with Lc393-rE290-VP2 or with Lc393:pPG612.1. An additional control group of pigs received only PBS. Intestinal lavage fluids and sera were collected on days 7, 14, 21, 28, 35, and 42 after the first immunization. The VP2-specific IgA (a) and IgG (b) levels were tested by ELISA using PPV strain LJL12 as the coated antigen. ST cell culture was used as a negative control antigen. Values are means ± standard errors of the means.
to recognize naturally processed epitope from the infectious
nized animals, which indicated that the effector cells were able
miniature swine vaccinated orally with the recombinant strain,
was analyzed. In the absence of any adjuvants, a CSFV-specific
stimulate CSFV-specific CTL responses
This is the basis for the development of vaccines to control
between the pathogen and the swine immune system (3, 8, 32).
the utmost importance for understanding the interaction be-
investigations have concentrated on the role of the humoral im-
by antigenic subunits against infection in the host. Most inves-
cation of immune responses and immune defenses stimulated
E290-VP2 showed good segregational and structural stability
antiserum. Moreover, the recombinant plasmid pPG612.1-
were not compromised, as it can be recognized by a specific
SDS-PAGE and Western blotting, and its antigenic properties
successfully expressed by Lc393-rE290-VP2, as determined by
response against CSFV and PPV in pigs. The fusion protein was
PPV-VP2 protein to induce a protective antiviral immune re-
vaccine vehicle to deliver a CSFV-specific CTL epitope and
This goal can be achieved only when the vaccination is admin-
only at the systemic but also at the mucosal level after vacci-
infection mainly occurs at the mucosa of the intestines (6).
Therefore, the elicitation of an efficient immune response not
only at the systemic but also at the mucosal level after vacci-
nation is highly desirable (7, 46) and would represent a signif-
advantage in preventing PPV infection via the mucosa.
This goal can be achieved only when the vaccination is admin-
istered by the mucosal route.
In this study, Lactobacillus casei was selected as the live oral
vaccine vehicle to deliver a CSFV-specific CTL epitope and
PPV-VP2 protein to induce a protective antiviral immune re-
sponse against CSFV and PPV in pigs. The fusion protein was
successfully expressed by Lc393-rE290-VP2, as determined by
SDS-PAGE and Western blotting, and its antigenic properties
were not compromised, as it can be recognized by a specific
antiserum. Moreover, the recombinant plasmid pPG612.1-
E290-VP2 showed good segregational and structural stability
in L. casei ATCC 393 following serial subcultures.
For virus vaccine research, one critical step is the identifi-
cation of immune responses and immune defenses stimulated
by antigenic subunits against infection in the host. Most invest-
igations have concentrated on the role of the humoral im-
une response in CSFV-infected animals (45, 47), while the
 cellular immune response is often ignored. The study of
the cellular immune response to CSFV in the natural host is of
the utmost importance for understanding the interaction be-
tween the pathogen and the swine immune system (3, 8, 32).
This is the basis for the development of vaccines to control
CSFV infection. In this work, the capacity of Lc393-rE290-VP2
to stimulate CSFV-specific CTL responses in vitro and in vivo
was analyzed. In the absence of any adjuvants, a CSFV-specific
CTL response was detected in the intestinal mucosal tissues of
miniature swine vaccinated orally with the recombinant strain,
and no measurable CTL activity was detectable in nonimmu-
nized animals, which indicated that the effector cells were able
to recognize naturally processed epitope from the infectious
virus and kill peptide 290-coated target cells. This result sug-
gests that the local mucosal immunity induced by the recom-
binant strain, in particular the induction of a local CD8+ cy-
totoxic T lymphocyte response, may control CSFV replication
within local tissues prior to systemic dissemination. We also
analyzed whether mucosal immunization with the strain could
induce antigen-specific CD8+ CTLs systemically, and a signif-
ificant cytolytic response was observed. The result is consistent
with previously described CTL activity in other studies (3).
Due to these promising results in vitro, the protective capacity
of Lc393-rE290-VP2 was tested. Pigs immunized orally with
this recombinant strain were effectively protected against
CSFV challenge through induction of CSFV-specific major
histocompatibility complex (MHC) class I-restricted CD8+ CTLs,
while control groups of pigs developed severe clinical
symptoms of CSF after viral challenge and were euthanized
when moribund. In addition, Lc393-rE290-VP2 was able to
induce long-term protective immunity against CSFV.
IgA is the predominant antibody at the mucosal surface, as
it is produced locally at a level that exceeds that of all other
immunoglobulins, and it plays an important role in preventing
the invasion of pathogens (7). Thus, an efficient PPV oral
vaccine will have to induce specific immune responses not only
at the systemic level but also at the mucosal level, in particular
the mucosal IgA response against virus invasion via the mu-
cosa. We evaluated the immunogenicity of Lc393-rE290-VP2
in pigs and found that both mucosal and systemic immune
responses could be efficiently elicited after intragastric admin-
istration. As experiments to test for protection of gravid pigs
against PPV challenge are difficult, in order to confirm the
efficacy of the induced antibodies in inhibiting the virus, we
determined whether intestinal lavage fluids and sera could
inhibit the infection of ST cells in a plaque reduction neutral-
ization assay. Serum and intestinal samples collected from
vaccinated pigs demonstrated statistically significant inhibition,
which would be a first step to give the proof of principle for the
protection efficacy in the target species. Moreover, the avail-
bility of a live oral vaccine that can colonize the intestinal
tract is important and desirable. In this study, we investigated
the colonization potential of Lc393-rE290-VP2 in pig intesti-
tines and showed that the recombinant strain was able to
persistently colonize the intestinal tracts, similar to its native
counterpart, L. casei ATCC 393. As it is a probiotic bacterium,
a long residence time in the intestinal tracts would prolong its
potential beneficial health effects.
In conclusion, our results clearly demonstrate that the re-
combinant strain Lc393-rE290-VP2, constructed in this study,
provides a safe and efficient vaccine to induce CSFV-specific
CTLs against CSFV and PPV-neutralizing activity with the
probiotic effects of L. casei ATCC 393, which suggests that this
is a promising expression and delivery system for presenting
different CTL epitopes or viral antigens to the immune system.

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