ORIGINAL PAPER

Systemic and local mucosal immune responses induced by orally delivered *Bacillus subtilis* spore expressing leucine aminopeptidase 2 of *Clonorchis sinensis*

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Abstract Human clonorchiasis caused by *Clonorchis sinensis* (*C. sinensis*) has been increasingly prevalent in recent years so that an effective measure is essential and urgent to control the infectious disease. Oral delivery of antigens from *C. sinensis* may be an important approach to effectively induce both systemic and local immune responses to anti-infection of the parasite. In the current study, we used *Bacillus subtilis* (*B. subtilis*) spores as a delivery vehicle to introduce leucine aminopeptidase 2 of *C. sinensis* (*Cs*LAP2), an excretory/secretory antigen with high immunogenicity, expressing on their surface. SDS-PAGE, western blotting, and flow cytometry indicated that *Cs*LAP2 was successfully expressed on the surface of *B. subtilis* spores (CotC-*Cs*LAP2 spores). BALB/c mice were treated with spores intragastrically. On day 31 after the treatment, we found that mice intragastrically treated with CotC-*Cs*LAP2 spores exhibited higher IgG, IgG1, IgG2a, and IgA level in sera as well as higher sIgA level in bile and intestinal lavage fluid compared to mice orally administrated with spores not expressing *Cs*LAP2 (CotC spores) and naïve mice. The peak titer of IgG/IgA presented on day 31/49 after oral administration. IgG1 level was lower than IgG2a in group administrated with CotC-*Cs*LAP2 spores. sIgA-secreting cells were obviously observed in intestinal epithelium of mice orally treated with CotC-*Cs*LAP2 spores. After incubated with CotC-*Cs*LAP2, the levels of IFN-γ, IL-6, IL-10, IL-17A, and TNF significantly increased in the supernatant of splenocytes isolated from mice orally treated with CotC-*Cs*LAP2 spores, while there was no statistically significant difference of IL-4 level representing Th2 response among the groups. Our study demonstrated that oral administration of *Cs*LAP2 delivered by *B. subtilis* spore elicited obvious systemic and local mucosal immunity. Secretory IgA and Th1-Th17 cellular immunity might involved in mechanisms of the immune response.

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Introduction

Clonorchis sinensis (*C. sinensis*) is an important food-borne parasite. The adult worm inhabits in intrahepatic bile duct and results in clonorchiasis. Clonorchiasis is currently a major health problem in most endemic areas, such as China, Korea, East Russia, and Vietnam (Lv et al. 2012; Huang et al. 2012). The disease is included in the control programs of neglected tropical diseases by WHO (Hong and Fang 2012). Most clonorchiasis cases are due to consumption of raw freshwater fish containing infective metacercariae of *C. sinensis*, which excyst in duodenum and then migrate into

bile ducts of the host (Rim 1986). During *C. sinensis* infection, immune molecules were secreted by bile duct epithelium and intestinal mucosa of the host, which play roles of immunotoxicity and growth inhibition on *C. sinensis* (Zhang et al. 2008). Worms recovered from the re-infected rats were immature (Wang et al. 2009; Chung et al. 2004). It demonstrated that the defense against infection of *C. sinensis* mainly resulted from mucosal immune response. Oral delivery of antigens from *C. sinensis* may be an important approach to effectively induce both systemic and local immune response to anti-infection of the parasite.

Most protein antigens are poorly immunogenic when delivered by intra-gastrointestinal route because of degradation in gastric secretions. Owing to extremely environmental resistance, immunological characteristics, and flexibility for genetic manipulation, *Bacillus subtilis* (*B. subtilis*) spores have been extensively employed as vehicles to carry heterologous antigens to extreme environments such as gastrointestinal tract (Hinc et al. 2013; Kim and Schumann 2009; Knecht et al. 2011). The spore can also be recognized by the Gut-associated lymphoid tissue (GALT) and exert an adjuvant effect, which increases secretory IgA (sIgA) and enhances the ability of antigen recognition (Huang et al. 2008). In the present study, *B. subtilis* spore was employed to deliver *C. sinensis* antigen by oral treatment.

It has been documented that leucine aminopeptidase 2 (LAP2) from *Fasciola hepatica*, *Paragonimus westermani*, *Toxoplasma gondii* and so on can elicit relatively effective immune response resisting infection of the corresponding parasite (Acosta et al. 2008; Jia et al. 2010; Song et al. 2008). In our previous study (Deng et al. 2012), LAP2 of *C. sinensis* (*Cs*LAP2) was identified to be one component of excretory and secretory products of *C. sinensis* (*Cs*ESP) and expressed abundantly at the life stage of excysted metacercaria of *C. sinensis* (the invasive stage). Additionally, *Cs*LAP2 could induce high level of compounded humoral and cellular immune responses by subcutaneous immunization. In the current study, mice were orally administrated with *B. subtilis* spores displaying *Cs*LAP2 on their surface and the systemic and local mucosal immune responses sponsored by the spores were evaluated. The involved immunological mechanisms were explored too.

Materials and methods

Ethics statement

Female BALB/c mice (6–8 weeks) were purchased from Animal Center of Sun Yat-Sen University and raised carefully in accordance with National Institutes of Health on animal care and the ethical guidelines. The experimental procedures were approved by the animal care and use committee of Sun Yat-Sen University (Permit Numbers: SCXK 2009-0011, Guangdong).

Construction of PEB03-CotC-*Cs*LAP2

To display *Cs*LAP2 on the surface of *B. subtilis* spore, we connected complete coding sequence (CDS) of CotC, a major component of spore coat proteins, to the upstream of CDS of *Cs*LAP2 to construct fusion gene of CotC-*Cs*LAP2. PEB03, a *B. subtilis* expression plasmid and shuttle vector, was stored in our laboratory. Owning to its limited sites of restrict endonucleases, we firstly employed pBluescript II SK (-) vector (Qiagen, USA) to construct the fusion gene. Briefly, CDS of CotC was amplified from genomic DNA of *B. subtilis* vegetative cells by polymerase chain reaction (PCR). The forward (5′-CAT<u>GTCGAC</u>TGTAGGATAAATCGTT-3′) and reverse (5′-CGG<u>AAGCTT</u>GTAGTGTTTTTTATGC-3′) primers containing restriction sites of *Sal* I and *Hind* III (underlined) were used. The specific PCR products were recovered, digested with *Sal* I and *Hind* III, and inserted into pBluescript II SK (-) to construct pBluescript II SK (-)-CotC.

CDS of *Cs*LAP2 was then amplified from pET28a (+)-*Cs*LAP2 plasmid previously constructed using specific primers (forward primer 5′-CGC<u>AAGCTT</u>ATGCAGTCTC CTCTGTTGAATGTAGAG-3′ and reverse primer 5′-CGT<u>GAGCTC</u>CTAGAGAAATCCAACACGGGGTAAG-3′ containing restriction sites of *Hind* III and *Sac* I (underlined). The purified PCR products were digested with *Hind* III and *Sac* I, inserted into pBluescript II SK (-)-CotC and transformed into *E. coli* DH5α (Promega, USA). The recombinant plasmid was selected with ampicillin (50 μg/ml) and verified by sequencing.

Finally, after digestion with *Sal* I and *Sac* I, the fusion gene of CotC-*Cs*LAP2 was sub-cloned into PEB03 and transformed into competent cells of *B. subtilis* WB600 by electroporation (Wu et al. 1991). PEB03-CotC was similarly constructed by the described method and employed as a control.

*Cs*LAP2 expression on the surface of *B. subtilis* spore

Sporulation of *B. subtilis* with PEB03-CotC or PEB03-CotC-*Cs*LAP2 was obtained in Difco Sporulation Medium (DSM, BD, USA) by exhaustion method as described (Harwood and Cutting 1990). After incubation for 24 h, spores were harvested by centrifugation and washed with 1 M NaCl, 1 M KCl, and distilled water successively. 1 mM phenylmethylsulfonyl fluoride (PMSF) was added to inhibit proteolysis. Finally, the spores were treated at 68 °C for 1 h to kill the residual viable vegetative cells. The number of spores was calculated by using a Burker-Turk counting chamber under an optical microscope.

Coat proteins of the spores were extracted from suspended spores (>1×10^10 spores per milliliter) using extraction buffer

(sodium dodecyl sulfate-dithiothreitol, SDS-DTT) as described (Harwood and Cutting 1990). To identify the expression of CsLAP2 on the surface of B. subtilis spore, extracted proteins were subjected to 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane for western blotting analysis. Serum from rat immunized with recombinant CsLAP2 purified from Escherichia coli was employed as primary antibody (1:500 dilutions). After incubation with primary antibody at 4 °C overnight and washed with PBS-0.05 % Tween 20 (PBST, pH 7.4), the membrane was incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-rat IgG (1:2000 dilutions, Sigma, USA) at 37 °C for 2 h. At last, the membrane was visualized by enhanced chemiluminescence (ECL) method.

Flow cytometry was also used to examine the expression of CsLAP2 on the surface of spores. A total of 10^5 spores were washed in PBST and incubated overnight at 4 °C with rat anti-CsLAP2 sera (1:200 dilutions). After washing four times, the spores were incubated with FITC-conjugated anti-rat IgG (Southern Biotech, USA) at 1:200 dilutions for 1 h at 37 °C. After washing thoroughly, the samples were then resuspended in 500 μl of PBS and analyzed using a FACSCalibur instrument (Beckman Coulter, USA).

Oral delivery of recombinant spores and samples collection

Thirty BALB/c mice were divided into three equal groups and treated with spores intragastrically on days 0, 1, 2, 15, 16, 17, 32, 33, and 34. Group I, II, or III was respectively treated with 5×10^5 spores expressing CotC-CsLAP2 (recombinant spores), 5×10^5 spores expressing CotC or 0.2 ml PBS as negative controls. Sera from each group were collected on days 1, 14, 31, 49, and 87. The mice were fasted and euthanized at 87 day after the first intragastric administration. Small intestines (between the distal end of stomach and ileum) were sterilely isolated from each mouse. A part of the intestine was fixed with 4 % paraformaldehyde for immunohistochemistry assay. Twenty centimeters of the other part was cut into small fragments of 2–3 cm and washed three times with 3 ml of PBS containing 0.1 % BSA, 50 mM EDTA, 1 mM PMSF, and 0.1 mg/ml of soybean trypsin inhibitor. The lavage fluids were centrifuged at $700 \times g$ for 2 min, and the supernatants were collected. At the same time, gallbladders were removed and 100 μl of bile was respectively collected from them. Splenocytes were sterilely isolated from each mice and single cell suspensions (1.2×10^7/ml) were respectively cultured with RPMI 1640 (Gibco, USA) supplemented with 10 % fetal calf serum (FBS, Invitrogen, USA) in triplicate. The cells were stimulated with recombinant CotC-CsLAP2 extracted from coat proteins of B. subtilis spores. After conventionally incubated for 48 h, the supernatants were collected for analysis.

Detection of specific antibodies by ELISA

Plates were coated with 100 μl of recombinant CotC-CsLAP2 (5 μg/ml in carbonate-bicarbonate buffer) per well and incubated at 4 °C overnight. After blocked with 5 % skim milk in PBST for 2 h at 37 °C, the plates were incubated with serum samples for 1 h at 37 °C (1:100 dilutions). After washing, the plates were incubated with HRP-conjugated rabbit anti-mouse IgG/IgG1/IgG2a as secondary antibody (1:2000 dilutions, Sigma, USA) for 1 h at 37 °C and followed by incubation with tetramethylbenzidine (TMB) solution. Finally, the reaction was stopped by 2 M H2SO4, and the absorbance of each well was detected at 450 nm using TMB as a blank control.

Using the same method, IgA levels in serum, intestinal lavage fluids, and bile were also detected. The samples were applied at 1:100 dilutions and HRP-conjugated goat anti-mouse IgA (1:2000 dilutions, Sigma, USA) was employed as secondary antibody.

Immunohistochemistry assay of IgA-secreting cells in intestine

Fixed intestinal fragments of mice were embedded in paraffin and sliced into 5-μm sections. After being deparaffinized by using graded ethanol and then immersed in distilled water, the sections were incubated with 3 % peroxide to inactivate the endogenous peroxidase. In order to retrieve antigens, the sections were incubated with 0.01 M citrate buffer (pH 6.0) at 95 °C for 20 min. Successively, the sections were washed with PBST and then blocked with normal goat serum at room temperature for 20 min. The slides were incubated with goat anti-mouse IgA (1:32 dilutions, CELLWAY-LAB, China) as primary antibody overnight at 37 °C. After washing, the slides were incubated with HRP-Protein A (1:1000 dilutions, GenScript, USA) at 37 °C for 1 h. The slides were developed color with 3, 3-diaminobenzidin (DAB) at room temperature under darkness for 10 min and stopped with distilled water. Finally, the slices were stained with hematoxylin. Positive cells were stained dark brown. Random thirty microscopic fields of each sample were captured. The images were analyzed using ImagePro Plus software (Media Cybernetics, Roper, USA). The numbers of IgA-secreting cells were indicated by integrated optical density (IOD). The sizes of areas of interest (AOI) were the same.

Cytokines analysis

The levels of IFN-γ, IL-4, IL-6, IL-10, IL-17A, and TNF in the supernatants of splenocytes treated with recombinant CotC-CsLAP2 were examined using mouse Th1/Th2/Th17 Cytokine Kit (BD, USA). The culture supernatants were added to tubes pre-coated with cytokine-specific antibodies

following the manufacturer's instructions. The results were reported in pg/ml as the means from triplicate wells.

Statistical analysis

Experimental values were expressed as means±standard deviation (SD) and obtained from three independent experiments with a similar pattern. Data were analyzed by one-way analysis of variance (ANOVA) using SPSS software package 10.0. Significance was set at p value <0.05.

Results

Construction of PEB03-CotC-CsLAP2

CDS of CotC were amplified from genome DNA of *B. subtilis* with expected length of 397 base pairs (bp). The fusion gene of CotC-CsLAP2 was constructed with expected length of 1,967 bp and successfully inserted into PEB03 vector (Figs. S1 and S2). Recombinant plasmid of PEB03-CotC-

CsLAP2 was confirmed by DNA sequencing and transformed into *B. subtilis* WB600 strain.

CsLAP2 expression on the surface of *B. subtilis* spore

About 0.3 mg of coat proteins was collected from 1×10^{10} spores. It meant that 0.03 pg of coat proteins was extracted from each spore.

In SDS-PAGE analysis, coat proteins extracted from recombinant spores expectedly showed a protein band at approximate 64.9 kDa (Fig. 1a) which was in accordance with the molecular mass of CsLAP2 plus CotC. While there was no corresponding band in coat proteins extracted from *B. subtilis* spore with PEB03-CotC. Western blotting showed that coat proteins of recombinant spores could be probed with CsLAP2-specific antiserum at the specific band (Fig. 1b).

When the recombinant spores were incubated with rat anti-CsLAP2 serum as primary antibody, the number of spores emitting mean fluorescence intensities was 110 while the number in control groups (group of recombinant spores incubated with serum from naïve rat and group of PEB03-CotC transformed *B. subtilis* spores treated with rat anti-CsLAP2
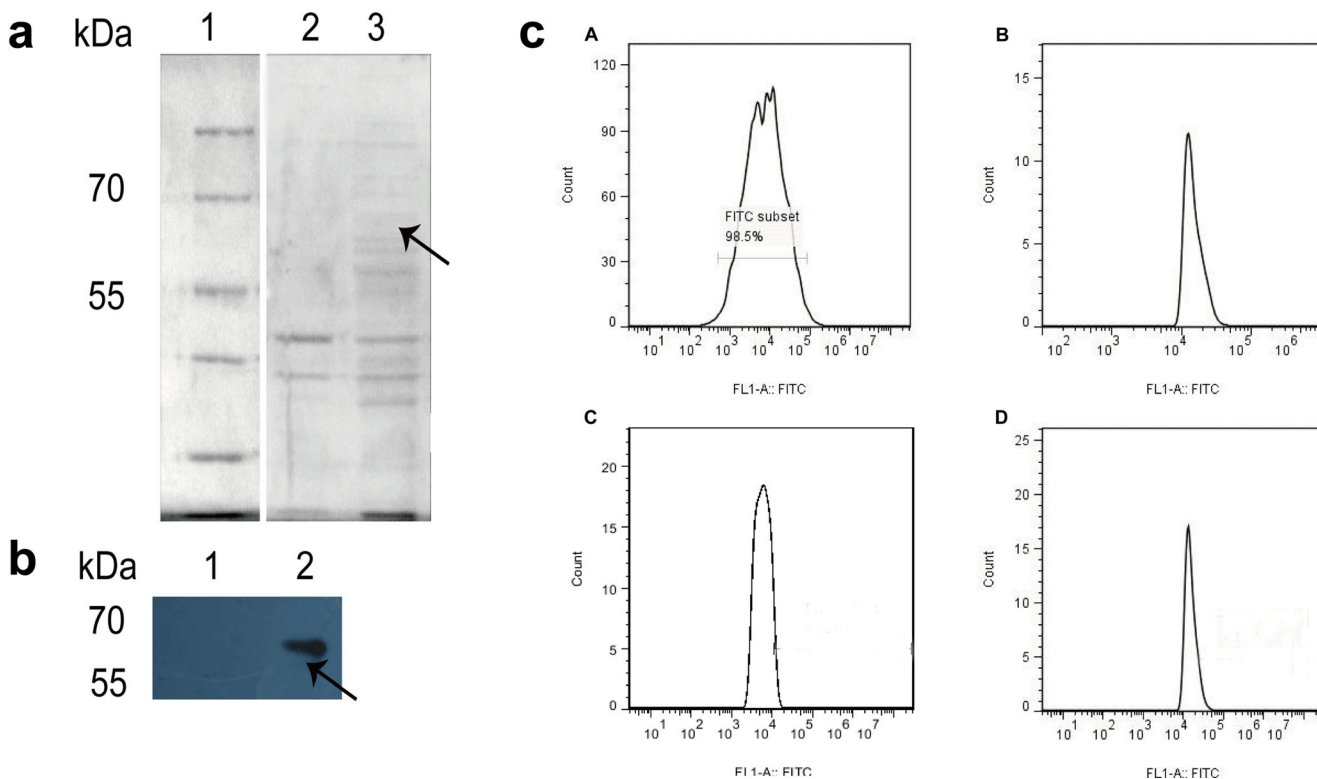

Fig. 1 Identification of recombinant CotC-CsLAP2 expressing on the surface of *B. subtilis* spore. **a** 12 % SDS-PAGE. Protein molecular weight markers (*lane 1*), coat proteins extracted from *B. subtilis* spore with PEB03-CotC (*lane 2*) or PEB03-CotC-CsLAP2 (*lane 3*). **b** Western blotting analysis. Coat proteins extracted from *B. subtilis* spore with PEB03-CotC (*lane 1*) or PEB03-CotC-CsLAP2 (*lane 2*) probed by

specific rat anti-CsLAP2 serum. *Arrows* indicate the recombinant protein. **c** Flow cytometric analysis. PEB03-CotC-CsLAP2 transformed *B. subtilis* spores incubated with rat anti-CsLAP2 serum (*panel A*) or serum from naïve rat (*panel B*), and PEB03-CotC transformed *B. subtilis* spores treated with rat anti-CsLAP2 serum (*panel C*) or serum from naïve rat (*panel D*) followed by FITC-labelled anti-rat IgG

serum or serum from naïve rat) were 12–18. Up to 98.5 % of recombinant spores which were incubated with anti-CsLAP2 serum emitted fluorescence intensity of 10^2–10^5 (Fig. 1c).

Detection of systemic antibodies

The levels of IgG (Fig. 2a) and IgA (Fig. 2d) in sera from mice of group I (intragastrically administrated with recombinant spores) were highest compared with those in mice of group II (intragastrically administrated with CotC spores) and group III (PBS treated). The peak titer of IgA/IgG presented on day 49/day 31. The level of specific IgA response to CotC-CsLAP2 increased significantly even on day 87, while the titer of specific IgG decreased to normal level. IgG1 and IgG2a levels in group I were significantly elevated simultaneously on days 31 and 49. IgG1 (Fig. 2b) level was lower than IgG2a (Fig. 2c) in group I at each time point.

Analysis of local mucosal antibodies

Compared with groups II and III, CsLAP2-specific sIgA levels in both intestinal lavage fluid and bile in group I were statistically higher on day 87 (Fig. 3).

Immunohistochemical analysis of IgA-secreting cells in intestine

By employing goat anti-mouse IgA and HRP-Protein A, DAB-stained (dark brown) cells were observed in intestinal epithelium of mice in group I (Fig. 4a), while there is no obviously positive cell in sections of group II and group III. By using of ImagePro Plus software, IOD of group I was significantly higher than that of group II or group III (Fig. 4b). There was no statistical difference between group II and group III.
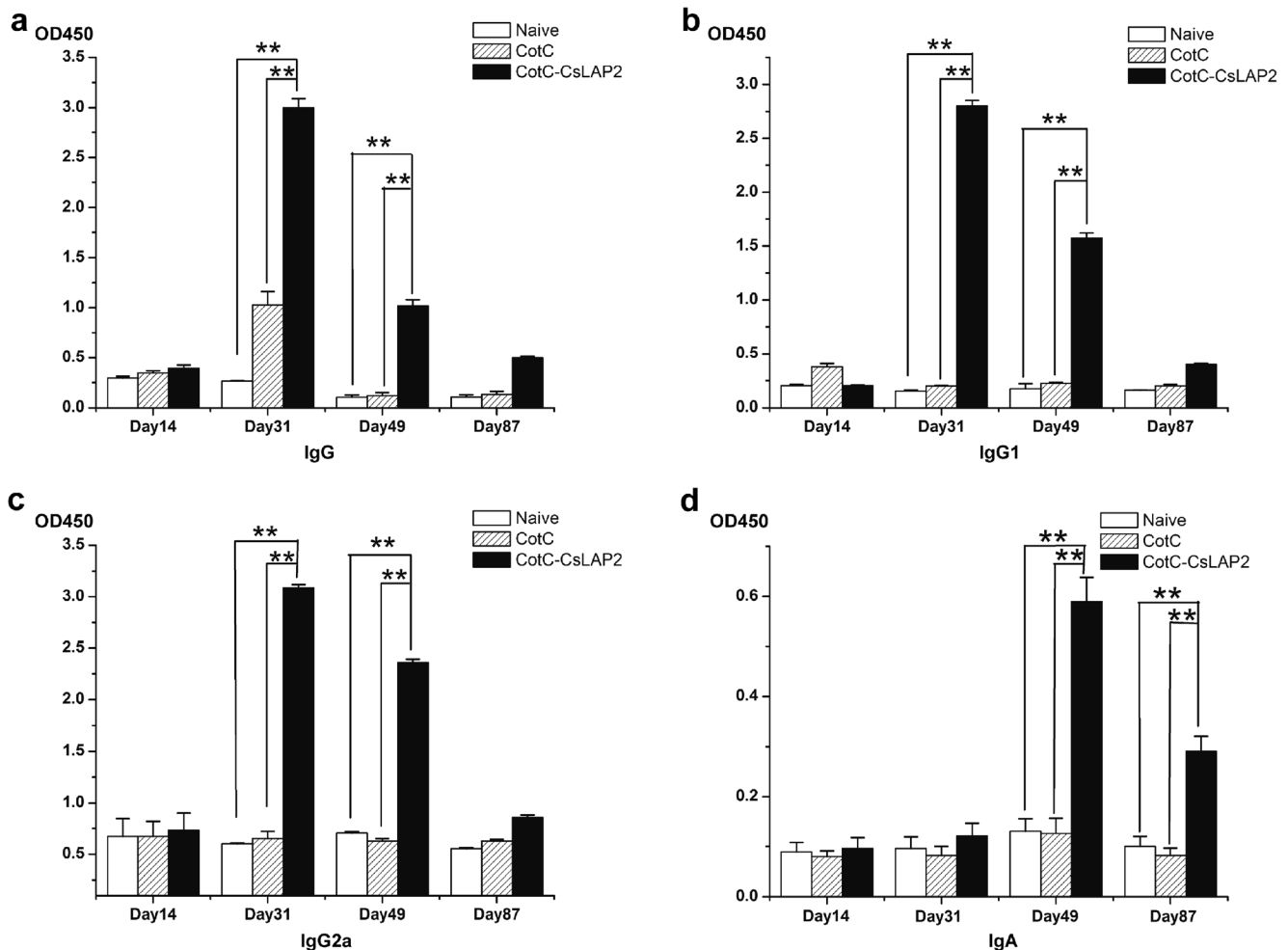

Fig. 2 Specific antibodies levels in sera from mice with oral immunization by ELISA analysis. Specific IgG (a), IgG1 (b), IgG2a (c), and IgA (d) levels in sera from mice orally treated with PEB03-CotC-CsLAP2 or PEB03-CotC transformed spores were detected. Each group was composed of 10 BALB/c mice. Serum from each mouse was analyzed and individual sample was performed in triplicate. Two asterisks mean significantly statistical differences (p<0.01)

Fig. 3 Specific sIgA levels in intestinal lavage fluid and bile of mice. Intestinal lavage fluid and bile were collected from 5 mice in each group on day 87 after the first oral administration. sIgA in intestinal lavage fluid (**a**) and bile (**b**) of individual samples were tested by ELISA. Statistically significant differences are indicated by two asterisks for $p<0.01$
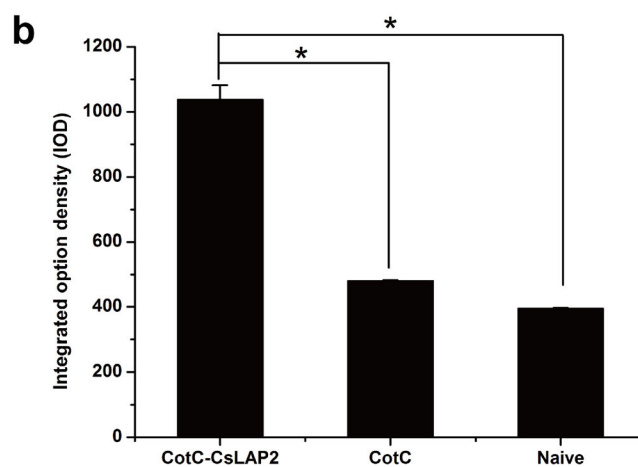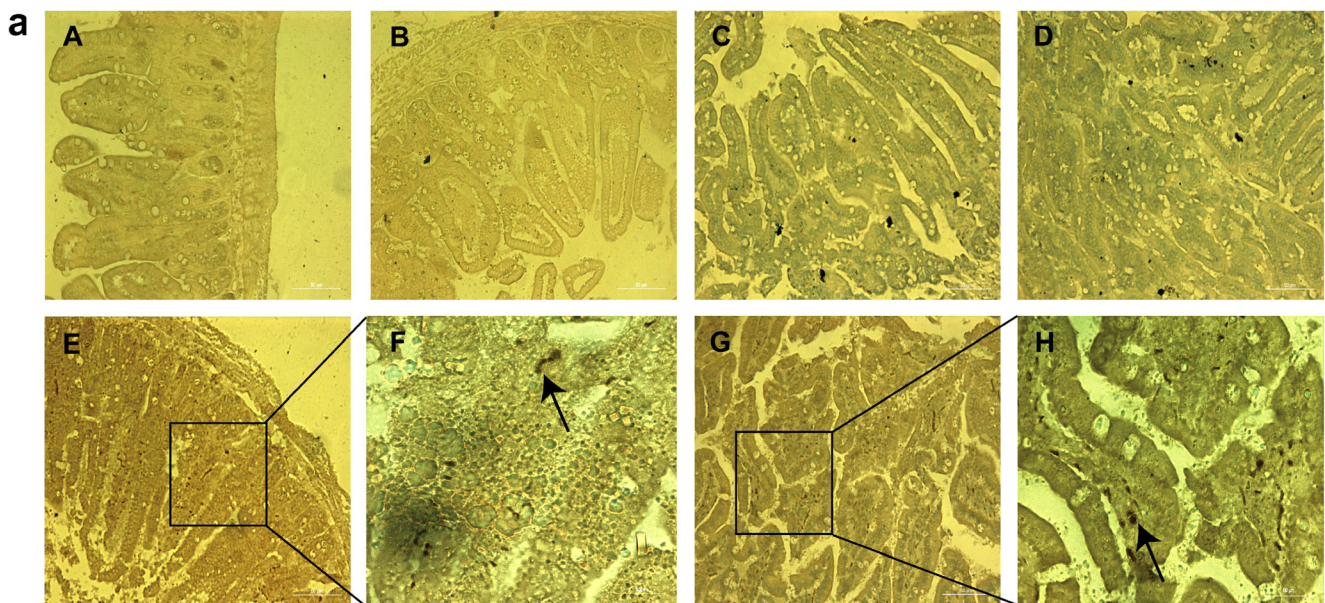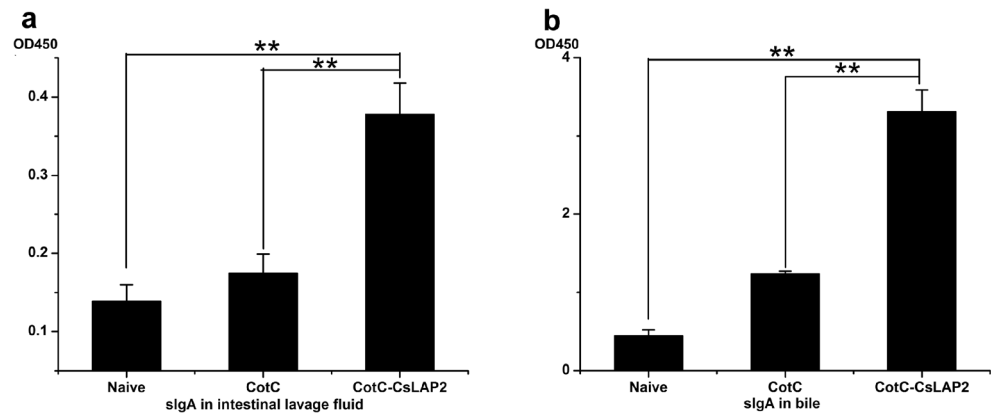

Fig. 4 IgA-secreting cells in intestinal epithelium of mice. IgA-secreting cells were examined by immunohistochemistry and the positive cells were stained dark brown. **a**, *panels A and B*, PBS-treated mice. *Panels C and D*, mice orally administrated with spores expressing CotC. Panels *E and G*, mice orally administrated with spores expressing CotC-*Cs*-LAP2. The images were magnified at ×200. Panels *F and H*, the indicated rectangle areas in panels *E* and *G* were magnified at ×400. The *arrows* indicated IgA-secreting cells. **b** Thirty random fields from each mouse were analyzed by using gray scale scanning software. The number of IgA-secreting cells in intestinal epithelium was presented by integrated option density (IOD). *$p<0.05$

Cytokines assays

After incubation with recombinant CotC-*Cs*LAP2 for 48 h, compared to groups II and III, IFN-γ level representing Th1 response dominantly increased in group I in the supernatants of splenocytes isolated from each group (Fig. 5a), while not IL-4 (Fig. 5b) representing Th2 response. In addition, IL-6, IL-10, IL-17A, or TNF level of group I was significantly higher than that of groups II and III (Fig. 5c–f). The concentrations of IL-6, IL-10, and TNF were respectively 3, 003± 80 pg/ml, 3, 234±324 pg/ml, and 11, 874±2,090 pg/ml.

Discussion

In the current study, we successfully expressed *Cs*LAP2 on the surface of *B. subtilis* spore. After mice were orally administrated with the recombinant spore, the systemic immune reactions including antibodies in sera and cytokines produced by splenocytes were detected. Local mucosal immune responses including IgA-secreting cells and sIgA levels in intestinal lavage fluid and bile were evaluated too.

In mammals, heterogenous antigens mainly elicit systemic immune response by subcutaneous or intramuscular injection, while antibodies in serum usually cannot offer effective protection against mucosal infection as well as gastrointestinal infection. Mucosal immunization can induce not only humoral

but also cell-mediated immune response as well as systemic and local immune responses, which protect the mucosa against invasion and colonization of pathogens (Holmgren and Czerkinsky 2005). During the invasion and parasitism of *C. sinensis*, both larva and adult worm interact with mucosa of gastrointestinal tract and bile ducts of the host, suggesting that mucosal immunization may trigger effective immune response against *C. sinensis* infection. Oral administration is considered an ideal strategy for mucosal immunization due to a few of advantages such as needle-free application, better compliance, and so on (Kim et al. 2012), but naked antigens are easy to be degraded by digestive juice. *B. subtilis* spores have been successfully used to immobilize heterologous protein on the outside layer (Kim and Schumann 2009). It has been documented that CotC, a coat protein expressing on the surface of *B. subtilis* spore, has been used as an anchoring motif for the display of tetanus toxin fragment C or heat-labile toxin from *E. coli* (Mauriello et al. 2004) so that CotC was utilized as a fusion partner to express *Cs*LAP2 on the surface of spore in our research.

Secretory antibodies are presumed to play a central role in clearance of parasites away from intestinal tract (Kanobana et al. 2003, 2002; Langford et al. 2002; Muller and von Allmen 2005), and sIgA is the predominant antibody isotype in mucosal secretion including bile. sIgA in bile can enhance immune protection of biliary tract and intestine, which can prevent the attachment of pathogens or their toxins to mucous membranes. sIgA can also aggregate pathogens, inhibit their
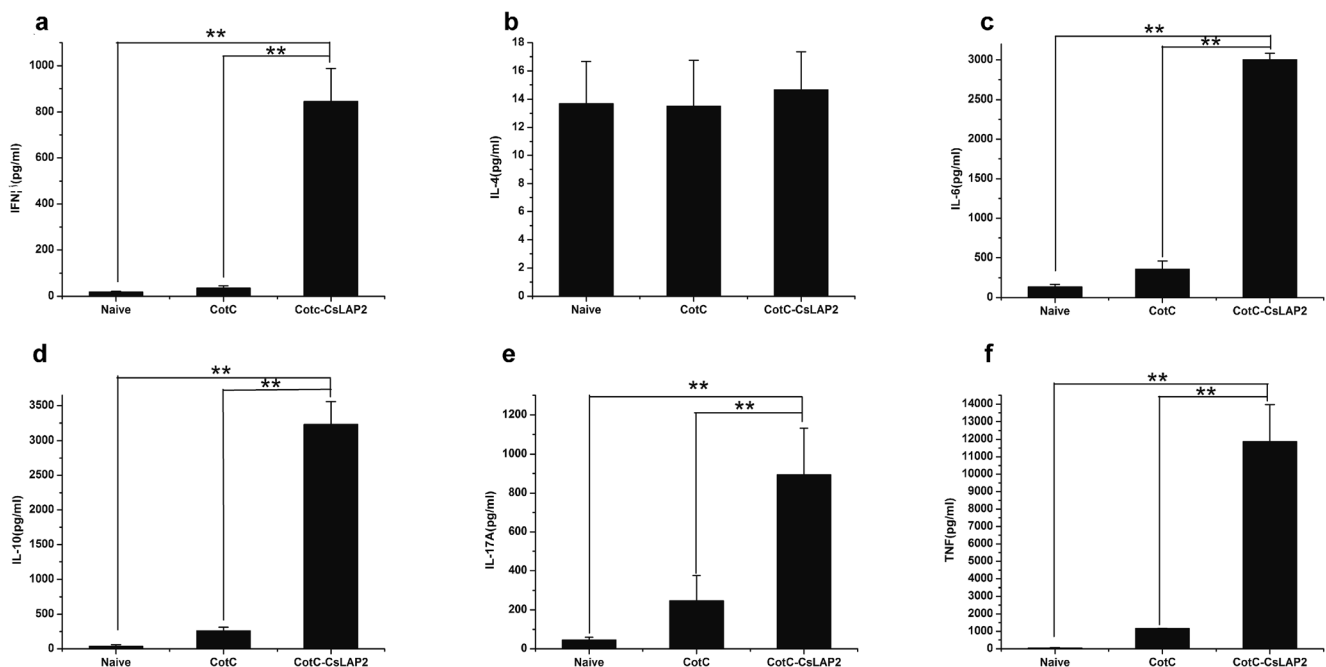

Fig. 5 Cytokines production of recombinant CotC-*Cs*LAP2 incubated splenocytes isolated from treated mice. Splenocytes were isolated on day 87 after the first immunization from five mice in each group and incubated for 48 h with recombinant CotC-*Cs*LAP2. IFN-γ (**a**), IL-4 (**b**), IL-6 (**c**), IL-10 (**d**), IL-17A (**e**), and TNF (**f**) levels in the supernatants of cultured cells were measured by flow cytometry. The results were showed as the mean concentrations in pg/ml from triplicate wells. Statistically significant differences are indicated by two asterisks for $p < 0.01$

motility, and prevent their adherence to epithelium (Reynoso-Paz et al. 1999). In our study, high titers of specific sIgA were generated in both intestine and bile of mice in group I (mice orally administrated with the recombinant spores). IgA-secreting cells were also seen in intestinal epithelium of the mice.

IL-17A was obviously elevated in mice from Group I. IL-17A is mostly secreted by Th17 cells which stem from naïve T cells during antigen priming in the presence of IL-6, transforming growth factor-β (TGF-β), IL-23 and so on. Th17 cells preferentially migrate to intestine and associated lymphoid tissues and then mediate host defense against microorganisms by releasing antimicrobial peptides by epithelial cells, recruiting neutrophils and macrophages, initiating humoral immunity, and augmenting other T helper subsets (Kumar et al. 2013). These results indicated that oral immunization with recombinant spores elicited local mucosal immune responses in mice.

IgG and IL-6 increased significantly in group I. IL-6 prominently regulates antibodies secretion of B cells (Jones 2005). It was indicated that the oral administration triggered systemic humoral immune response. The levels of IgG and IgG subclass declined on day 49 from the peak titers on day 30. IgA level in serum as well as sIgA titer in bile or intestine was still high on day 87. It was indicated that systemic humoral immune response was elicited by oral administration rapidly but did not last for long time. The increase of IgG2a level was more obvious than IgG1 in group I. It was indicated that systemic cell-mediated immune response was produced. After cultured with recombinant CotC-CsLAP2 in vitro, IFN-γ rather than IL-4 level increased in the supernatants of splenocytes isolated from mice of group I. IFN-γ, an indicator of Th1 immune response, increased in accordance with the rise of IgG2a. It was suggested that CotC-CsLAP2 spores induced Th1 immune response by oral immunization. The increased Th1 immune response can lead to the reduction of host adaptation and elicit enhanced protective immune response in host against helminths infection (Choi et al. 2003).

TNF and IL-10 levels increased dominantly in group I. TNF can enhance innate immunity via its effect on epithelial cells of gastrointestinal tract and activate dendritic cells to promote T cell proliferation and differentiation by engulfing antigen into mature dendritic cells (Waters et al. 2013). IL-10 as an anti-inflammatory factor can reduce the damages caused by excessive inflammatory responses. Generation of effective immune responses against pathogenic microbes depends on a fine balance between pro- and anti-inflammatory responses (Carey et al. 2012). Collectively, the cytokines mentioned above could initiate diverse cellular effects to promote immunological responses.

In conclusion, we showed that oral delivery of *B. subtilis* spores displaying *Cs*LAP2 on the surface induced both systemic and local mucosal immune response. sIgA and Th1/ Th17 cellular immunity were involved in the immunological mechanisms. Oral administration of *B. subtilis* spore displaying *Cs*LAP2 on the surface can be further considered as a strategy for vaccine development to prevent and control clonorchiasis.

References

Acosta D, Cancela M, Piacenza L, Roche L, Carmona C, Tort JF (2008) *Fasciola hepatica* leucine aminopeptidase, a promising candidate for vaccination against ruminant fasciolosis. Mol Biochem Parasitol 158:52–64

Carey AJ, Tan CK, Ulett GC (2012) Infection-induced IL-10 and JAK-STAT: a review of the molecular circuitry controlling immune hyperactivity in response to pathogenic microbes. JAKSTAT 1: 159–167

Choi YK, Yoon BI, Won YS, Lee CH, Hyun BH, Kim HC, Oh GT, Kim DY (2003) Cytokine responses in mice infected with *Clonorchis sinensis*. Parasitol Res 91:87–93

Chung BS, Zhang H, Choi MH, Jeon D, Li S, Lee M, Hong ST (2004) Development of resistance to reinfection by *Clonorchis sinensis* in rats. Korean J Parasitol 42:19–26

Deng C, Sun J, Li X, Wang L, Hu X, Wang X, Chen W, Lv X, Liang C, Li W, Huang Y, Li R, Wu Z, Yu X, Xu J (2012) Molecular identification and characterization of leucine aminopeptidase 2, an excretory-secretory product of *Clonorchis sinensis*. Mol Biol Rep 39:9817–9826

Harwood CR, Cutting SM (1990) Molecular biological methods for *Bacillus*. Wiley, New York

Hinc K, Iwanicki A, Obuchowski M (2013) New stable anchor protein and peptide linker suitable for successful spore surface display in *B. subtilis*. Microb Cell Factories 12:22

Holmgren J, Czerkinsky C (2005) Mucosal immunity and vaccines. Nat Med 11:S45–S53

Hong ST, Fang Y (2012) *Clonorchis sinensis* and clonorchiasis, an update. Parasitol Int 61:17–24

Huang JM, La Ragione RM, Nunez A, Cutting SM (2008) Immunostimulatory activity of *Bacillus* spores. FEMS Immunol Med Microbiol 53:195–203

Huang Y, Li W, Huang L, Hu Y, Chen W, Wang X, Sun J, Liang C, Wu Z, Li X, Xu J, Yu X (2012) Identification and characterization of myophilin-like protein: a life stage and tissue-specific antigen of *Clonorchis sinensis*. Parasitol Res 111:1143–1150

Jia H, Nishikawa Y, Luo Y, Yamagishi J, Sugimoto C, Xuan X (2010) Characterization of a leucine aminopeptidase from *Toxoplasma gondii*. Mol Biochem Parasitol 170:1–6

Jones SA (2005) Directing transition from innate to acquired immunity: defining a role for IL-6. J Immunol 175:3463–3468

Kanobana K, Ploeger HW, Vervelde L (2002) Immune expulsion of the trichostrongylid *Cooperia oncophora* is associated with increased eosinophilia and mucosal IgA. Int J Parasitol 32:1389–1398

Kanobana K, Koets A, Kooyman FN, Bakker N, Ploeger HW, Vervelde L (2003) B cells and antibody response in calves primary-infected or re-infected with *Cooperia oncophora*: influence of priming dose and host responder types. Int J Parasitol 33:1487–1502

Kim J, Schumann W (2009) Display of proteins on *Bacillus subtilis* endospores. Cell Mol Life Sci 66:3127–3136

Kim SH, Lee KY, Jang YS (2012) Mucosal Immune system and m cell-targeting strategies for oral mucosal vaccination. Immune Netw 12: 165–175

Knecht LD, Pasini P, Daunert S (2011) Bacterial spores as platforms for bioanalytical and biomedical applications. Anal Bioanal Chem 400: 977–989

Kumar P, Chen K, Kolls JK (2013) Th17 cell based vaccines in mucosal immunity. Curr Opin Immunol 25:373–380

Langford TD, Housley MP, Boes M, Chen J, Kagnoff MF, Gillin FD, Eckmann L (2002) Central importance of immunoglobulin A in host defense against *Giardia spp*. Infect Immun 70:11–18

Lv X, Chen W, Wang X, Li X, Sun J, Deng C, Men J, Tian Y, Zhou C, Lei H, Liang C, Yu X (2012) Molecular characterization and expression of a cysteine protease from *Clonorchis sinensis* and its application for serodiagnosis of clonorchiasis. Parasitol Res 110:2211–2219

Mauriello EM, le Duc H, Isticato R, Cangiano G, Hong HA, De Felice M, Ricca E, Cutting SM (2004) Display of heterologous antigens on the *Bacillus subtilis* spore coat using CotC as a fusion partner. Vaccine 22:1177–1187

Muller N, von Allmen N (2005) Recent insights into the mucosal reactions associated with *Giardia lamblia* infections. Int J Parasitol 35: 1339–1347

Reynoso-Paz S, Coppel RL, Mackay IR, Bass NM, Ansari AA, Gershwin ME (1999) The immunobiology of bile and biliary epithelium. Hepatology 30:351–357

Rim HJ (1986) The current pathobiology and chemotherapy of clonorchiasis. Korean J Parasitol 24(Suppl):1–141

Song SM, Park JH, Kim J, Kim SI, Hong YC, Kong HH, Chung DI (2008) Identification and characterization of *Paragonimus westermani* leucine aminopeptidase. Parasitol Int 57:334–341

Wang X, Liang C, Chen W, Fan Y, Hu X, Xu J, Yu X (2009) Experimental model in rats for study on transmission dynamics and evaluation of *Clonorchis sinensis* infection immunologically, morphologically, and pathologically. Parasitol Res 106:15–21

Waters JP, Pober JS, Bradley JR (2013) Tumour necrosis factor in infectious disease. J Pathol 230:132–147

Wu XC, Lee W, Tran L, Wong SL (1991) Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases. J Bacteriol 173:4952–4958

Zhang H, Chung BS, Li S, Choi MH, Hong ST (2008) Changing patterns of serum and bile antibodies in re-infected rats with *Clonorchis sinensis*. Korean J Parasitol 46:17–22