Construction of *Bifidobacterium infantis* as a live oral vaccine that expresses antigens of the major fimbrial subunit (CfaB) and the B subunit of heat-labile enterotoxin (LTB) from enterotoxigenic *Escherichia coli*

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We sought to develop *Bifidobacterium infantis* (BI) as a vehicle for the expression of heterologous antigens. Two proteins of enterotoxigenic *Escherichia coli* (ETEC) were expressed in BI: CfaB, a major fimbrial subunit protein, and LTB, the B subunit of heat-labile enterotoxin. The expression of CfaB and LTB in BI was verified by electrophoretic analysis. Sprague–Dawley rats were then subjected to intragastric immunization with BI-CfaB and BI-LTB systems both separately and together. ELISA was used to characterize the serum and mucosal immune responses against ETEC antigens. The immunized rats were intraperitoneally challenged with wild-type ETEC H10407 to study the immune response *in vivo*. The serum titres of IgG and faecal IgA antibodies in the BI-CfaB plus BI-LTB mixed vaccination group were significantly greater than those in the other two groups, which were immunized with a single vaccine ($P<0.05$). However, no significant difference was seen between the two groups that received a single immunization. These results suggest that expressing CfaB and LTB in BI provides a probiotic system with immunogenic properties. Furthermore, the expression of LTB in BI preserved its mucosal adjuvant effect. So this study confirms that BI can be used as a novel oral vaccine expression system for a heterologous antigen and BI-LTB can provide mucosal adjuvant properties.

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INTRODUCTION

Bifidobacterium infantis (BI) is a Gram-positive, anaerobic probiotic bacterium that is a component of the normal human intestinal microflora (Guarner & Malagelada, 2003). Due to the symbiotic relationship of BI and humans, it is likely that BI will be safe for human oral vaccine development. Given its ability to colonize in the intestine, BI may be appropriate for use as an antigen expression vector that directly and efficiently presents antigen to the intestinal mucosa. We therefore hypothesize that the use of a BI-based oral vaccine will be safe for the expression of heterologous antigens. However, to our knowledge, BI has not been reported as a vaccine development system to date.

Enterotoxigenic *Escherichia coli* (ETEC) infections are a major cause of diarrhoeal disease worldwide. ETEC is the

chief cause of diarrhoeal morbidity and infant mortality in developing countries, and it is also perennially associated with diseases like traveller's diarrhoea in developing countries (DuPont, 2008). Therefore, a safe and effective vaccine against ETEC is essential for public health (Daley *et al.*, 2007; Gaastra & Svennerholm, 1996).

CFAs (colonization factor antigens) are subdivided into three major groups: CFA/I, CFA/II (CS3 alone or CS3 with CS1 or CS2) and CFA/IV (CS6 alone or with CS4 or CS5) (Gaastra & Svennerholm, 1996). ETEC induces diarrhoea by colonizing the intestine with the help of CFAs. In the intestine, the bacteria release LT (heat labile) and/or ST (heat stable) enterotoxins, which induce the secretion of fluid and electrolytes from the small intestinal epithelium (Sack, 1975). The major virulence determinants for ETEC are LT and ST and the colonization of the gut, which requires involvement of CFAs. Previous research has indicated that, for prolonged protection from the symptomatic effects of ETEC, immunity against surface antigens present on the bacteria is more important than immunity against LT and/or ST (Boedeker, 2005; Girard *et al.*, 2006).

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Abbreviations: BI, *Bacteroides infantis*; CFA, colonization factor antigen; ETEC, enterotoxigenic *Escherichia coli*; HIA, haemagglutination inhibition assay; HRP, horseradish peroxidase; LT, heat-labile toxin; LTB, B subunit of LT; ST, heat-stable toxin.

LT is encoded by a heteromeric operon *eltAB* and it consists of a toxic A subunit (LTA) and a pentamer of receptor-binding B subunit (LTB). LTBs, which are functionally similar to CTB (cholera toxin B subunit), are powerful immunogens and adjuvants (Levine *et al.*, 1979; Pizza *et al.*, 2001).

Due to the presence of CFAs on the bacterial surface, CFAs may be advantageous as antigenic targets for inducing a prolonged mucosal immune response to ETEC, if incorporated in a vaccine (Girard *et al.*, 2006; Steinsland *et al.*, 2002). Therefore, oral administration of a live ETEC vaccine may be more effective than a killed vaccine, although this is currently debatable. With oral administration of a live vaccine, the vaccine is directly exposed to the intestinal mucosal surface, where antigen presentation takes place. Furthermore, a live vaccine requires fewer doses than a killed one. Therefore, oral administration of a live ETEC antigen expression vector is a reasonable strategy for vaccine development. Previously, *Vibrio cholerae* and *Salmonella typhimurium* were used in developing ETEC vaccines (Barry *et al.*, 2003; López-Gigosos *et al.*, 2007; Pascual *et al.*, 1999).

In this study we aimed to develop BI as a vehicle for the expression of ETEC antigens CfaB and LTB as an oral vaccine candidate, in the hope of inducing a protective immune response against human ETEC.

METHODS

Bacterial strains and growth conditions. ETEC strains H10407 (E44813) and E44815 were propagated in CFA agar medium [Luria–Bertani (LB) agar with 1 % Casamino acids, pH 7.4, 0.4 mM MgSO₄, 0.04 mM MnCl₂]. *E. coli* DH5 α was used as host for molecular cloning; pBEX was constructed from pGEX-5X-1 (see below) and used as the expression vector in BI.

The BI strain (China Center of Industrial Culture Collection, CICC no. CICC6069) was cultured in MRS broth (Difco) containing 0.25 % (w/v) L-cysteine.HCl (pH 7), at 37 °C under anaerobic conditions. Ampicillin (50 μ g ml⁻¹) was added to both recombinant BI and *E. coli* strains when required.

Plasmid construction. The recombinant plasmid used for heterologous antigen expression in BI was constructed as follows. The promoter of the amylase gene in BI (*pamyB*, GenBank no. AY240946), containing a *Sac*II site at the 5'-terminus and a *Bam*HI site at the 3'-terminus, and the replicase B gene of *Bifidobacterium longus* (*repB*, GenBank no. AF139129), containing an *Aat*II site at both termini, were designed and purchased from Sangon (Shanghai, China). The *bla-ori* fragment (β -lactamase gene and pBR322 *ori*) was amplified from plasmid pGEX-5X-1 by using PCR with forward primer 5'-ATTAGGATCCCCGAATTCCCG-3' (*Bam*HI site underlined) and reverse primer 5'-TCGACCGCGGATTACACACCTG-AATTGAC-3' (*Sac*II site underlined). The PCR was performed at 95 °C for 4 min, followed by 30 cycles of 95 °C for 40 s, 52 °C for 30 s and 72 °C for 1 min. Then the PCR products of the *bla-ori* and *pamyB* fragments were digested with *Bam*HI and *Sac*II, respectively. Subsequently, the *pamyB* fragment was ligated into the *bla-ori* fragment with T4 DNA ligase at 4 °C and the primary recombinant, pBEX-pre, was amplified in *E. coli* DH5 α . The third step involved digesting pBEX-pre and the *repB* fragment with *Aat*II, followed by ligating them together. Thus the final recombinant shuttle vector, pBEX, was constructed.

pBEX-CfaB construction. The major fimbrial subunit (CfaB) of ETEC was amplified from the H10407 plasmid (GenBank No: FN649418.1) by PCR with forward primer 5'-TGGGTCGACT-AAGGAGTTCTAATG-3' (*Sal*I site underlined) and reverse primer 5'-TGGCTCGAGCTAGAGTGTGTTGACT-3' (*Xho*I site underlined). The PCR products and pBEX were digested with *Sal*I and *Xho*I, respectively. After purifying, the digested *cfaB* fragment was ligated into pBEX with T4 DNA ligase (TaKaRa) and the recombinant plasmid was transformed into *E. coli* DH5 α . Colonies were selected on LB agar plates, and those containing the correct insert were confirmed by restriction digestion.

pBEX-LTB construction. The gene encoding LTB (*eltB*) of ETEC was amplified from ETEC E44815 by PCR with primers 5'-AAGG-TCGACGGATCCATGAATAAGTAAAATG-3' and 5'-ACGGAGC-TCGAATTCTTAGACATGCTTTTAAAG-3' (*Bam*HI and *Eco*RI sites underlined). The PCR products and the pBEX plasmid were digested with *Bam*HI and *Eco*RI, respectively. Then the purified *eltB* fragment was ligated into pBEX and the product was transformed into *E. coli* DH5 α as described above. The positive transformant was verified as described above.

BI transformation. BI cells were grown to OD₆₀₀ 0.4–0.5 and washed four or five times with 10 % glycerol (containing 0.25 % L-cysteine.HCl, pH 7). Plasmids pBEX, pBEX-LTB and pBEX-CfaB were mixed with the prepared BI cells and incubated on ice for 5 min. Then the plasmids were transformed into competent BI by a Gene Pulser apparatus with a 0.2 cm cuvette (Bio-Rad) at 2.0 kV, 25 μ F and 200 Ω . The BI transformants, namely BI-LTB and BI-CfaB, were plated onto MRS agar containing 40 μ g ampicillin ml⁻¹, and were incubated (as described earlier) until colonies appeared after 72 h. The colonies were isolated and cultured in MRS-ampicillin broth. Plasmid DNA from BI was isolated using the method of O'Sullivan & Klaenhammer (1993). The plasmid was verified by electrophoresis.

Protein expression and Western blotting analysis. The primary antibodies of CfaB and LTB were prepared from BALB/c mice immunized subcutaneously with purified 6xHis-CfaB and 6xHis-LTB (each 5 μ g), both expressed in *E. coli* BL21 (Wang *et al.*, 1999). The transformed BI (pBEX, BI-LTB and BI-CfaB) were inoculated in MRS broth with ampicillin (50 μ g ml⁻¹), grown to OD₆₀₀ 1.5 and then collected by centrifugation (5000 g for 5 min at 4 °C). The pellets were resuspended in 1 $\times$ loading buffer. Lysates were prepared by sonicating the pellets (three times for 15 s each, 0–4 °C) followed by boiling for 5 min. Protein samples of both LTB and CfaB were subjected to 12 % SDS-PAGE at 80 V. Proteins were visualized by Coomassie blue R250 staining.

For Western blotting, the proteins were separated by 12 % SDS-PAGE and then transferred onto a nitrocellulose membrane (BIO WORLD) using a Semi-Dry Transfer System (Bio-Rad). Non-specific protein binding was blocked by incubating the membrane in TBS containing 0.05 % Tween 20 and 5 % skim milk powder at room temperature overnight. After washing with TBS-Tween 20, the membrane was then incubated with either polyclonal mouse anti-CfaB (1:800) or anti-LTB antiserum (1:800) for 2 h at 37 °C with constant shaking. The membrane was washed four times for 10 min with TBS-Tween 20, incubated at 37 °C with the appropriate horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG for 1 h, and subsequently washed four times for 10 min with TBS-Tween 20. Bound antibody was revealed by the addition of the peroxidase substrate 3,3'-diaminobenzidine (DAB) (BOSTER, China).

Immunization. Male Sprague–Dawley rats 3–4 weeks of age were obtained from the Experimental Animal Center, Chongqing Medical University, China, and divided into four groups, A–D. After treatment with saturated sodium bicarbonate (0.3 ml per rat), the

rats were intragastrically vaccinated three times, on days 0, 10 and 17, with 1.0×10^{10} c.f.u. ml^{-1} of BI-CfaB, BI-LTB, BI-CfaB with BI-LTB, and BI (pBEX) (Table 1).

Immunological assay. Blood samples were individually collected from immunized rats by a tail bleed on days 0, 10, 17 and 27 for the analysis of systemic LTb- and CfaB-specific antibodies.

Fresh faecal pellets were individually collected from the same rat groups on days 0, 10, 17 and 27, and lyophilized. Samples (1 mg) of lyophilized faecal material were resuspended in 500 μl PBS (containing 0.2 mg trypsin inhibitor ml^{-1} and 0.05 % sodium azide), left to soak at 4 °C overnight, and then vortexed and micro-centrifuged at 20 000 g for 10 min. The supernatants were analysed for sIgA levels to study the secretory mucosal immune response.

ELISAs. CFA/I antigen used for ELISAs was prepared from ETEC H10407 as previously described and LTb expressed in BI (named rLTb) was prepared from BI-LTB (Adachi *et al.*, 2000; Uesaka *et al.*, 1994). Antigens ($1.0 \mu\text{g ml}^{-1}$ of rLTb or CFA/I) were diluted in a coating buffer (50 mM sodium carbonate, 30 mM sodium bicarbonate, pH 9.6) and allowed to adsorb to 96-well plates ($1 \mu\text{g}$ each well) overnight at 4 °C. The plates were blocked with 3 % BSA for 1 h at room temperature and a twofold serial dilution of serum was added. After incubation, the plates were washed three times with PBS containing 0.05 % Tween 20 (PBS-Tween), then 1.0 mg ml^{-1} of goat anti-rat IgG mAb or anti-rat IgA mAb conjugated to HRP ($1.0 \mu\text{g ml}^{-1}$, BOSTER, China) was added and incubated again for 1 h.

The plates were then washed with 2 mM PBS-Tween and incubated with TMB/UHP (1:1, v/v). TMB solution contained 0.56 mM TMB (3,3',5,5'-tetramethylbenzidine); UHP solution contained 0.1 M sodium citrate, 0.2 M Na_2HPO_4 and 1.2 mM urea-hydrogen peroxide (Sangon, China). After 1 h incubation with the conjugated antibody, the A_{450} value was measured on a Molecular Devices SpectroMax Plus spectrophotometer. End-point titres were determined as the dilution of each sample showing a 2.1-fold higher A_{450} than that of the negative control samples. Mean A_{450} values for the animals were calculated.

CFA/I inhibition assay (chemiluminescence immunoassay).

The chemiluminescence immunoassay was performed with haemagglutination inhibition assay (HIA) (Bhaskaran *et al.*, 2005). Before HIA, the H10407 cells were adjusted to 5.0×10^9 cells ml^{-1} in PBS (pH 7.4). The bacterial suspension (30 μl) and PBS (30 μl) were added to well A1 in a 96-well round-bottomed microtitre plate. Twofold serial dilutions of the suspension were then made from well A1 to A8. D-Mannose (0.1 M, 30 μl) and washed human erythrocyte suspension (30 μl , type A) were added to wells A1 to A8 and the plate was incubated for 10 min at 25 °C. The highest dilution that showed clear haemagglutination with human erythrocytes was used for the HIA as the minimal bacterial density.

For the HIA, 30 μl PBS was added to the round-bottomed wells from well A1 to A8 and 30 μl immune serum from the rats was added to

wells A1 and A2. Then, serial double dilutions of the immune serum were made from wells A2 to A7, and 30 μl solution was discarded from well A7. Another 30 μl PBS was added to well A8 to act as serum-negative control and all wells were adjusted to a final volume of 60 μl with PBS. Then 30 μl D-mannose (0.1 M)-blocked H10407 bacterial suspension was added to wells A1 to A8, gently mixed, and the plate was incubated for 10 min at room temperature. Finally, 30 μl erythrocyte suspension (3.0 %) was added and the plate was incubated at 4 °C for 90 min.

Diarrhoea protection assay. The immunized rats were subjected to a rat ileal loop test after injection of ETEC H10407 or LT (Adachi *et al.*, 2000). After 12 h fasting, one of the vaccinated rats randomly selected from each group was anaesthetized with chloral hydrate (10 %, 0.25 ml per 100 g rat) and the ileum of the rat was ligated to form four 3 cm loops. The rat from group B was injected with 0.2 ml (250 μg) prepared ETEC LT into the three ileal loops (3.0 cm) and 0.2 ml PBS was injected into the fourth loop as negative control. The rats of groups C and D were injected with 0.2 ml ETEC H10407 (containing 1.0×10^5 c.f.u. ml^{-1} bacteria) into the first three loops, and with PBS into the fourth loop. The rat of group A received 0.2 ml (250 μg) purified LT in the first loop; the second and third loops were injected with 0.2 ml ETEC H10407 cells, and the fourth loop with 0.2 ml PBS (Fig. 1). The rats were euthanized 18 h after the injection, and the fluid weight to length ratio [fluid accumulated (mg)/loop length (cm)] was determined; it was defined as diarrhoea positive when the ratio was more than 0.2 mg cm^{-1} .

Lethal challenge test. In order to measure the mucosal immune response, the vaccinated rats were lethally challenged with ETEC H10407 by use of the intraperitoneal challenge model (Bühler *et al.*, 1991). On day 28, six rats from each vaccinated group were challenged with lethal doses (1.0 ml) of ETEC H10407 (2.0×10^{10} c.f.u. ml^{-1}) administered intraperitoneally. The ETEC deletion rate in the abdominal cavity was detected by abdominocentesis 24 h post-challenge. The rats in groups B, C and D were each injected with 2 ml PBS into the abdominal cavity and the abdomen was kneaded gently for 1–2 min. Then the liquid was taken in a sterile syringe from the abdominal cavity of the rats and cultured overnight on LB agar at 37 °C to detect ETEC.

Statistical analysis. The data were statistically evaluated by variance analysis (ANOVA) and the comparison of means (one-tailed Student's *t*-test) was done at a significance level of $P < 0.05$.

Table 1. Rats and vaccination groups

Group (no. per group)	Vaccination	
	Bacterium	Dose (c.f.u. ml^{-1})
A (12)	BI (pBEX)	1.0×10^{10}
B (10)	BI-LTB	1.0×10^{10}
C (10)	BI-CfaB	1.0×10^{10}
D (10)	BI-LTB plus BI-CfaB	Each 1.0×10^{10}

Fig. 1. Administration of the ileal loop test in the different rat groups post-immunization. The first three loops were injected with 0.2 ml ETEC H10407 (containing 1.0×10^5 c.f.u. ml^{-1}) or 0.2 ml (250 μg) purified LT; an equal volume of PBS was injected in the fourth loop as negative control.

RESULTS

Vector and target proteins

Following construction of the new cloning vector pBEX, the target genes, *eltB* and *cfaB*, were ligated into the appropriate sites. After verification by sequencing, the plasmids were transformed into BI, and the expression of LTB and CfaB was confirmed by Western blotting assay (Fig. 2).

Serum and faecal antibody response after vaccination

The immune response due to each vaccine strain was tested in Sprague–Dawley rats. Serum and mucosal antibody levels were measured by ELISA. High levels of serum LTB-specific antibodies were observed on day 17 after the second booster immunization, with an end-point titre of 1 : 245 in group B. Serum CfaB-specific IgG levels increased following another booster dose on day 17 with an end-point titre of 1 : 200 in groups C and D. Both the LTB- and CfaB-specific IgG titres were greatly increased after the third booster and reached a higher titre on day 27

Fig. 2. Analysis of the proteins expressed in BI by SDS-PAGE (see Methods for details). Lanes 1 and 2 show CfaB bands and lane 4 shows the LTB band. Lane 3 was a negative control of LTB. Lanes M1 and M2 contain size markers. The Western blotting results are shown below the SDS-PAGE; see Methods for details. Pure LTB and CfaB were included as positive controls.

Table 2. Serum IgG levels in the different vaccinated groups

Means $\pm$ SE are shown. There were no significant differences in IgG titres between groups B and C; the titres of group D were significantly different from those of group C ($P < 0.05$).

Group	IgG titre (end point) on day:			
	0	10	17	27
A	0	0	0	0
B	0	9.2 $\pm$ 2.80	245.5 $\pm$ 52.22	772.7 $\pm$ 64.7
C	0	10 $\pm$ 0	200 $\pm$ 0	708.3 $\pm$ 50.6
D	0	300 $\pm$ 0	1433.3 $\pm$ 119.34	2950 $\pm$ 124.3

($P < 0.05$) in groups B and C. The serum CfaB-specific IgG titre in group D also reached a higher titre on day 27. Statistically, there was no significant difference in the IgG titre between groups B and C for the two vaccines immunized separately. From day 10 onwards, vaccination induced a significant increase in the IgG level in group D, which was co-immunized with the two vaccines ($P < 0.05$). That is, LTB expressed in BI contributed to increasing the CfaB-specific serum IgG level as adjuvant (Table 2).

Before day 17, the faecal IgA titre showed no significant difference between group C and group D. High levels of faecal anti-CfaB were observed on day 27, after the third booster dose. Similar to the serum IgG titre variation, the faecal IgA titre in group D was greater than that in group C. The results clearly indicate that LTB expressed in BI had a strong mucosal adjuvant activity in group D. Faecal samples from group D had high CfaB-specific IgA titres from day 10 onwards ($P < 0.05$). No such significant difference was observed in IgA titre between groups B and C (Table 3).

Protection assay

HIA analysis. The HIA results showed that the CfaB-specific antibody strongly reduced adherence of ETEC to erythrocytes via CFA/I fimbriae (Table 4). The CfaB-specific serum IgG titre in group D was fourfold higher than that in group C. Therefore, the elicitation of

Table 3. Faecal IgA levels in the different vaccinated groups

Means $\pm$ SE are shown. There were no significant differences in IgA titres between groups B and C; the titres of group D were significantly different from those of group C ($P < 0.05$).

Group	IgA titre (end point) on day:			
	0	10	17	27
A	0	0	0	0
B	0	3.3 $\pm$ 0.98	15.3 $\pm$ 5.61	93.1 $\pm$ 33.42
C	0	3.5 $\pm$ 2.36	16 $\pm$ 4.82	85.3 $\pm$ 31.53
D	0	8.5 $\pm$ 2.11	96 $\pm$ 33.42	362.7 $\pm$ 131.8

Fig. 3. Results of the ileal loop test in the different rat groups post-immunization. The ratio of fluid weight to length was determined after 18 h and defined as diarrhoea positive when the ratio was more than 0.16 mg cm^{-1} . Compared with the negative group (A), the vaccination protected the rats against LT-induced fluid accumulation in all three groups: there was a large significant difference in ileal fluid accumulation between group A and groups B, C and D ($P < 0.05$). No significant difference was observed between groups B, C and D.

antibodies was more effective in rats immunized with mixed vaccine (BI-CfaB plus BI-LTB) than in those vaccinated with BI-CfaB alone.

Ileal loop assay. The mean ratio of ileal fluid accumulation in the positive groups (B, C and D) was significantly less ($P < 0.05$) than that in the negative (group A). This indicated that both the BI-LTB and BI-CfaB vaccination gave the rats immunity against diarrhoea from ETEC LT (Fig. 3). There was no significant difference between groups B, C and D.

Lethal challenge analysis. First, we determined the mortality rate of the rats. In group A, we observed 50 % death on the first day and 100 % on the third day post-challenge. In group B, 50 % of the rats died on the second day and 100 % died over the subsequent 5 days. For group C the mortality rate was only 17 % on the sixth day. Most importantly, in group D, 100 % of the rats survived to the end of the experiment on day 9 (Fig. 4). The test for bacterial deletion showed that ETEC was deleted completely by 24 h post-challenge in groups C and D. However, for group B we could still detect ETEC when 1000-fold dilution of abdominal fluid was plated on LB agar (not shown). These

Fig. 4. Mortality rate of rats in different immune groups after peritoneal challenge. Six rats from each group were challenged with lethal doses (1.0 ml) of H10407 ($2.0 \times 10^{10} \text{ c.f.u. ml}^{-1}$) via peritoneal cavity injection. Co-immunization with BI-LTB and BI-CfaB elicited a more powerful protection, than immunization with BI-CfaB alone, while the BI-LTB immunized group showed no immunity against ETEC mucosal infection.

results confirmed that BI-LTB plus BI-CfaB vaccination is more effective than BI-CfaB alone.

DISCUSSION

Lactic acid bacteria have been used as a live oral vaccine expression system for their safety, weak immunogenicity and adjuvant properties (Bermúdez-Humarán, 2009; Hanniffy *et al.*, 2007; Liu *et al.*, 2009; Medina *et al.*, 2010; Wei *et al.*, 2010). As a specific lactic acid bacterium in the human intestinal tract, BI is an advantageous vehicle for mucosal vaccination purposes because it is considered safer than other live vaccine carriers, which have pathogenic variants (e.g. *Salmonella*, *Shigella* and *Listeria* spp.). BI can be an efficient system for delivery of pathogen antigens to the intestinal mucosa. Mucosal immunization is more convenient than other routes and can induce both local and systemic immune responses (Bermúdez-Humarán, 2009). BI, a probiotic, resides in the colon and plays an important role in regulating intestinal homeostasis and in inhibiting the growth of pathogenic micro-organisms in the intestine. Therefore, a BI-based oral vaccine has many potential benefits in comparison with other delivery systems (Barry *et al.*, 2003; López-Gigosos *et al.*, 2007; Pascual *et al.*, 1999).

Table 4. Haemagglutination inhibition levels of serum IgG in different vaccinated groups

Group	Haemagglutination inhibition titre (end point)						
	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400
C	+	+	–	–	–	–	–
D	+	+	+	+	–	–	–

In this study, in order to deliberately express the antigens CfaB and LTb outside the BI cell, we maintained the signal peptide coding sequences in their genes. However, there is a discrepancy in the SDS-PAGE data in that a large number of the CfaB and LTb proteins were detected in cell lysates as insoluble expression. No visible CfaB and LTb bands were detected in BI supernatant by SDS-PAGE. Nevertheless, the oral live vaccine elicited both systemic and mucosal immune responses in animal tests. So we infer that CfaB and LTb may be constantly secreted into the intestinal mucosa at low levels but sufficient to trigger an immune response. However, we do not discount the possibility that insoluble CfaB and LTb proteins are released after lysis of BI cells, thereby eliciting an immune response.

The antibodies from BI-CfaB-vaccinated rats reduced fluid accumulation to the same degree as the BI-LTb plus BI-CfaB co-immunization group. The ability of ETEC to adhere to and colonize the intestinal epithelium is a prerequisite for pathogenicity, in addition to the ability to produce LT and/or ST (Gaastra & Svennerholm, 1996). A previous study showed that enterotoxin production is not sufficient to enable ETEC to cause diarrhoea without CFA colonization (Lund *et al.*, 2000).

Another theory states that LT may be involved in promoting the adherence of ETEC to intestinal epithelial cells (Johnson *et al.*, 2009). However, LT does not alter the surface localization of host CFA receptors. We observed that rats vaccinated with BI-LTb were protected from pure LT-induced fluid accumulation, but were not immune from colonization of ETEC in the intraperitoneal cavity. Rats (groups C and D) immunized with BI-CfaB, or BI-CfaB plus BI-LTb, produced CfaB-specific antibodies, blocked ETEC adherence to intraperitoneal mucosa, and cleared ETEC infection. These data indicate that CFAs are important antigenic targets for BI-based ETEC oral vaccine development.

The major aim of ETEC live vaccines is to achieve colonization in the host. However, direct antigen delivery via the oral route generally leads to weak elicitation of an immune response (Lee *et al.*, 1997; Byrd & Cassels, 2006). BI resides in the colon, so a BI-based vaccine can improve the efficacy of oral vaccination for expressing the antigens on the mucosal surface. This strategy can be extended to the expression of other ETEC colonization factors such as CS3 and CS6 fimbriae, which are capable of protecting against infection with the most prevalent forms of ETEC. The BI-LTb vaccine strain is a significant achievement in the progression towards synthesizing a nontoxic and ready-to-use BI-based mucosal adjuvant. Our future study will confirm whether BI-LTb has a universal oral adjuvant property and can be combined with any kind of oral vaccine.

In summary, we have developed BI as an oral vaccine expression system. ETEC antigen proteins (CfaB and LTb) expressed in this vehicle can elicit both systemic and

mucosal immune responses. Therefore, a BI-based vaccine may be used in protecting against a variety of pathogens that invade the human body.

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