Curr Microbiol (2017) 74:854–862
DOI 10.1007/s00284-017-1259-1

Yeast Surface Display of *Escherichia coli* Enterotoxin and Its Effects of Intestinal Microflora and Mucosal Immunity

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Received: 16 March 2017 / Accepted: 26 April 2017 / Published online: 2 May 2017
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Abstract Enterotoxigenic *Escherichia coli* (ETEC) is a significant cause of infectious diarrhea in animals. In this study, yeast surface display technology was employed to investigate the effects of ETEC enterotoxin fusion protein on the intestinal flora and mucosal immunity of rats. ETEC *estA*, *estB*, and *eltAB* (heat-labile and heat-stable toxins) were expressed on the surface of yeast. Rats were divided into normal saline, yeast and display yeast groups. Fecal and jejunal content samples were collected on the 7th, 14th, and 21st days. Rats were then fed ETEC for 3 days before again collecting these samples. Levels of SIgA, IL-2, IL-4, IFN-γ, and microbial population density and diversity were documented by ELISA, T-RFLP and real-time PCR. The results demonstrated that *estA*, *estB*, and *eltAB* fusion proteins were expressed on the surface of yeast. Following ETEC challenge, levels of SIgA, IL-2, IL-4, IFN-γ, and, the numbers and variety of intestinal microbes were significantly increased in rats receiving display yeast and yeast. These factors were significantly decreased in rats given normal saline and yeast. Our results indicate that display yeast and yeast can increase the number and diversity of intestinal microbes in rats and improve intestinal immune function. After ETEC challenge, the display yeast can better maintain the balance of intestinal bacteria and mucosal immunity.

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Introduction

Diarrhea in animals may be acute or chronic, and may be caused by bacterial infection [18], viral infection, or other factors. The majority of cases occur in young animals, and the condition presents a serious threat to good animal husbandry [33]. Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of diarrhea in animals. It can produce several toxins, including heat-labile and heat-stable enterotoxins (LT and ST, respectively) [34], leading to water and electrolyte loss into the intestinal lumen through intestinal cell dysfunction, resulting in cholera-like watery diarrhea [23]. In recent years, excessive use of antibiotics has resulted in high concentrations of veterinary drug residues in animals and the surrounding environment, to the detriment of water and soil ecosystems [24]. With the emergence of resistant strains, traditional antibiotics often do not achieve their expected effect.

Microecologic therapy involves utilizing microbes such as yeast and bacteria to benefit the microecology of the gut without harm to the host [27]. Livestock and poultry microecologics as feed additives are now the focus of

research because of advantages such as increased beneficial gut bacteria, reduction in pathogenic bacteria, non-toxic side effects (as these are non-chemical agents), and absence of drug residues and drug tolerance [25]. To date, microecologics have been applied in various fields including nutrition, agriculture, medicine and health care, and food production [16]. Lactic acid-producing bacteria and yeast are the most commonly employed microecologics in scientific experiments, for example in surface display technology. Yeast cell surface display technology is a eukaryotic expression system which uses a signal peptide to guide and then anchor a foreign target protein to the yeast cell surface [26]. *S. cerevisiae* is a commonly used display carrier. This technology has the characteristics of small expression error, variety of presentation proteins, large number of molecules, and ease of screening, purifying, and activity determination [19]. This technology has already been applied in many research fields, and has broad application prospects. Therefore, developing microecologics with good safety and high immunogenicity has become a hot research topic. ST is a small-molecule protein, often used for vaccine development because of its good immunogenicity [15]. LT is often used as an adjuvant to stimulate mucosal immunity by virtue of its large molecular weight and strong immunogenicity [30].

Materials and Methods

Yeast Strains and Culture

S. cerevisiae EBY100 strain was purchased from Invitrogen Life Technologies. ETEC H10407 and genetically engineered *E. coli* DH5α were cultured in LB medium (0.5% yeast extract, 1.0% peptone, 1.0% NaCl, optional 1.5% agar). Resistant media and plates (LB with 0.1% ampicillin, optional 1.5% agar) were used to culture the recombinant *E. coli* strain. EBY100 yeast was cultured in YPD medium (2.0% peptone, 1.0% yeast extract, 2.0% glucose). YNB medium plates (0.67% YNB, 2.0% glucose, 0.01% leucine, 0.01% tryptophan, optional 1.5% agar) were used to screen EBY100 S. *cerevisiae* cells containing the pYD1 plasmid. YNB-CAA medium (0.67% YNB, 2.0% galactose, 0.5% casamino acids) was used to induce the expression of the target protein on the EBY100 yeast cell surface.

DNA Manipulation

Ex Taq DNA Polymerase (TaKaRa Biotechnology Co., Ltd, Changchun, China) was used to generate polymerase chain reaction (PCR) products for cloning and gene insertion, and Reddy Mix (Thermo Fisher Scientific, Beijing, China) was used to screen colonies by PCR, according to the standard PCR protocols. pYD1 Yeast Display Vector Kit (Invitrogen Life Technologies Co., Ltd.), restriction enzymes (Takara Biotechnology Co., Ltd.), T4 DNA ligase (TaKaRa Biotechnology Co., Ltd.), and PCR Purification Clean-Up Kit (TaKaRa Biotechnology Co., Ltd.) were used according to the manufacturers' instructions. Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse and FITC-labeled goat anti-rabbit fluorescent IgG antibodies (Kangwei Biotechnology Co., Ltd, Beijing, China) and anti-V5 antibody (Shifeng Biotechnology Co., Ltd, Shanghai, China) were used to screen display proteins by detection of immunofluorescence.

Primers

Primers for this study are shown in Table 1.

Amplification of *estA*, *eltAB*, and *estB* Genes from ETEC H10407

Genomic DNA of ETEC H10407 was prepared according to the methods described by Sambrook et al. [21]. Primers are listed in Table 1. The PCR procedure was as follows: initial denaturation at 94 °C for 10 min, followed by 30 cycles of 1 min at 94 °C; 30 s at the appropriate temperature for *estA, eltAB, and estB* (48, 45, 53 °C, respectively); a 72 °C extension period (estA: 20 s, eltAB: 70 s, estB: 105 s), followed by an additional 1 min at 72 °C. The resultant 218, 1148, and 1861 bp PCR products were prepared by the following process. *estA* and *eltAB* genes were transformed by PCR in order to reduce target protein toxicity. TGT and TGC (Cys) at the 3′ end of the *estA* gene were mutated to encode serine residues AGT and AGC (Ser), AGA (Arg) at position 192 of eltAB was mutated to GGA (Gly), and AAG (Ser) at position 63 was mutated to TCT (Lys) [17]. Transformed *estA, eltAB*, and original *estB* genes were linked by a seven amino acid linker (Asp-Pro-Arg-Val-Pro-Ser-Ser) [7]. The *estA-eltAB-estB* gene was cloned into the pYD1 vector.

Transformation of Yeast Strain

pYD1-estA-eltAB-estB and pYD1 were transformed into competent cells according to the manufacturer instructions for the pYD1 Yeast Display Vector Kit. The transformants were grown in selective YNB-CAA medium at 30 °C for 48 h. The derived transformants containing pYD1/estA-eltAB-estB and pYD1 were confirmed by colony PCR technique and named EBY100/pYD1-estA-eltAB-estB and EBY100/pYD1.

Table 1 Primers used in this study

Gene	Primer	Product length
eltAB	F:5′- ATGAAAAATATAACTTTCAT -3′	1148 bp
	R:5′-CTAGTTTTCCATACTGATTG-3′	
estB	F:5′-ATGGTGGCAATCATTGGAGC-3′	1836 bp
	R:5′-TTCGCTGGCCAGTGAAATAA-3′	
estA	F:5′-ATGAAAAAATCAATATTATT-3′	216 bp
	R:5′-ATAGCACCCGGTACAAGCAG-3′	
estA mutation	F:5́-CGGGGTACCATGAAAAAATCAATATTATTTA-3́	216 bp
	R:5́-AGATGGAACACGTGGATCATAGCTCCCGGTACTAGC-3́	
estB mutation	F:5́-TCCACGTGTTCCATCTTCTATGGTGGCAATCATTGGAGC-3	1836 bp
	R:5́-CCGCTCGAGTTCGCTGGCCAGTGAAATAA-3́	
eltAB+linker	F:5′-TCCACGTGTTCCATCTTCTATGAAAAATATAACTTTCATTT-3′	1170 bp
	R:5′-CCGCTCGAGCTAGTTTTCCATACTGATTGC -3′	
estA+linker	F:5′-CGGGGTACCATGAAAAAATCAATATTATTA-3′	240 bp
	R:5′-AGATGGAACACGTGGATCATAGCTC CCGGTACTAGC-3′	
estB+linker	F:5′-GCTACTAGGGACTCTCGGGTCCTAGTTTTCCATACTGATTG -3′	1858 bp
	5′-CCGCTCGAGTTCGCTGGCCAGTGAAATAA-3′	
63 mutation	F:5′-GACGGATATGTTTCCACTAAGCTTAGTTTGAGAAGTGCT -3′	189 bp
	R:5′-AGCACTTCTCAAACTAAGCTTAGTGGAAACATATCCGTC -3′	
192 mutation	F:5′-GTGGAAATTCATCAGGAACAATTACAGGTG -3′	576 bp
	R:5′-CACCTGTAATTGTTCCTGATGAATTTCCAC -3′	
	R:5′-CCGCTCGAGTTCGCTGGCCAGTGAAATAA -3′	
Eubacteria	F:5′-CCTACGGGAGGCAGCAG-3′	189 bp
	R:5′-ATTACCGCGGCTGCTGG-3′	
Bacteroides	F:5′-GAAGGTCCCCCACATTG-3′	550 bp
	R:5′-CAATCGGAGTTCTTCGTG-3′	
Fusobacterium	F:5′-CGCAGAAGGTGAAAGTCCTGTAT-3′	100 bp
	R:5′-TGGTCCTCACTGATTCACACAGA-3′	
Bifidobacterium	F:5′-GCGTGCTTAACACATGCAAGTC-3′	117 bp
	R:5′- CACCCGTTTCCAGGAGCTATT-3′	
Lactobacilli	F:5′- CACCGCTACACATGGAG-3′	341 bp
	R:5′- AGCAGTAGGGAATCTTCA-3′	
Enterococcu	F:5′- CCCTTATTGTTAGTTGCCATCATT-3′	140 bp
	R:5′- ACTCGTTGTACTTCCCATTGT-3′	
IL-2	F:5′- CTCGGAGCTCTGCAGCGTGT-3′	164 bp
	R:5′- TCCACCACAGTTGCTGGCTCATC-3′	
IL-4	F:5′- CCACGGAGAACGAGCTCATC-3′	101 bp
	R:5′- GAGAACCCCAGACTTGTTCTTCA-3′	
IFN-γ	F:5′- ACAACCCACAGATCCAGC-3′	103 bp
	R:5′- TCAGC ACCG ACTCCTTTT-3′	

Expression of *estA-eltAB-estB* Gene and Bioassay of Surface-Displayed Protein

Gene expression in EBY100/pYD1-estA-eltAB-estB and hemolysin display on the yeast cells were carried out according to the pYD1 Yeast Display Vector Kit manufacturer's instructions. The cells were induced by growing the cells in YNB-CAA medium for 0, 12, 24, 36, and 48 h.

Immunofluorescence Microscopy Assays

Immunostaining was performed as follows. 100% acetone was used to immobilize *S.* cerevisiae cells at 37 °C for 15 min. Anti-V5-FITC antibody was used as the primary antibody at a dilution rate of 1:500. Cells and the antibody were then incubated at 37 °C for 2 h. After the cells had been washed with PBS, a second antibody, FITC-

conjugated goat anti-mouse IgG, was diluted at 1:600, and reacted with the cells at 37 °C for 1.5 h. After washing with PBS, the cells were observed with a fluorescence microscope.

Scanning Electron Microscopy

Yeast scanning was conducted as described by Balikoglu et al. [4].

Animals and Sampling

Six-week-old Sprague–Dawley (SD) rats, purchased from the Jilin University Animal Center (Jilin, China), were housed in stainless steel wire cages (three or four per cage) with a 12 h light/dark cycle. Rats were allowed access to laboratory rodent chow and water ad libitum. After 1 week of acclimatization, all rats were randomly assigned to one of three groups; control group ($n = 24$), display group ($n = 12$), or yeast group ($n = 12$). The rats were gavaged with normal saline, 10^7 cfu/mL display yeast liquid, and 10^7 cfu/mL S. *cerevisiae*, 2 mL/rat, once a day for 21 days. After 21 days they were gavaged with 10^8 cfu/mL *E. coli* H10407 5 mL/rat for 3 days. Rats were euthanized after 7, 14, and 21 days, and 3 days after ETEC challenge. Feces and jejunal tissue were collected from each group, labeled and stored at −80 °C.

SIgA Assay

SIgA assay was performed using a Rat SIgA ELISA Kit (Lengton Bioscience Co., LTD, shanghai, China), according to the manufacturer's protocol for intestinal tissues.

Microbial Composition

Feces was assessed using terminal restriction fragment length polymorphism (T-RFLP) analysis as described by Sepehri et al. [22].

Real-Time PCR

Real-time was performed as described by Wang et al. [28]. Primers used for this study are listed in Table 1. The $2^{-\Delta\Delta C_T}$ method was used to normalize the data [20].

Statistical Analysis

Results were expressed as mean ± standard deviation (SD) and analyzed using SPSS 20.0 software (SPSS Incorporated, Chicago, IL, USA). Group differences were compared with Student's *t* test. *P*-values of 0.05 were considered statistically significant, and those of 0.01 and below were considered highly statistically significant.

Results

Cloning of *estA, eltAB, estB* gene from ETEC H10407, estA-eltAB-estB gene was obtained by overlap PCR. The agarose gel result is shown in Fig. 1.

Transformation of Yeast Strain

Plasmid was extracted from the EBY100/pYD1-estA-eltAB-estB display strain (Fig. 2). The similarity between the sequencing results and those referenced in GenBank was 100%, demonstrating that pYD1-estA-eltAB-estB was transformed into the EBY100 yeast cell.

Immunofluorescence Microscopy Assays

The results of immunofluorescence showed that EBY100/pYD1-estA-eltAB-estB could be detected as fluorescence on the cell surface, indicating that the target protein was successfully displayed on the surface of S. *cerevisiae*. However, neither the EBY100/pYD1 nor EBY100 strains fluoresced (Fig. 3).

Scanning Electron Microscopy Results

Scanning electron microscopy demonstrated that the EBY100 yeast surface was smooth, but that yeast surfaces expressing estA-eltAB-estB fusion protein had many protuberances (Fig. 4).

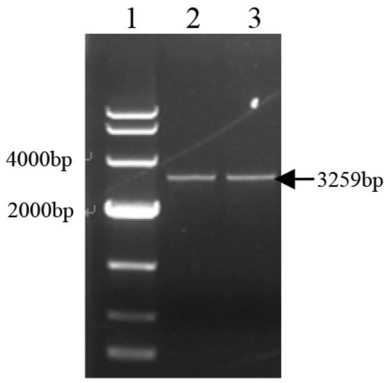

Fig. 1 Agarose gel results of *estA-eltAB-estB* gene overlap PCR. *Lane 1* is the DNA marker of 10,000 bp. *Lane 2–3* are the results for *estA-eltAB-estB* gene overlap PCR

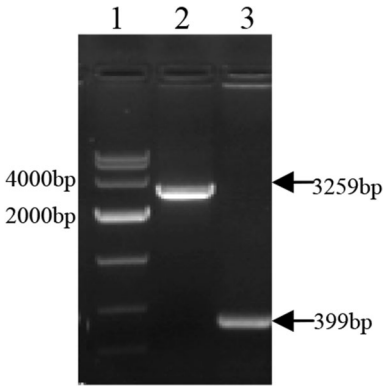

Fig. 2 Agarose gel results of recombinant plasmid PCR. *Lane 1* is the DNA marker of 10,000 bp. *Lane 2* is the result for recombinant plasmid PCR. *Lane 3* is the result for pYD1 plasmid PCR

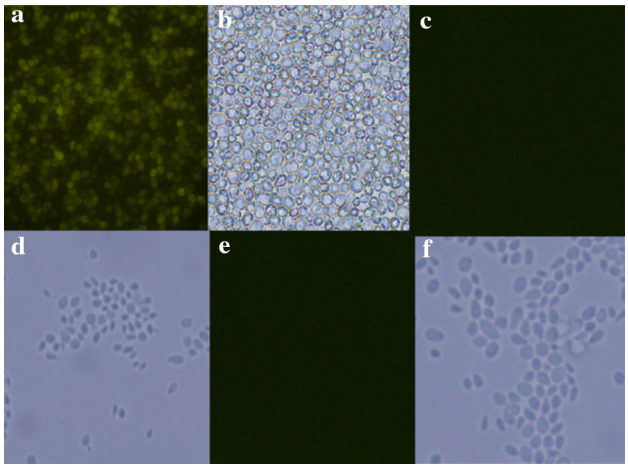

Fig. 3 The results of immunofluorescence. (10 × 40) **a** EBY100/pYD1-estA-eltAB-estB display yeast under light fluorescence. **b** EBY100/pYD1-estA-eltAB-estB display yeast under light microscopy. **c** EBY100/pYD1 *S. cerevisiae* under fluorescence. **d** EBY100/pYD1 *S. cerevisiae* under light microscopy. **e** EBY100 *S. cerevisiae* under fluorescence. **f** EBY100 yeast under light microscopy

SIgA Assay

Compared with the control group, the SIgA level of the EBY100 and display yeast groups were significantly increased in the first 3 weeks. Following ETEC challenge, the SIgA levels of all the groups decreased, but were significantly higher in the EBY100 and display yeast groups than in the control group (Fig. 5).

Real-Time PCR Assay for Intestinal Mucosal Cytokines

There were no significant differences between the IL-2, IL-4, and IFN-γ levels of any of the groups at the beginning of the experiment. After 3 weeks, compared with the control group, the display yeast group and yeast group

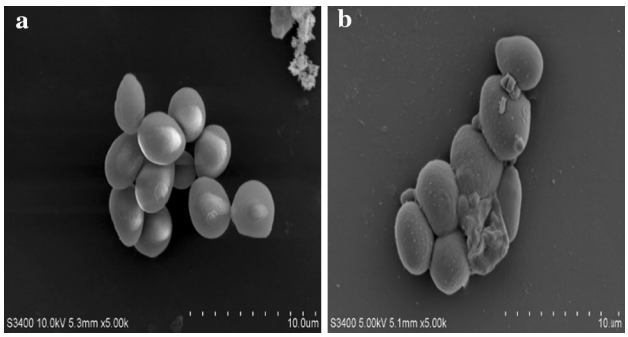

Fig. 4 Scanning electron microscopy. **a** EBY100 yeast, **b** estA, estB, and eltAB fusion protein expressed on the surface of EBY100 yeast

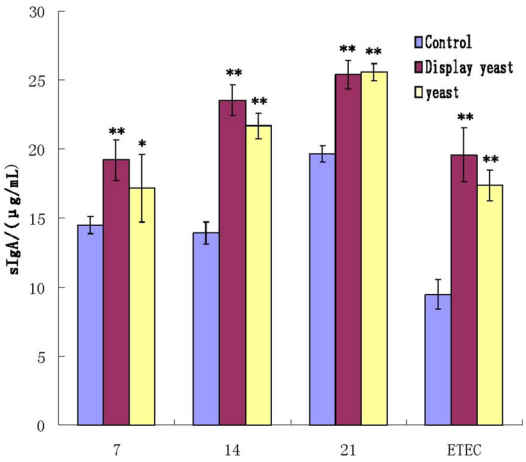

Fig. 5 Levels of ileal intestinal SIgA. The * sign indicates statistical significance at a *P* value of <0.05; ** represents $P < 0.01$. *Error bars* show standard errors (Color figure online)

demonstrated significantly increased levels of all three factors ($P < 0.01$). Following ETEC challenge, the levels of IL-2 and IL-4 were significantly decreased in the control group, while IFN-γ was significantly increased. These three factors were not significantly altered in display yeast and yeast groups (Fig. 6).

Real-Time PCR Assay of Bacteria

After 3 weeks, intestinal microbial quantity in display yeast and yeast groups was increased significantly compared with control group. After ETEC challenge, the number of intestinal flora in the control group was decreased significantly, while in the display yeast and yeast groups, it was not significantly changed (Fig. 7).

T-RFLP Analysis

PCR products with a fragment size of approximately 400 bp were obtained (Fig. 8). The OTU and statistical

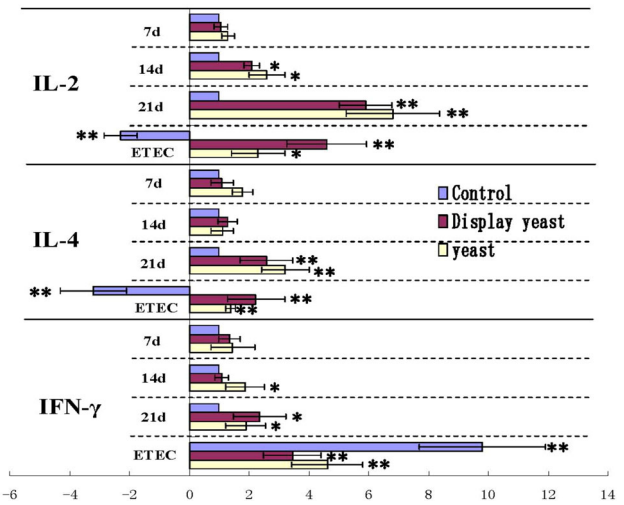

Fig. 6 Results of real-time PCR for intestinal mucosal cytokines. The * sign indicates statistical significance at a P value of <0.05; ** represents $P < 0.01$. *Error bars* show standard errors (Color figure online)

analysis showed that the control group had 37 OTU, the display yeast group 45 OTU, and the yeast group had 44 OTU after 3 weeks. Following ETEC challenge, the control group had 28 OTU, the display yeast group had 43, and the yeast group had 36 (Fig. 9).

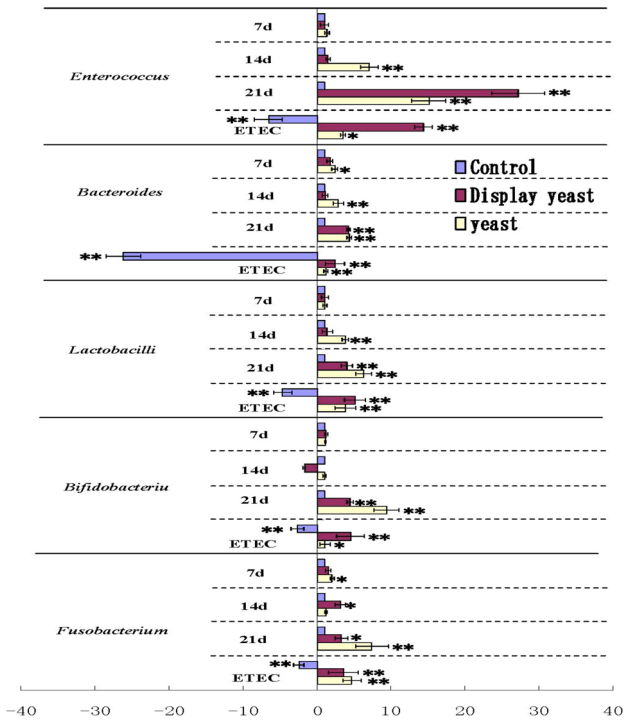

Fig. 7 Results of real-time PCR for bacteria. The * sign indicates statistical significance at a P value of <0.05; ** represents $P < 0.01$. *Error bars* show standard errors (Color figure online)

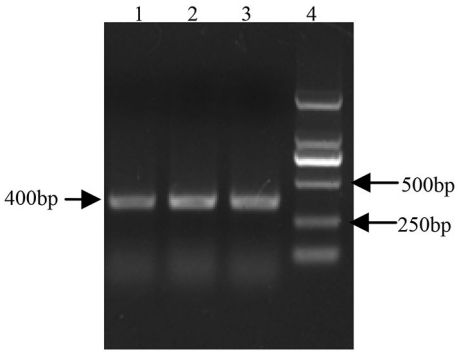

Fig. 8 Agarose gel result for PCR with universal primers. *Lanes 1–3* represent PCR products. *Lane 4* is the 2000 bp DNA marker

Discussion

ETEC, which can produce two types of enterotoxins, LT and ST, is a major cause of diarrhea in animal [34], resulting in cholera-like watery diarrhea [23]. Mature ST is a small peptide consisting of 18 or 19 amino acids, with strong toxic activity and poor immunogenicity [13]. LT exhibits good immunogenicity [11], and is often used as an immunogenic adjuvant in vaccine production [14]; however, its toxicity poses a potential threat to human and animal health. Therefore, we proposed to use yeast surface display technology to express ST-LT fusion enterotoxin in order to prevent diarrhea in our test animals.

In this study, the *estA, estB, and eltAB* genes of ETEC were cloned by PCR. Naturally occurring enterotoxins have strong toxic effects; we therefore must reduce the toxicity of these proteins before using them experimentally. The three intramolecular disulfide bonds of ST are closely related to its toxicity, which is reduced by a factor of at least 250 by the loss of one of these bonds, and may be

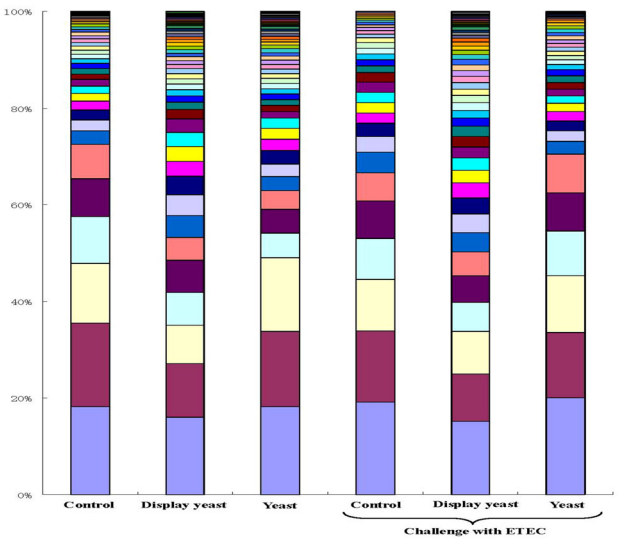

Fig. 9 Diversification results of the OTUs (Color figure online)

completely abolished by the destruction of two [2]. Therefore, when the fusion gene was constructed, we mutated the cysteine (Cys) residues, TGT and TGC, of the *estA* gene into the serine (Ser) residues AGT and AGC, using site-directed mutagenesis to remove ST toxicity. LT toxicity is closely related to ADP-ribosyltransferase subunit activity. Based on this fact, researchers have created many varieties of attenuated LT, including LTG63 and LTG192. This study combined the above two mutant forms and mutation sites to minimize LT toxicity, with results similar to those previously reported.

Yeast cells, being large eukaryotes, are easily recognized by the immune system, so we used EBY100 as the carrier for the surface display system to display smaller molecular weight proteins. The small STa and STb protein molecules are weakly immunogenic, but by linking to LT with a flexible linker (Asp-Pro-Arg-Val-Pro-Ser-Ser) to form the fusion gene expressed on the yeast cell surface, we increased their affinity for antibody binding.

SIgA is the major mucosal immunoglobulin and is an important component of the intestinal immune barrier [29]. Perdigon et al. found that lactic acid-producing bacteria enhanced SIgA-producing phagocytic activity and specific immune responses [10]. Studies have shown that microecologics have non-specific immunomodulatory effects [12], and enhance non-specific intestinal immune function. SIgA, which accounts for 80% of all mucosal tissue-generated antibodies, is expressed on the surface of the intestinal mucosa in response to stimulation from the normal intestinal flora. In addition, IFN-γ, IFN-α, IL-1, IL-2, and other cytokines promote the activation and proliferation of mucosal lymphoid B cells, and enhance secretion of antigen-specific SIgA to improve intestinal immunity [10]. Our results demonstrate that both display and non-display yeast can act as probiotics to stimulate intestinal lymphocytes, thereby increasing the level of SIgA. However, the response of the display yeast group was much higher than that of the yeast group. The reason may be that display yeast not only exert a probiotic effect, but the immunogenic surface-displayed protein as an antigen may stimulate the intestinal specific immune response to increase SIgA secretion. After being administered as a challenge, ETEC adhered to small intestinal epithelial cells and secreted enterotoxin, leading to epithelial cell osmotic pressure changes and hence the occurrence of acute diarrhea. ETEC greatly damaged the intestinal environment, caused intestinal flora imbalance, and decreased both cytokine secretion and the number of microecologics present. This scenario of weakened intestinal immune capacity was more conducive to the growth of ETEC. Our results demonstrate that the levels of SIgA in the control and yeast groups were significantly reduced after ETEC challenge, but levels in the display group were not significantly changed. This result shows that display yeast not only act as intestinal microecologics, but also reduce ETEC-induced damage by occupying enterotoxin-binding sites.

Cytokines are polypeptides or proteins secreted by immunologically active cells and other cells with a variety of biological activities [31]. They are involved in the transmission of information and play an important role in vital biological processes of the organism [8]. IL-2 (secreted by Th1 lymphocytes) promotes T cell, B cell, and NK cell proliferation and differentiation, and promotes antibody production, enhancing the body's cellular immune response [5]. Studies have shown that intestinal bacteria can affect the level of IL-2 secretion [6]. IL-4 is secreted by Th2 cells and is responsible for their characteristic activities. It inhibits cytokine production by Th1 cells and performs other auxiliary functions, promoting the proliferation and differentiation of B cells and increasing the secretion of SIgA [1]. IFN-γ can enhance the ability of cytotoxic T cells and NK cells to kill target cells and induces cells to express IL-2 receptors, promoting T cell proliferation. Our results showed that the yeast effectively promoted the secretion of IL-2, IL-4, and IFN-γ and improved mucosal immune function. After ETEC challenge, the levels of IL-2 and IL-4 in the display yeast group were not significantly changed, demonstrating that display yeast could effectively reduce ETEC-induced intestinal mucosal damage. However, the levels of IFN-γ in the control and yeast groups were significantly increased. One possible explanation for this may be the high concentration of ETEC introduced into the body, stimulating T cells and NK cells to secrete large amounts of IFN-γ. This disturbance to the Th1/Th2 balance has adversely affected intestinal mucosal immune function [9].

Approximately, 400 species of bacteria constitute the normal intestinal flora, with *Bifidobacterium*, *Lactobacillus*, *Enterobacter*, and *Enterococcus predominating* [32]. Yeast and other microecologics have the potential to adhere to host intestinal epithelial cells and play a vital role in preventing colonization by pathogens [3]. Our study showed that numbers of the five dominant intestinal bacteria were significantly increased in the display yeast and yeast groups after 3 weeks of administration. This is not an immediate effect; it takes some time for the yeast to regulate the intestinal flora. Intestinal bacterial numbers began to increase significantly on the 14th day, representing the prebiotic effect of the yeast. After the ETEC challenge, the intestinal microflora population was significantly reduced in the control and yeast groups, but display group was not significantly changed. This proves that the display yeast not only acted as a microecologic to increase intestinal flora numbers, but also maintained the balance of the intestinal flora when challenged by ETEC. T-RFLP and real-time PCR yielded similar results. OTU values in the display

yeast and yeast groups were greater than in the control group after 3 weeks. The OTU values of both the control and yeast groups decreased significantly after challenged.

In conclusion, yeast surface display technology can accurately express estA-eltAB-estB and other exogenous genes on yeast surfaces. Both display yeast and yeast can increase the number and variety of intestinal flora in rats and improve intestinal immune function. In the face of challenge with ETEC, the display yeast can better maintain the balance of intestinal bacteria and mucosal immunity.

Acknowledgement I thank Xiaoxu Wang, Zhou sha, and Xinglong Wang for excellent technical assistance. This work was supported by National key R & D plan [Grant Numbers: 2016YFD0501004].

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