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An Effective Cloning, Heterologous Expression and Physiological Activity in *Lactococcus lactis* NZ9000 of Catalase Gene from *Escherichia coli* DH5α

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Authors' contributions

This work was carried out in collaboration among all authors. Author Chongbi L designed the study, performed the analysis of the sequence and completed the whole manuscript writing. Author SL completed expression and identification of the recombinant L. lactis. Author Chen L completed cloning and activity examination. Author YL completed sequencing and analysis. Author GF completed detection of physiological activity and statistical analysis of the recombinant catalase. All authors read and approved the final manuscript.

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ABSTRACT

In order to clone active Catalase gene from bacteria, we introduced a method of shotgun integrating specific screen from *Escherichia coli* DH 5α. Genome DNA was extracted from *E. coli* DH5 and partially digested with Sau3AI. Then some fragments more than 2.26kb were collected and ligated with T4 DNA ligase into the BamHI-cleaved plasmid pUC18 and transformed competent *E. coli* TG1 cells. The transforments were incubated anaerobically on brain heart infusion (BHI) containing tannic acid. The method could detect catalase activity and screen the catalase-positive clones. The

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catalase protein can be identified by SDS-PAGE. The results showed that the recombinant plasmid pUC18-kat was constructed successfully by PCR identification and restriction enzyme digestion. Opening the reading frame from DNA Sequence DH5a katE, the sequence length is 2262nt and coding protein are 753 Amino Acids, Molecular Weight is 84198.72 Daltons. This method is simple, and possess of popularization and application value. It would lay a foundation for cloning an active catalase later. Further, in this study, the fragment of 2,262 bp catalase gene katE was cloned into the expression vector pQE30 and transformed into *Escherichia coli* M15, and KatE protein was expressed after the induction with Isopropylthio-β-D-galactoside. The KatE protein was separated on SDS-PAGE and recovered using a His-tag affinity. New Zealand white rabbits were immunized with the purified protein to harvest polyclonal antibodies. Since *L. lactis* has no catalase, katE was inserted into *Escherichia coli - L. lactis* shuttle vector pMG36e and electro-transformed into *L. lactis* NZ9000. The expression of the KatE protein was confirmed by SDS-PAGE analysis and Western blot. Further experiment demonstrated that the expression of the KatE gene in *L. lactis* NZ9000 is able to produce active catalase that can provide efficient antioxidant activity. Additionally, to understand the import of catalase katE gene of *Lactococcus lactis* on the body's physiological changes of immune function in mice, Enzyme-linked immunoassay (ELISA) was used on the blood of mice, to measure IgG, IgE, CD4 and CD8 levels, and find out whether there is a difference between Mice Groups eating the recombinant *L. lactis* NZ9000 and other groups i.e. (recombinant *E. coli* DH5α and *L. lactis* NZ9000; *E. coli* DH5α and saline). The experiments showed that recombinant *L. lactis* NZ9000 was significantly higher than the other on IgG concentrations; IgE, CD4 or CD8 levels are not significant. Mice with increased IgG levels of IgG after the intake of recombinant *L. lactis* NZ9000 helps to explain the recombinant *L. lactis* NZ9000 regulatory role in humoral immunity of mice; IgE level did not change, CD4 and CD8 levels were also not changed, thus suggesting that there is no significant effect of cellular immunity on the body in a short time.

Keywords: Lactococcus lactis NZ9000; screening; catalase-positive; transforments; Immunity.

1. INTRODUCTION

Lactococcus lactis (*L. lactis*) is the model organism of lactic acid bacteria which are widely used for the dairy industry and other food fermentations but also for an increasing number of biotechnological applications. During industrial processes, this bacterium has to cope with various stresses including low pH, high temperature, osmotic shock, metal stress, acidity, and oxygen (O2) as well as carbon limitation [1-2].

Catalases are antioxidant metalloenzymes that divide H_2O_2 into water (H_2O) and O_2 [3]. Therefore, in microbiology, the catalase test is also used to differentiate between bacterial species in the lab (Percy, 1984). Catalase is a very highly conserved enzyme that is ubiquitous in bacteria, fungi, plants and animals. Bacterial catalases are widespread in aerobes such as *Escherichia coli* (*E. coli*). And Catalases are classified into three major families including Mn-catalases, catalase-peroxidases and monofunctional catalases [4]).

Catalase is a tetramer of four polypeptide chains, each over 500 amino acids long [5]. The optimum pH for human catalase is approximately 7. Almost all aerobic microorganisms use catalase. It is also present in some anaerobic microorganisms, They are widely used in food either alone in milk pasteurization and cheese production or its combination with glucose oxidase in the production of dietetic beverages [6-9].

It was shown in a study that gallate decarboxylase activity was elevated in the presence of tannic acid in *S. gallolyticus* (Chung K T et al. 1998). This shows that some enzyme activity could make the organism have a selective advantage over *S. bovis* when grown in the presence of tannins [10].

At present, many methods are used to clone genes. Such as those obtained from genome bank, PCR, shotgun, RT-PCR and so on [11]. To clone the active catalase from bacteria, an effective approach was assayed [12]. In this study, we cloned the active catalase of *E. coli* DH5a. We used methods including cloning, vector constructed, sequencing, and expression. We also screened the selected activity of catalase by the presence of tannic acid in recombinant strains in vitro. Additionally, to understand the import of catalase katE gene of *Lactococcus lactis* on the body's physiological

changes of immune function in mice, the levels of IgG, IgE antibody and CD4 and CD8 cells in the blood of experimental animals were examined by enzyme-linked immunoassay (ELISA) for laying the foundation of further study of recombinant *L. lactis*.

2. MATERIALS AND METHODS

A. DNA manipulations and plasmid construction.

2.1 Cloning and Transformation

In order to clone the catalase gene, total genomic DNA was isolated and purified with DNA purfing kit (Qiagen). Then it was partially digested with Sau3AI, and the DNA fragments of 1.5 to 2.5, 2.5 to 3.5, 3.5 to 5, and 5 to 7 kb were purified from the agarose gel using the QIAEX II kit (Qiagen). DNA fragments ligated with T4 DNA ligase (Qiagen) into the BamHI-cleaved plasmid pBluescript SK+/− were used to transform electrocompetent *E. coli* Top 10 cells [11]. The gene library was constructed to about 1,500 clones. The transformants were screened for catalase activity on brain heart infusion (BHI) containing tannic acid (BHI-tannic acid medium) and ampicillin (150 μg/ml), which is the same medium used for screening for tannase activity [12]. This medium was used as a plate-screening assay which could detect catalase activity when cloning into catalase-positive bacteria such as *E. coli*. First, the plates were incubated anaerobically for 2 days to allow the colonies to grow and to prevent darkening of the medium; the plates were then kept aerobically for a further 24 h at 37°C to induce the catalase gene [13]. A zone of clearing surrounding the colonies which developed during aerobic incubation indicated tannic acid degradation as a result of catalase activity. Plasmid DNA of one clone was isolated using the Midiprep plasmid purification kit (Qiagen Cat. Nos: 130708), and the insert was sequenced bi-directionally at GATC Biotech (Konstanz, Germany) using M13 universal oligonucleotide primers and custom primers. Plasmid DNA (pMD18-T-kat) exhibiting presumptive catalase activity on BHI-tannic acid medium was shown to harbor a 2.26kb DNA fragment insert. The expression vector (pET28a (+)) was constructed as followed.

E. coli was transformed by the method of electroporation [14] which was pre-digested with SalI and XbaI restriction enzymes. After ligation, the reaction mixture was employed to transform DH5α competent cells, and the recombinant plasmid having a size larger than pET28a was then screened out. It was confirmed that the cloned DNA fragment of 2.2 kb was the katE gene and the construction of expression plasmid pET 20b/katE had thus been accomplished.

2.2 Amplification by PCR and Oligonucleotide Synthesis

PCRs were performed with a Perkin-Elmer Cetus (Norwalk, Conn.) apparatus using Taq DNA polymerase (Promega) as recommended by the manufacturer. Oligonucleotides were synthesized with a DNA synthesizer (Applied Biosystems, San Jose, Calif.). A 2262-bp fragment was PCR amplified from the pUCkat matrix.

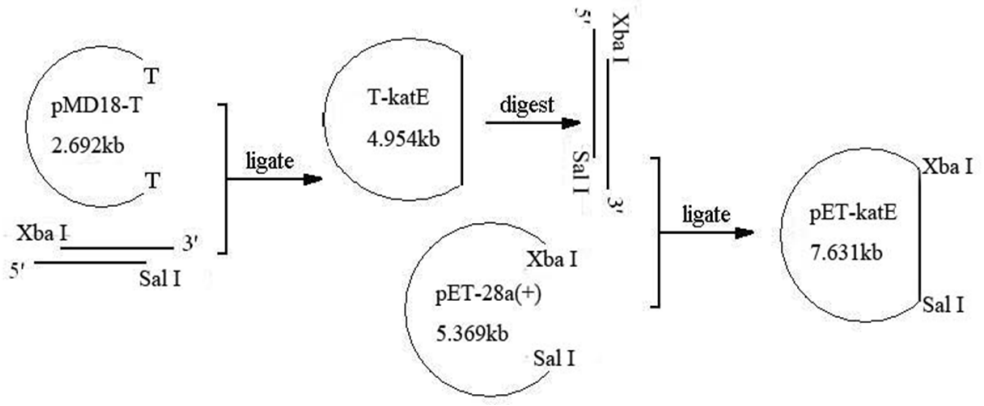

Fig. 1. Construction of recombinant plasmid pET-katE

The oligonucleotides used were XbaI-K-UP：5'-TCTAGAATGTCGCAAKATAACGAAAAGAACCCAKATCAGC-3' (oligo 1) for the coding strand and SalI-K-Down：5'-GTCGACTTACGCCGGGATTTTGTCAATCTTAGGAATGCGTG-3' (oligo 2) for the complementary strand.

The reaction conditions 94 degree Celsius for 3 min, then 30 cycles of 94 degree Celsius for 0.5 min, 50 degree Celsius for 50 sec, 72 degree Celsius for 1 min, followed by a final extension of 5 min at 72 degree Celsius. The amplified fragment was purified using the QIA quick PCR purification Kit (QIAGEN Cat Nos: 130708), and digested with EcoR I and Not I. The resulting fragment was respectively ligated into the contained T7 promoter plasmid pET28 digested with EcoR I and Not I. This DNA fragment was then cloned on pET28 vector in *E. coli* TG1, resulting in pET28 kat. Then katE was also inserted into *Escherichia col*i - *L. lactis* shuttle vector pMG36e and electro-transformed into *L. lactis* NZ9000.

2.3 Protein Detection of Catalase

In order to assay the production as well as the expression of catalase induced by IPTG in *E. coli* BL21 (DE3) cells, the cell extracts by ultrasonic crashing and supernatants of the different *E. coli* recombinant clones were examined by SDS-PAGE [13,15]. Preparation of cellular and supernatant protein fractions of *L. lactis* was confirmed by SDS-PAGE analysis and Western blot. For fractionation between cell and supernatant fractions, 2 ml samples of nisin-induced *L. lactis* cultures were centrifuged for 5 min at 6000 g at 4 degree Celsius Protein extracts were then prepared as previously described [16]. Further experiment demonstrated that the expression of the KatE gene in *L. lactis* NZ9000 is able to produce active catalase that can provide efficient antioxidant activity.

2.4 Recombinant Protein Expression

The recombinant catalase plasmid was placed into *Lactococcus lactis* NZ9000 competent cells by electrophore transformation, at 30 degrees celsius after being cultured for 24 hours, then a single colony of bacteria was picked out in fresh GM17 medium for incubation. When A600 is up to 0.5, the inducer nisin (shanghai 1414-45-5) was added to a final concentration of 40 ug/L, with the strains without induction as a control, after incubation for 4h, the protein were detected by SDS-PAGE.

2.5 Bacterial Cultivation and the Sample Prepared

Microbial strains of recombinant *Lactococcus lactis* and normal NZ 9000 were obtained from the lab of Dr. Li (Central of biopharmaceutical engineering in Zhaoqing University, China). They were cultured in 100 mL of GM17 broth overnight at 30 degree Celsius. In order to induce recombinant *Lactococcus lactis* producing exo-protein a compound nisin was added as a promoter was used to stimulate for 4 h. The bacteria count was done by plate count method under a microscope. The bacterial cells were centrifuged for 10 min at 3,000×g and washed twice with the sterilized PBS (pH 7.4). Finally, bacteria were resuspended in GM17 medium (pH 7.4) under gentle vortex mixing. This preparation was made for oral consumption of the experimental mice.

2.6 Sequence Alignment and Analysis of the Kat Gene

The cloned catalase gene was sequenced by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. Sequence alignment and analyses of the kat gene were performed by DNA star software. A number of *kat* homologs have been aligned and analyzed with DNA star package. Sequence similarities were analyzed with the BLAST and FASTA programs.

2.7 The Mice Fed with the Preparations

Female BALB/c and C57BL/50 mice (7–8 weeks old) were gifted by Center of experimental animals in Sunyat University (China) and were divided into 5 groups with 10 mice in each group. Non-induced cultures and *L. lactis* NZ strains harbouring the control plasmid pVE3655 (Table 1) were used as negative controls. Groups consisting of at least 10 mice each were inoculated intragastrically with 0.5 ml of sampling fluid of recombinant *L. lactis* and recombinant *E. coli* (test groups) and of normal *L. lactis* and *E. coli* (control groups). The animals were kept in separated cages and had free access to an autoclaved pelleted diet and sterile water during the experimental period. Four weeks after inoculation, the animals were killed under ether

Table 1. The bacterial strains and plasmids used in this study

Bacterial strain or plasmid and kit	Description	Source or reference
Bacterial strains	*E. coli* Top10,TG1,DH5a Plasmidless *Lactococcus lactis* NZ9000	Invitrogen and this lab.
pMD18-T	Cloning vector, Ap^r, 2.692 kb, pBluescript SK+/	New England Biolabs
Midiprep plasmid purification kit	Midiprep plasmid purification kit	Qiagen Catalogue No./ID: **130708(NeXtal Tubes Suites)**
Examing kit	Examing for T and B cells kit	Shanghai and Jianglai Biotechnology limited company Cat Nos: EPX070-20815-901)
pET-28a(+)	Expression vector, Ap^r, 5.369 kb	Gibco BRL
pTkatE	MD18-T containing 2.5-kb *Bam*HI insert *of catalaseE* DH5a genomic fragment	This work
pVE3655 and pMG36e	PET28a(+) containing 2.262-bp *Xba*I-*Sph*I PCR product insert of catalase gene, plasmids pVE3655 and pSEC(donated by Dr.Gruss)	This work

anesthesia and bloods of the experimental mice were obtained for microbiological and immunological studies. The spleen was gently disrupted through a nylon mesh and the cells analyzed for in vitro cellular proliferative response and T and B lymphocyte subset profile in response to *Helicobacter pylori* (*H. pylori*) antigens.

2.8 Immune Function Analysis of Experimental Mice

Spleen cells (1.0 x 106 cells in 900 _l of RPMI 1640 medium containing 10% heat inactivated fetal calf serum, 200 mM L-glutamine and 50 g/ml gentamycin) from the experimental mice were cultured for 48 hr at 37 degree Celsius in 24 well microplates (Nunc). The cells were stimulated with 100 ul of sonicated suspension of each *H. pylori* antigen preparation at a final concentration of 4 ng/ml, stained with phycoerythrin-conjugated antibodies anti-IgG anti-CD4, anti-CD8 (Shanghai Jianglai Biotechnology limited company, China). The percentages of CD4 (helper), CD4/ CD8_ (suppressor) T and B cells were determined by the kit (48T/96T, Shanghai Jianglai Biotechnology limited company). A total of 104 viable cells were analyzed after cell debris had been gated out. The results were expressed as percentage of blast cells (CD4, CD8, T and B cells) or lymphocytes (CD4, CD25, T cells). ELISA for katE-specific IgG and IgE antibodies was performed by porcine anti-rotavirus A IgG and IgE antibodies (IgG, IgA, IgE, IgM Invitrogen ProcartaPlex Mouse Antibody examing kit Cat Nos: EPX070-20815-901) as described in the manufacturer's instructions but the secondary antibody was exchanged by peroxidise-labelled goat anti-porcine IgE (Thermo Fisher Scientific, Bonn, Germany) at a dilution of 1/10 000. The absorbance of each sample was measured at 450 nm.

2.9 Salt Stress

Salt stress experiments were performed in TYG broth. Early-exponential-phase cultures were harvested and resuspended in TYG broth containing 20% NaCl and incubated at 30 degree Celsius for 8h, plating serial dilutions at 2h intervals.

2.10 Statistical Analysis

The results were analyzed with statistical software SPSS 11.5 as median percentage values and analyzed by the variance analysis. The level of significance was set at $P< 0.05$.

3. RESULTS

3.1 Genome DNA Extraction

E. coli W3110 genome DNA was extracted and identified by 1percent agarose (see Fig. 2).

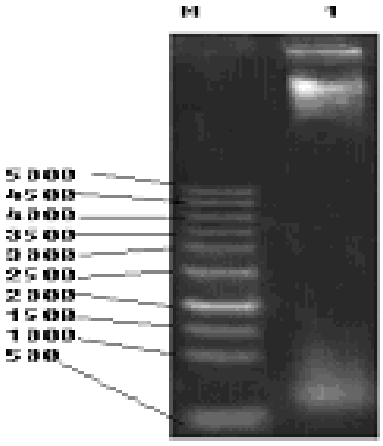

Fig. 2. DNA elecircphoresis from *E. coli*
M: Marker. 1 Genome DNA

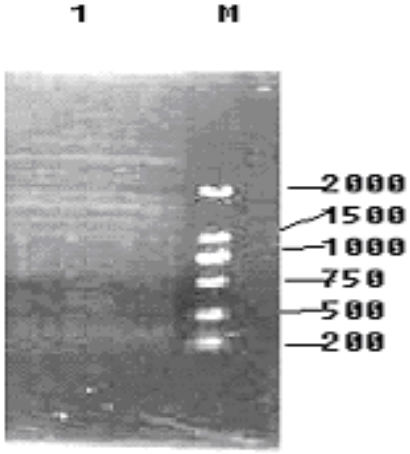

Fig. 3. Electrophoresis from E. coli total genomic DNA Partially digested with Sau3AI
M: Marker; 1: Genomic DNA Partially digested with Sau3AI

3.2 Positive Clone Selection

The positive clones more than 1500bp molecular weight with catalase activity on brain heart infusion (BHI) containing tannic acid and ampicillin for screening for tannase activity after transformation were selected (see Figs. 4,5).

3.3 Kat Gene Identification

The kat gene was identified correctly by PCR and restrictive enzymes Xbal / Sal (see Figs. 6,7). The recombinant *L. lactis* containing kat gene was identified by PCR and restriction enzyme analysis. The recombinant plasmid was transformed into *E. coli* by electrophoration. The results from the Figs. 7 and 8 showed that the kat gene isolated in the *E. coli* was correct.

Fig. 4. A control plate without catalase activity

Fig. 5. A plate-screening assay with catalase activity

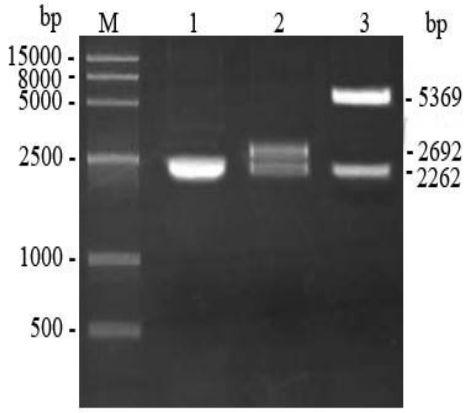

Fig. 6. Identification of PCR product of *katE*, recombinant plasmid T-katE and pET-katE
M: DNA marker; 1: PCR product; 2: T-katE digested with Xbal / Sal I; 3: pET- katE digested with Xbal / Sal I.

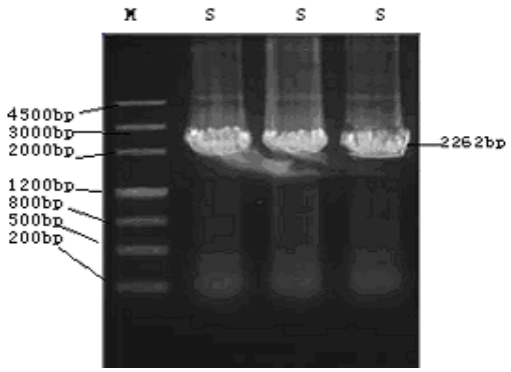

Fig. 7. Kat gene identification by PCR
M: Marker, S: product by PCR

3.4 Expression of Kat Gene in *E. coli*

The recombinant *E. coli* were incubated and induced by IPTG in LB medium for 6 hours. The KAT protein was identified by SDS-PAGE and Western-blot with the antibody prepared from the rabbits immuned by recombinant KAT protein (prepared in our laboratory). The results confirmed that KAT protein had been expressed obviously in *E. coli*. The molecular weight is 84.2kD. The plasmid containing kat gene was called pET-katE. And it was proved by Western-blot. The quantity of expression was about 10 mg/ml (Figs. 8, 9).

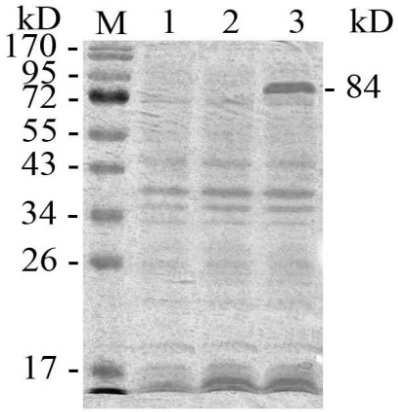

Fig. 8. SDS- PAGE analysis of KATE expressed in *E. coli* BL21（DE3）
M: protein marker; 1: BL21 (DE3) with IPTG induction; 2: pET-katE clones transformed E. coli BL21 (DE3) without IPTG induction; 3: pET-katE clones transformed E. coli BL21 (DE3) with IPTG induction

The catalase activity of *L. lactis* was also dependent on the concentration of salt. Salt stress experiments showed that after 8 h incubation, 5·1% of NZ9000 were viable. However, only the recombinant *L. lactis* in TYG broth, containing 20% NaCl and incubated at 30 degree Celsius for 8h exhibited greater salt sensitivity as compared to the parent culture NZ9000 strains surviving. From this result it would appear that this recombinant strain may be involved in responding to salt or osmotic stress. The recombinant *L. lactis* revealed growth difference in different physiological environment. It survived and revived surpassing normal *L. lactis* NZ9000 at low temperature (4-0 degree Celsius). The result suggests that recombinant *L. lactis* has ability against environment salt stress.

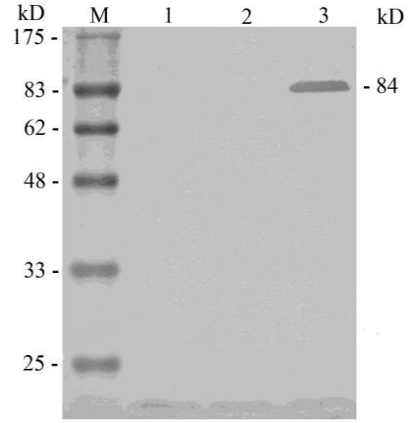

Fig. 9. Western blot analysis of the Kat protein expressed in *E. coli* BL21 (DE3) using Ni-NTA conjugate antibody
M: prestained protein marker; 1: BL21 (DE3) with IPTG induction; 2: pET-katE clones transformed E. coli BL21 (DE3) without IPTG induction; 3: pET-katE clones transformed E. coli BL21 (DE3) with IPTG induction

3.5 Sequence Analysis

The open reading frame of pET-katE containing kat gene is 2262nt long，and translating protein is 753 amino acids, and its' isoelectric point is 5.67. The nucleotides and amino acids are shown in Fig. 10 (See appendix). The protein sequence was translated as shown in Fig. 11 (See appendix).

It is known that the kat gene is katE, because the sequence is homology to katE genes from *Escherichia coli* O157:H7 strain and *Escherichia coli* strain K-12 sub-strain, the homology is 98, 99.8 percent, respectively. Analysis of the amino acid sequence of katE of *E. coli* (cloned) revealed that it contains all the typical features of the KAT proteins (Table 2).

3.6 Phylogenetic Analysis

In order to root a tree of katE from 8 bacteria sequenced to date, a DNA polymerase phylogeny was inferred using the kat genes from those bacteria representing different Phyla which had a high score and the lowest e-values in tblastx searches against bacteria kat gene (Fig. 12).

Pair Distances of Untitled ClustalW (Weighted), Percent Similarity in upper triangle in Table 2.

3.7 Detection of Immunization in the Blood of Experimental Animals

The results were expressed as percentage of blast cells (CD4, CD8, T and B cells). ELISA for katE-specific IgG and IgE antibodies was performed by porcine anti-rotavirus A IgG and IgE antibodies as described in the manufacturer's instructions. But the secondary antibody was exchanged by peroxidise-labelled goat anti-porcine IgE (Thermo Fisher Scientific, Bonn, Germany) at a dilution of 1/10 000. The absorbance of each sample was measured at 450 nm.

The concentration of *L. lactis* NZ9000 is 8.4×10^{8}cfu/ml, and that of *E. coli* DH5α is 7.2×10^{6}cfu/ml.

3.8 Comparison of Statistical Analysis

The level of IgG from the group with recombinant *L. lactis* NZ9000 treatment was higher than that of other treatments (see Table 3). There is a significant difference between them. Variation of IgG of the sera from mice with recombinant *L. lactis* NZ9000 was higher than that of other treatments, and the difference is significant ($p<0.05$; see Figs. 13, 14 and Table 3). However, the level of IgE was not difference between them ($p>0.05$; see Figs. 15, 16 and Table 4).

Table 2. Homology comparison of DNA sequences among different strains

	1	2	3	4	5	6	7	8	9
1.*E. coli* katE(cloned) seq.	100	98	99.8	24.9	26.2	26.5	25.5	25.0	25.1
2.*E. coli* katE seq#2 seq	98	100	97.9	24.8	26.0	26.1	25.7	25.1	25.6
3.*E. coli* CK2 katE Seq #4 seq	99.8	97.9	100	24.9	26.2	26.5	25.5	25.0	25.1
4.*Bacillus* subtilis katE #5 seq	24.9	24.8	24.9	100	24.4	26.5	27.1	24.4	26.7
5.Hay bacillus cot(ORF)seq	26.2	26.0	26.2	24.4	100	26.4	25.9	58.3	27.5
6.*Pseudomonas* katE #8seq	26.5	26.1	26.5	26.5	26.4	100	29.0	26.1	27.3
7.*Salmonella* katE #6seq	25.5	25.7	25.5	27.1	25.9	29.0	100	24.6	27.0
8.*Staphylococcus* cornosuscat (ORF)seq	25.0	25.1	25.0	24.4	58.3	26.1	24.6	100	27.0
9.Yersnio katE#7seq	25.1	25.6	25.1	26.7	27.5	27.3	27.0	26.5	100

Table 3. Comparison of IgG among different treatment groups (u/ml)

Groups	N	2h	4h	6h	8h
Recombinant *L. lactis* NZ9000	10	1.628±0.012	2.967±0.008	2.424±0.009	2.745±0.010
Recombinant *E. coli* DH5α	10	1.483±0.021·	1.431±0.011·	1.079±0.013·	2.010±0.00·9
L. lactis NZ9000	10	1.119±0.011·	1.745±0.013·	1.443±0.009·	1.893±0.008·
E. coli DH5α	10	1.881±0.010	1.732±0.010	2.075±0.008	0.758±0.009
N.S	10	1.443±0.009·	1.939±0.011·	1.516±0.009·	1.589±0.012·

Table 4. Comparison of IgE among different treatment groups (u/ml)

Groups	n	2h	4h	6h	8h
Recombinant *L. lactis* NZ9000	10	1.619±0.006	2.30±0.013	2.178±0.0010	2.245±0.011
Recombinant *E. coli* DH5α	10	2.211±0.010	2.061±0.015	2.100±0.015	2.521±0.010
L. lactis NZ9000	10	2.049±0.011	1.917±0.011	1.444±0.009	2.425±0.011
E. coli DH5α	10	3.101±0.009	2.283±0.011	2.749±0.011	2.069±0.012
N.S	10	1.957±0.012	2.270±0.009	2.405±0.013	2.120±0.012

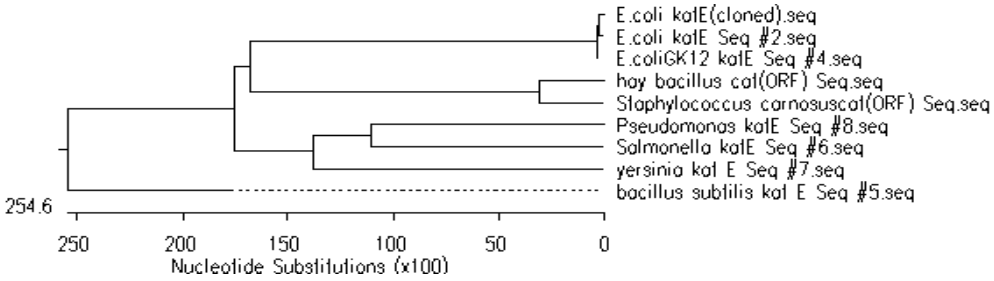

Fig. 12. Phylogenetic tree analysis from kat genes of bacteria strains

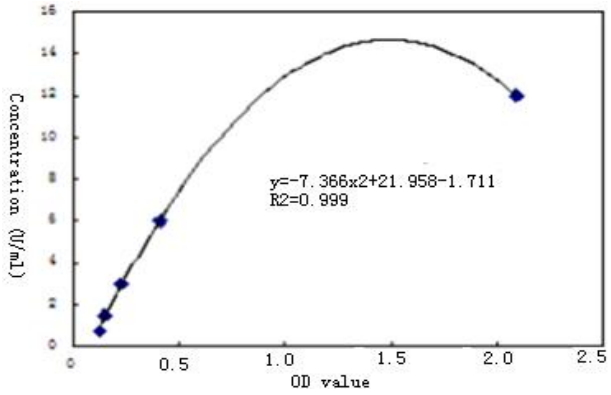

Fig. 13. Standard curve of IgG

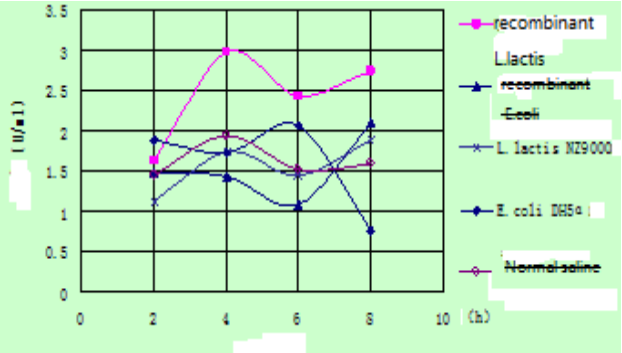

Fig. 14. Comparison of IgG among the different treatments

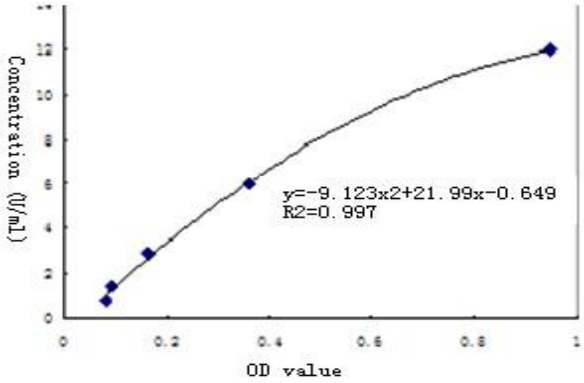

Fig. 15. Standard curve of IgE

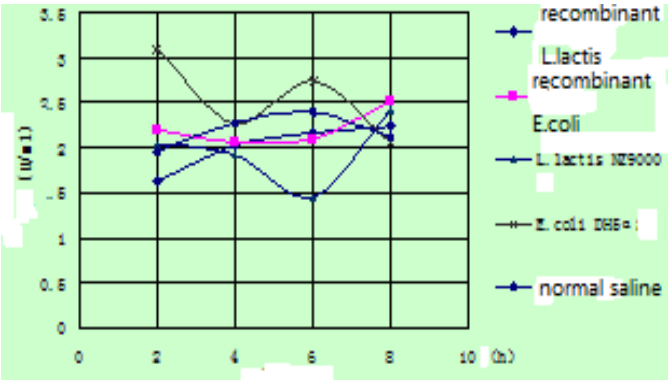

Fig. 16. Comparison of IgE among the different treatments

The results showed that the concentration of CD4 and CD8 from experimental animals with recombinant *L. lactis* NZ9000 was not higher than that of the other groups. There was no significant difference between them (P>0.05, see Tables 5,6 and Figs. 18,20).

Table 5. Comparison of CD4 among groups (u/ml)

Groups	n	2h	4h	6h	8h
Recombinant *L. lactis* NZ9000	10	2.175±0.012	1.358±0.012	1.739±0.011	1.739±0.012
Recombinant *E. coli*	10	2.008±0.009	1.783±0.011	2.609±0.011	2.883±0.014
L. lactis NZ9000	10	3.2256±0.014	1.975±0.013	2.350±0.008	2.803±0.015
E. coli H5α	10	3.378±0.012	2.849±0.011	2.211±0.013	2.289±0.011
NS	10	2.240±0.013	2.289±0.013	1.805±0.012	2.375±0.013

Table 6. Comparison of CD8 among groups (u/ml)

Groups	n	2h	4h	6h	8h
Recombinant *L. lactis* NZ9000	10	1.601±0.013	2.281±0.010	1.317±0.007	2.377±0.019
Recombinant *E. coli*	10	1.823±0.011	2.272±0.013	1.174±0.015	2.481±0.018
L. lactis NZ9000	10	2.385±0.016	1.945±0.011	2.322±0.020	2.397±0.016
E. coli H5α	10	2.773±0.012	1.954±0.013	2.483±0.021	1.734±0.023
NS	10	2.059±0.010	1.907±0.012	1.317±0.022	1.907±0.022

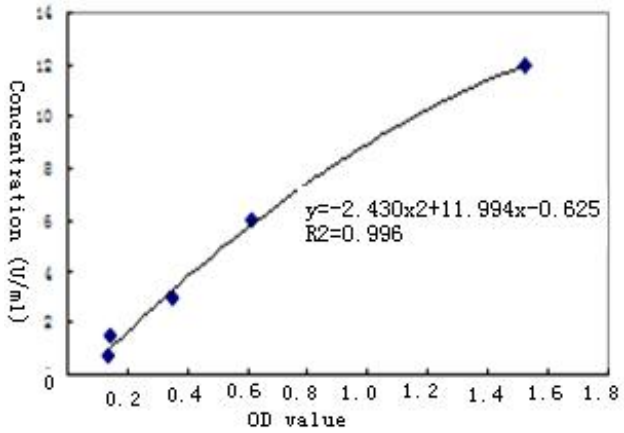

Fig. 17. Standard curve of CD4

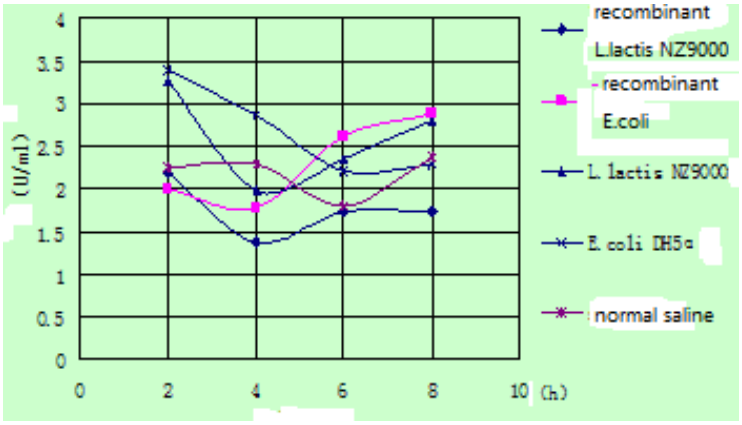

Fig. 18. Variation of CD4 concentration

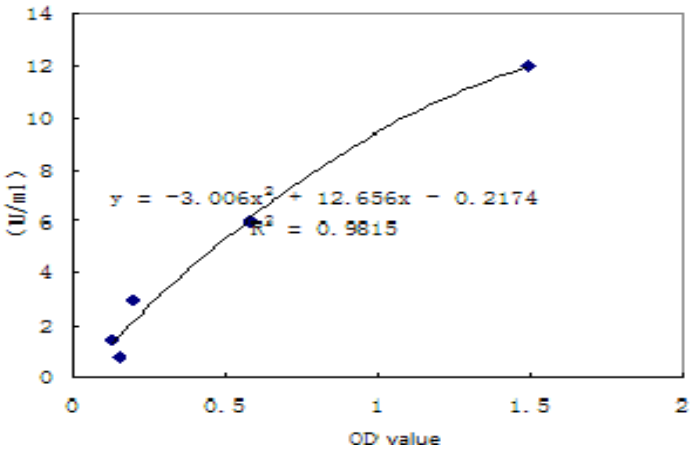

Fig. 19. Standard curve of CD8

The ratio of CD4 and CD8 cells from the animals were constant relatively through comparison. And there is no different between them (see Table 7). The trend of variation is shown in Fig. 21.

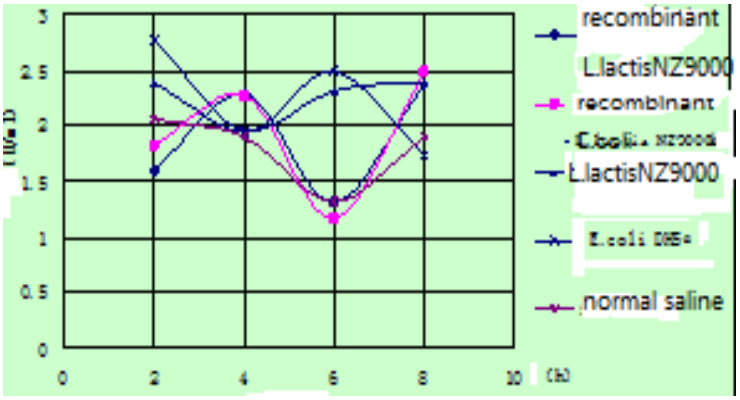

Fig. 20. Variation of CD8 concentration

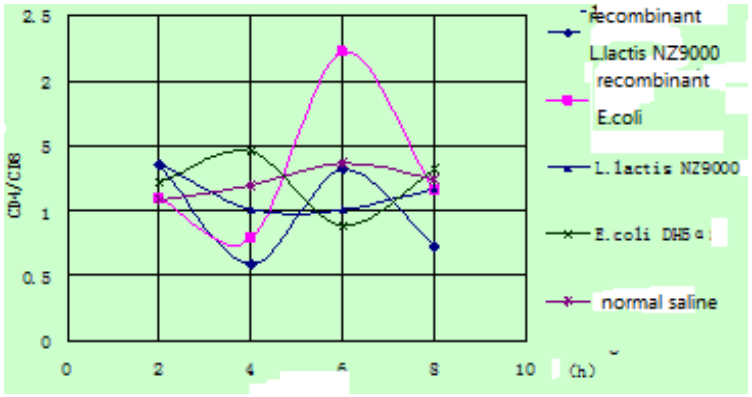

Fig. 21. Variation of CD4/CD8

Table 7. Comparison of CD4/ CD8 among groups (u/ml)

Groups	n	2h	4h	6h	8h
Recombinant *L. lactis* NZ9000	10	1.359±0.075	0.595±0.053	1.320±0.075	0.732±0.091
Recombinant *E. coli*	10	1.044±0.081	0.785±0.069	2.222±0.083	1.162±0.076
L. lactis NZ9000	10	1.369±0.073	1.015±0.080	1.012±0.081	1.70±0.085
E. coli H5α	10	1.218±0.036	1.458±0.079	0.887±0.064	1.667±0.078
NS	10	1.088±0.054	1.200±0.062	1.543±0.053	1.245±0.081

3.9 Survival of the Mice

Five days after the treatment, the mice with *L. lactis* NZ9000 and recombinant *L. lactis* NZ9000 were more active than that with *E. coli* DH5α.

4. DISCUSSION AND CONCLUSION

Report demonstrated the parameter requirement for successful transformation of *L. lactis* NZ9000 strain with electroporation [17]. Electroporation transformation was used in the study. It is better than chemical transformation in this study, and the concentration of 2-3% glycine in the media also will provide the optimal growth for the transformation efficiency. Also we investigated the cloning and expression of catalase from *E. coli* in *L. lactis* and the role of oxidation and salt stress in the expression of the catalase of *L. lactis* subsp. The transformants were screened for catalase activity on brain heart infusion (BHI) containing tannic acid (BHI-tannic acid medium) and ampicillin (150 µg/ml), Tannica cid could induce the activity of catalase it is identified in the experiment [18], and this method for cloning catalase gene is feasible.

Many problems were found when genome of *E. coli* was cut with Sau3AI. The intact catalase gene was to be obtained only by random partly cutting. The fragments molecular weights more than 2000Dt were insured to be collected. And the restricted enzyme BamHI which could produce the same cohesive 5'ends as Sau3A was used in the test. For many fragments, the time should be controlled when the plasmid of pUC18-kat was cut with BamHI because of the sites of BamHI in the kat gene in order to ensure the integrity of gene. In this work, catalase is induced either by nisin. Moreover, this induction occurs at the appropriate time in anaerobic culture. It may be transcriptional time and results in enhanced catalase expression. The kat gene was successfully expressed in the heterologous host *E. coli*. It was also identified by western-blot. Of the recombinant strain tested, only recombinant *L. lactis* exhibited greater salt sensitivity compared to the parent culture NZ 9000. This observation suggests that engineering bacteria may be involved in the response of *L. lactis* to salt stress. After 20 min incubation, 70% of the NZ9000 cells remained viable. The effects associated with the catalase insertion were due specifically to changes in recombinant *L. lactis*, strain which was exposed to salt, like the other strains. This strain was more resistant to salt than normal strain NZ9000. This may be the result of high catalase levels as a consequence of the high copy number of the plasmid harboring the catalase gene [13].

In some cases, catalase genes exhibit sequence homology between the different bacteria strains

but they are regulated differently [19]. It was shown in this study.

The genetic improvement of *L. lactis* is a matter of biotechnological interest in the food industry and in the pharmaceutical and medical fields. The main metabolism of *L. lactis* is through the anaerobic pathway and fermentation, which produces lactic acid from the available carbohydrates and is used for industrial food production [8].

The report demonstrated that antigen transport would be increased in the absence of the intestinal microflora; it indicates that the gut microflora is an important constituent in the intestines defense barrier. For example production of IgE antibodies, is based upon oral antigen administration.

Abrogation of oral tolerance was due to the absence of intestinal flora. The aberrant IgE response could be corrected by reconstitution of the microflora at the neonatal stage, but not at a later age [20]. However, the interest in gastric bacteria has increased since the isolation of *H. pylori* from the gastric mucosa of human patients with gastro duodenal diseases [17]. While immunization with recombinant *L. lactis* containing catalase gene merely induces slightly higher levels of B-cell epitope specific IgG, It is suggest that recombinant *L. lactis* NZ9000 containing catalase gene could stimulate humoral immunity of the animals. Whether contribution, if any, of the T cell response to humoral response leads to qualitatively superior specific IgG antibodies is an interesting consideration to explore in future studies.

Lymphocytes are anti infection and tumor immune cells, which mainly contain CD4 molecules and CD8 molecules. CD4 cells play a messenger role while CD8 cells can kill infected cells and cancer cells. In the study, the relative number of CD4 cells was not significantly ($p>0.05$) increased in the probiotic group. No difference was observed between mouse strains in regard to the presence only in the blood of the experimented animals. It was shown that no influence of recombinant *L. lactis* NZ9000 containing catalase gene for cellular immunity of animal could be detected in the blood. It is known that the immune type of humoral and cellular immunity, complement each other, but different diseases showed different types of immune cells. Only the enzyme changes in the cells and this change is not a foreign antigen, so that the humoral and cell mediated immunity level will not change. This shows that some enzyme activity could make the organism have a selective advantage over *S. bovis* when grown in the presence of tannins [10]. The researchers found that genetically modified mice with catalase deficiency were still normal phenotypes, suggesting that catalase is essential for animals only under certain conditions.

The genetic improvement of *L. lactis* is a matter of biotechnological interest in the food industry and in the pharmaceutical and medical fields. The main metabolism of *L. lactis* is through the anaerobic pathway, fermentation, which produces lactic acid from the available carbohydrates and is used for industrial food production [8,5,3,21]. The vast majority of aerobic microorganisms contain catalase, However *Lactococcus lactis* do not have catalase activity or little, resulting in the presence of oxygen decreased antioxidant capacity [5,3]. The purpose of this study is to insert the catalase gene from the bacteria into *Lactococcus lactis* by gene engineering methods to induce its expression in order to improve the utilization degree of *Lactococcus lactis*.

The result in genetic performance of *L. lactis* reveals another industrial advantage for producing new protein by genetic engineering. It is shown that the recombinant *L. lactis* containing catalase is beneficial by the test of animals' survival. It is not only important in dairy production, but also has potential for use as oral vaccine, and foreign protein production.

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My colleagues and I have no financial and non-financial competing interests in relation to this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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APPENDIX

Sequence Detail

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Fig. 10. Determination of nucleotide sequence of kat katE gene (bt sequence)

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Fig. 11. Sequence of amino acids from KAT protein*. The sequence was from Blast search

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The peer review history for this paper can be accessed here:
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