

An Effective Cloning, Heterologous Expression and Physiological Activity in *Lactococcus lactis* NZ9000 of Catalase Gene from *Escherichia coli* DH5 α

Abstract:

In order to clone active Catalase gene from bacteria, we introduced a method of shotgun integrating specific screen from *Escherichia coli* DH 5 α . 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 transformants were incubated anaerobically on brain heart infusion (BHI) containing tannic acid. The method could detect catalase activity and screen the catalase-positive clones. The 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 DH5 α 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* DH5 α and *L.lactis* NZ9000; *E.coli* DH5 α 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.

Key words: *Lactococcus lactis* NZ9000 , screening, catalase-positive, transformants, Immunity

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 (Rochat *et al.*, 2012; van de Guchte *et al.*, 2002).

Catalases are antioxidant metalloenzymes that divide H₂O₂ into water (H₂O) and O₂ (Abriouel *et al.*, 2004). 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 (Arockiaraj *et al.*, 2012).

Catalase is a tetramer of four polypeptide chains, each over 500 amino acids long (Boon EM, *et al.* 2007). 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 (Yuzugullu Y *et al.* 2001, Boucher, I. *et al.* 2002, Martirosyan A.O., *et al.* 2004, Jacek S, and Loewen P C. 2002).

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 (Engesser, D. M *et al.* 1994).

At present, many methods are used to clone genes. Such as those obtained from genome bank, PCR, shotgun, RT-PCR and so on (Sambrook, J. *et al.* 1989). To clone the active catalase from bacteria, an effective approach was assayed (Chung K T *et al.* 1998). 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*.

Materials and methods

A. DNA manipulations and plasmid construction

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

Bacterial strain or plasmid and kit	Description	Source or reference
Bacterial strains	<i>E. coli</i> Top10, TG1, DH5a Plasmidless <i>Lactococcus lactis</i> NZ9000	Invitrogen and this lab.
pMD18-T Qiagen Midiprep plasmid purification kit Examining kit	Cloning vector, Ap ^r , 2.692 kb, pBluescript SK+/ Midiprep plasmid purification kit Examining for T and B cells kit	New England Biolabs Qiagen Shanghai and Jianglai Biotechnology limited company)
pET-28a(+)	Expression vector, Ap ^r , 5.369 kb	Gibco BRL
pTkatE	MD18-T containing 2.5-kb <i>Bam</i> HI insert of <i>catalaseE</i> DH5a genomic fragment	This work
pVE3655 and pMG36e	PET28a(+) containing 2.262-bp <i>Xba</i> I- <i>Sph</i> I PCR product insert of catalase gene, plasmids pVE3655 and pSEC(donated by Dr.Gruss)	This work

Cloning and transformation.

In order to clone the catalase gene, total genomic DNA was isolated and purified with DNA purifying kit (Qiagen). Then it was partially digested with *Sau*3AI, 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 *Bam*HI-cleaved plasmid pBluescript SK+/- were used to transform electrocompetent *E. coli* Top10 cells (Sambrook, J *et al.* 1989). 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 (Chung K T, *et al.* 1998). 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 (Hikmate A *et al.*2002). A zone of clearing surrounding the colonies which developed during aerobic incubation indicated tannic acid degradation as a result of catalase activity. Plasmid DNA (pHA01) of one clone was isolated using the Qiagen Midiprep plasmid purification kit (Qiagen), 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.

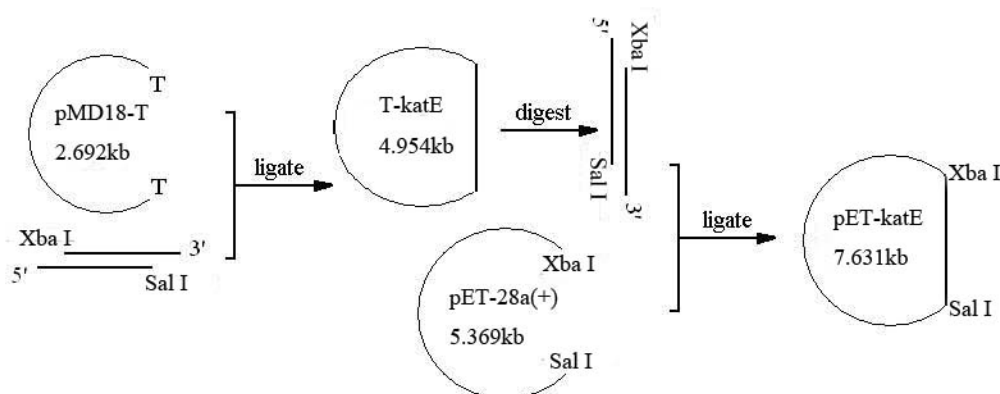


Fig. 1:Construction of recombinant plasmid pET-katE

E. coli was transformed by the method of electroporation (Powell IB *et al.*1988) which was pre-digested with SalI and XbaI restriction enzymes. After ligation, the reaction mixture was employed to transform DH5a 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.

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.

The oligonucleotides used were XbaI-K-UP:

5'-TCTAGAATGTCTCGCAAKATAACGAAAAGAACCCAKATCAGC-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 Inc), 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 coli* - *L. lactis* shuttle vector pMG36e and electro-transformed into *L. lactis* NZ9000.

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 (Hikmate Abriouel *et al.*, 2004, Bernhard T *et al.*, 2004). 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 (Le Loir *et al.*, 1998). 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.

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 to stimulate 2-3 h *E.coli* DH5 α cultured in 100 mL of LB broth overnight at 37 degree Celsius. The bacteria count was done by plate count method under a microscope. The bacterial cells were centrifuged for 10 min at 3,000 \times 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.

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.

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 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.

Immune function analysis of experimental mice.

Spleen cells (1.0×10^6 cells in 900 μ 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 100ul of sonicated suspension of each *H. pylori* antigen preparation at a final concentration of 4ng/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 (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 as described in the manufacturer's instructions but the secondary antibody was exchanged by peroxidase-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.

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.

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 $P < 0.05$.

Results

Genome DNA extraction

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

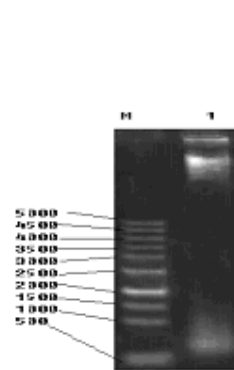


Fig 2 .DNA electrophoresis from *E. coli*

M:Marker, 1 Genome DNA

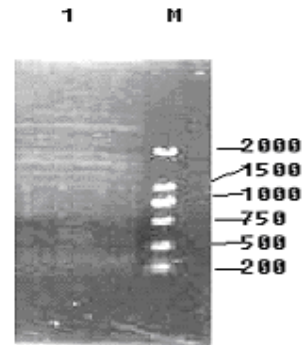


Fig3 .Electrophoresis from *E. coli* total genomic DNA partially digested with *Sau3AI*
M:Marker;1:genomic DNA partially digested with *Sau3AI*

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 Fig 4,5).



Fig 4 a control plate without catalase activity



Fig 5 a plate-screening assay with catalase activity

Kat gene identification

The *kat* gene was identified correctly by PCR and restrictive enzymes *XbaI* / *Sal* (see Fig 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 electroporation. The results from the Figure 7 and 8 showed that the *kat* gene isolated in the *E. coli* was correct.

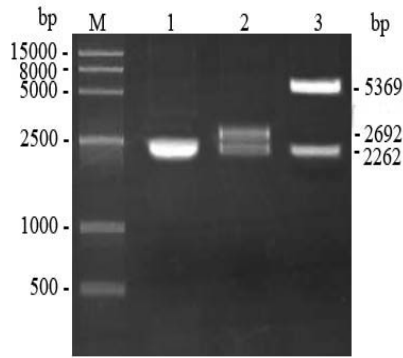


Fig.6 Identifikation of PCR product of *katE*, recombinant plasmid T-*katE* and pET-*katE*
M: DNA marker; 1: PCR product; 2: T-*katE* digested with *Xba*I / *Sal* I; 3: pET- *katE* digested with *Xba*I / *Sal* I.

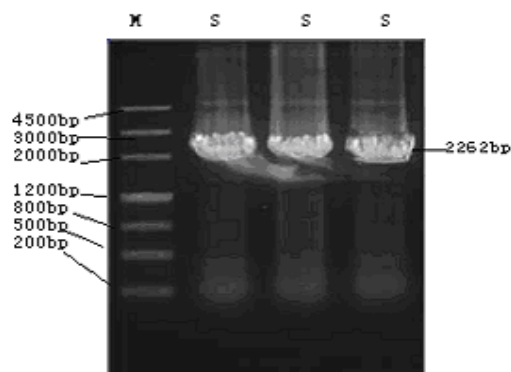


Fig 7: *kat* gene identifikationby PCR
M: Marker, S: product by PCR

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 immunized 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 (Figure8, 9).

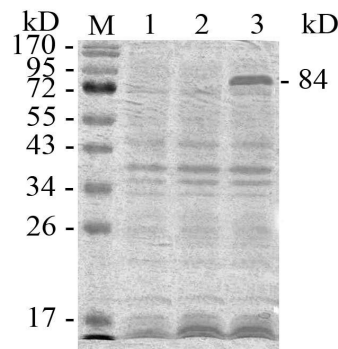


Fig. 8: SDS- PAGE analysis of KATE expressed in *E. coli* BL21 (DE3)
M: protein marker; 1: BL21 (DE3) with IPTG induction; 2: pET-*katE* clones transformed *E.coli* BL21 (DE3) without IPTG induction; 3: pET-*katE* clones transformed *E.coli* BL21 (DE3) with IPTG induction

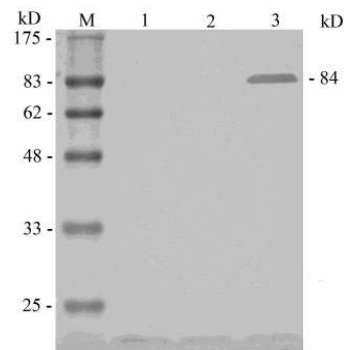


Fig. 9: Western blot analysis of the Kat protein expressed in *E.coli* BL21 (DE3) using Ni-NTA conjugate antibody
M: prestained protein marker; 1: BL21 (DE3) with IPTG induction; 2: pET-*katE* clones transformed *E.coli* BL21 (DE3) without IPTG induction; 3: pET-*katE* clones transformed *E.coli* BL21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

Sequence analysis

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).

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

Percent Identity									
1	2	3	4	5	6	7	8	9	
■	98.0	99.8	24.9	26.2	26.5	25.5	25.0	25.1	1
	■	97.9	24.8	26.0	26.1	25.7	25.1	25.6	2
		■	24.9	26.2	26.5	25.5	25.0	25.1	3
			■	24.4	26.5	27.1	24.4	26.7	4
				■	26.4	25.9	58.3	27.5	5
					■	29.0	26.1	27.3	6
						■	24.6	27.0	7
							■	26.5	8
								■	9
									E.coli katE(cloned).seq
									E.coli katE Seq #2.seq
									E.coliCK12 katE Seq #4.seq
									bacillus subtilis kat E Seq #5.seq
									hay bacillus cat(ORF) Seq.seq
									Pseudomonas katE Seq #8.seq
									Salmonella katE Seq #6.seq
									Staphylococcus carnosuscat(ORF) Seq.seq
									yersinia kat E Seq #7.seq

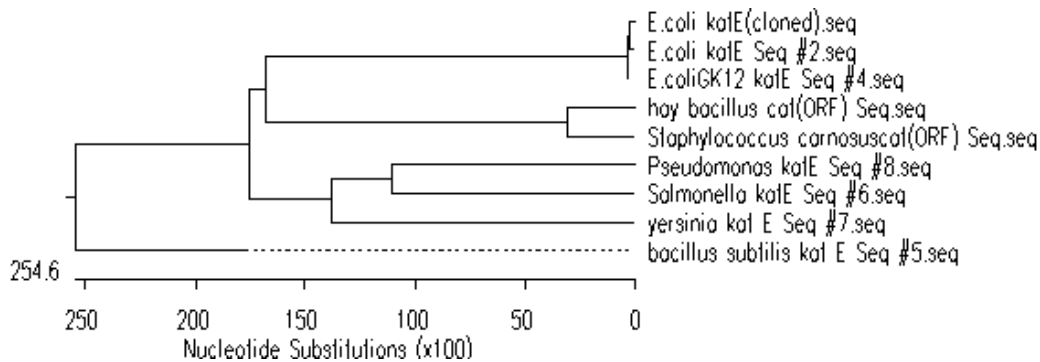


Fig 12. Phylogenetic tree analysis from kat genes of bacteria strains

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* DH5 α is 7.2×10^6 cfu/ml.

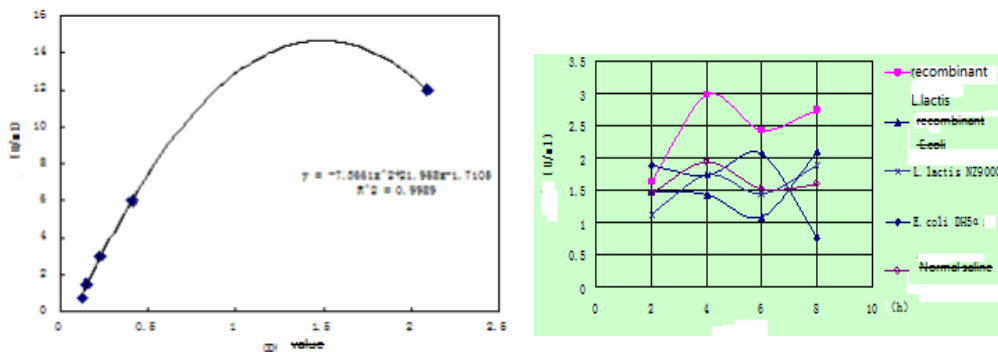


Fig 13. Standard curve of IgG Fig 14. Comparison of IgG among the different treatments

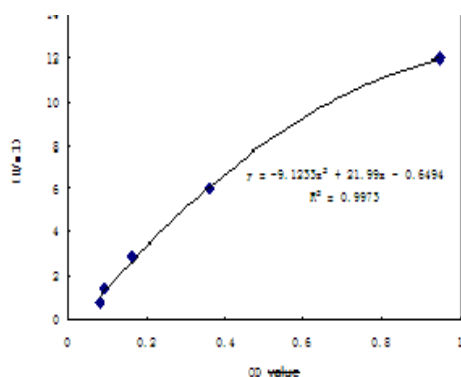


Fig 15. Standard curve of IgE

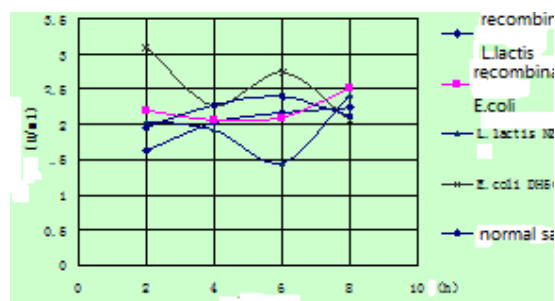


Fig 16. Comparison of IgE among the different treatments

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 Tab 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 Fig 13, 14 and Tab3). However, the level of IgE was not difference between them ($p > 0.05$; see Fig15, 16 and Table 4).

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

groups	N				
		2h	4h	6h	8h
Recombinant <i>L. lactis</i> NZ9000	10	1.628±0.012	2.967±0.008	2.424±0.009	2.745±0.010
Recombinant <i>E. coli</i> DH5 α	10	1.483±0.021	1.431±0.011	1.079±0.013	2.010±0.009
<i>L. lactis</i> NZ9000	10	1.119±0.011	1.745±0.013	1.443±0.009	1.893±0.008
<i>E. coli</i> DH5 α	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 <i>L.lactis</i> NZ9000	10	1.619±0.006	2.30±0.013	2.178±0.0010	2.245±0.011
Recombinant <i>E.coli</i> DH5α	10	2.211±0.010	2.061±0.015	2.100±0.015	2.521±0.010
<i>L.lactis</i> NZ9000	10	2.049±0.011	1.917±0.011	1.444±0.009	2.425±0.011
<i>E.coli</i> 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

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 Tab 5,6 and Fig 18,20).

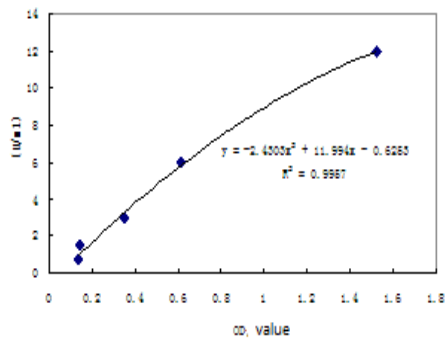


Fig.17 Standard curve of CD4

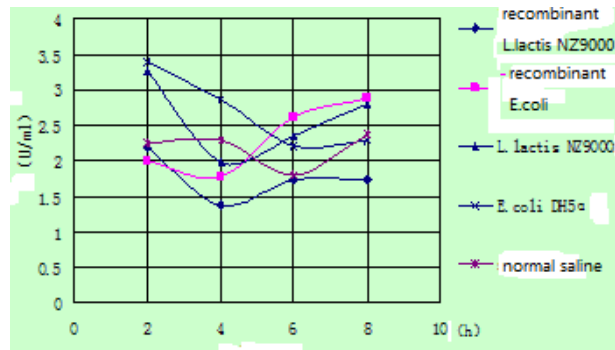


Fig.18 Variation of CD4 concentration

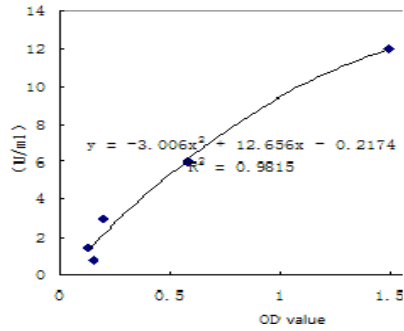


Fig.19 Standard curve of CD8

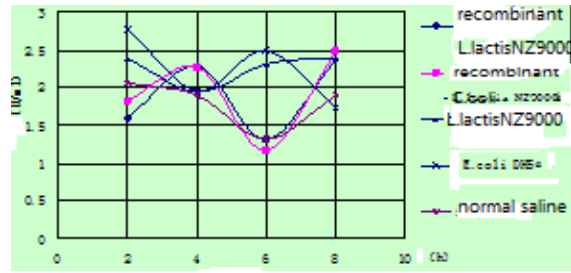


Fig.20 Variation of CD8 concentration

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

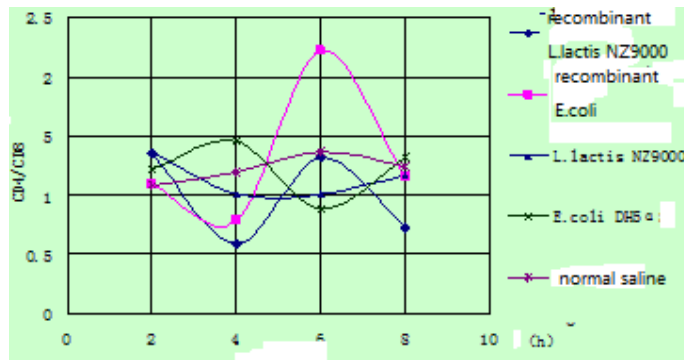


Fig.21 Variation of CD4/CD8

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

Groups	n	2h	4h	6h	8h
Recombinant <i>L.lactis</i> NZ9000	10	2.175±0.012	1.358±0.012	1.739±0.011	1.739±0.012
Recombinant <i>E.coli</i>	10	2.008±0.009	1.783±0.011	2.609±0.011	2.883±0.014
<i>L.lactis</i> NZ9000	10	3.2256±0.014	1.975±0.013	2.350±0.008	2.803±0.015
<i>E.coli</i> H5a	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

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

Groups		2h	4h	6h	8h
Recombinant <i>L.lactis</i> NZ9000	10	1.601±0.013	2.281±0.010	1.317±0.007	2.377±0.019
Recombinant <i>E.coli</i>	10	1.823±0.011	2.272±0.013	1.174±0.015	2.481±0.018
<i>L.lactis</i> NZ9000	10	2.385±0.016	1.945±0.011	2.322±0.020	2.397±0.016
<i>E.coli</i> 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

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

Groups		2h	4h	6h	8h
Recombinant <i>L.lactis</i> NZ9000	10	1.359±0.075	0.595±0.053	1.320±0.075	0.732±0.091
Recombinant <i>E.coli</i>	10	1.044±0.081	0.785±0.069	2.222±0.083	1.162±0.076
<i>L.lactis</i> NZ9000	10	1.369±0.073	1.015±0.080	1.012±0.081	1.70±0.085
<i>E.coli</i> 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

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.

Discussion

Report demonstrated the parameter requirement for successful transformation of *L. lactis* NZ9000 strain with electroporation (Gerber, S.D. *et al.* 2007). 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), Tannic acid could induce the activity of catalase it is identified in the experiment (Osawa, R. 1990.), 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 2000D_t 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 (Hikmate A, and Anette H, 2002).

In some cases, catalase genes exhibit sequence homology between the different bacteria strains but they are regulated differently (Schellhorn & Hassan, 1988). 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 (Martirosyan A.O. *et al.*, 2004).

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 (Sudo *et al.* 1997). 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 (Gerber, S.D, *et al.*, 2007). 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.

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.

Acknowledgements

We thank Dr. Alexandra Gruss from Institute of National Agriculture Research in France for providing the plasmids. This research was supported by Guandong Natural Science Fund Committee (06029354) .

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

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Appendix: sequence detail

atgtcgcaakataacgaaaagaacccakatcagcaccagtcaccactacacgattccagc... ..60

gaagcgaaaccgggatggactcactggcacctgaagacggatctkatcgccagcggt... ..120

gaaccaacaccgcctggtgcacaacctaccgcccaggagcctgaaagcccctgatacg.....180

cgtaacgaaaaacttaattctctggaagacgtacgcaaaggcagtgaaaattatgcgctg.....240
accactaatcagggcgtgcgkatcgccgacgatcaaaactcactgcgtgccgtagccgt..... 300
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aatctcggatcgaaactgactgatgaccagctgaatacacccacctccggacgtcaac... ..1740
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cgcgtgtagcaattttgcttaatgatgaagtgaatcggcagaccttctggckattctc..... ..1860
aaggcgctgaaggccaaggcggtkatgccaactgctctactcccgaatgggggaagtg.1920
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acggtcgatgcagtkattgtcccttgcggcaatattgcggatatcgctgacaacggcgat... ..2040
 gccaaactactatctgatggaagcctacaaacaccttaaaccgattgcgctggcaggagac..... 2100
 gcgcgcaagttaaagcaacaatcaaggtcgctgaccagggtgaagaagggtgattgcggaa... 2160
 gctgacagcgccgatggtagttttatggatgaactgttaacgctgatgacagcacaccgc..... 2220
 gtgtggtcacgkattcctaagattgacaaaatcccggcgtaa.....2262

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

MSQHNEKNPHQHQSPLHDSSEAKPGMDSLAPEDGSHRPAAEPTPPGAQPTAPGSLKAPDT
 RNEKLNSLEDVRKGSENYALTTNQGVRiADDQNSLRAGSRGPTLLEDFILREKITHFDHERIPER
 IVHARGSAAHGYFQPYKSLSDITKADFLSDTNKITPVFVRFSTVQGGAGSADTVRDIRGFATKF
 YTEEGIFDLVGNNTPIFFIQDAHKFPDFVHAVKPEPHWAIPQGQSAHDTFWDYVSLQPETLHN
 MWAMSDRGIPRSYRTMEGFGIHTFRLINAEGKATFVRFWKPLAGKASLVWDEAQKLTGRDP
 DFHRRELWEAIEAGDFPEYELGFQLIPEEDEFKFDLDDPTKLIPEELVPVQVRVGKMLNRNP
 DNFFAENEQVAFHPGHIVPGLDFTNDPLLQGRIFS YTD TQISRLGGPNFHEIPINRPTCPYHNFQ
 RDGMHRMGIDTNPANYEPNSINDNWPRETTPGPKRGGFESYQERVEGNKVRERSPSFGYYSH
 PRLFWLSQTPFEQRHIVDGFSELSKVVRPYIRERVVDQLAHIDLTLAQAVAKNLGIELTDDQLN
 ITPPPDVNGLKKDPSLSLYSIPDGDVKGRVVAILLNDEVRSADLLAILKALKAKGVHAKLLYSR
 MGEVTADDGTVLPiAATFAGAPSLTVDAVIVPCGNIADIADNGDANYYLMEAYKHLKPIALAG
 DARKFKATIKVADQGEEGIAEADSADGSFMDELLTLMTAHRVWSRIPKIDKIPA.

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