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

Abstract:

In order to clone active Catalase gene from bacteria, we introduced a method of shotgun integrating specific sceen from Escherichia coli DH 5α. Genome DNA was extracted from E.coli DH5 and partially digested with Sau3AI. Then some fragments more than2.26kb 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 screened the catalase-positive clones. And 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. Open reading frame from DNA Sequence DH5a katE. The sequence long is 2262nt and coding protein are 753 Amino Acids, Molecular Weight 84198.72 Daltons. This method is simple, and possessed 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. As L. lactis has no catalase, katE was inserted into Escherichia coli - L. lactis shuttle vector pMG36e and electrotransformed 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 physiological changes of immune function in mice. Enzyme-linked immunoassay (ELISA) was the blood of mice, IgG, IgE, and CD4 and CD8 levels, find out whether the difference between Grouping more mice eating the recombinant L.lactis NZ9000 and other groups (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, the difference was significant; IgE of CD4 of CD8 levels are no significant difference. Mice after the intake of recombinant L. lactis NZ9000 increased IgG levels explain recombinant L. lactis NZ9000 regulatory role of humoral immunity in mice; IgE level did not change CD4, CD8 levels were also no change, suggesting that there is no significant effect on the body of cellular immunity in a short time.

Key words: an effective method, active catalase gene, cloning and screening, tannin resistance

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 disproportionate H2O2 to water (H2O) and O2 during aerobic growth (Abriouel et al., 2004). And when the concentration of H2O2 is low, catalase can catalyze the oxidation of electron donors, such as ethanol or phenols. 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: Mn-catalases, catalase-peroxidases and monofunctional catalases, the monofunctional catalases are the best characterized heme-containing enzymes (Arockiaraj et al., 2012).

Catalase is used in the food industry for removing hydrogen peroxide from milk prior to cheese production and preventing food from oxidizing ((Boucher, I.et al.2002, Martirosyan A.O., et al. 2004 ,Jacek S, Loewen P C.2002). Additionally catalase has also been used in the aesthetics industry.

It was proved that gallate decarboxylase activity was elevated in the presence of tannic acid in S. gallolyticus (Chung K T et al.1998). It demonstrated that some enzyme activity could make the organism with 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 they are got by 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 selected the 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 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 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	**Description**[a]	**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
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

Cloning and transformation.

In order to clone the catalase gene, total genomic DNA was isolated and purified with DNA purfing kit (Qiagen). And 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 Top10 cells (Sambrook, Jet al. 1989). The gene library was constructed that of 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 purifikation kit (Qiagen), and the insert was sequenced

bidirectionally 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.

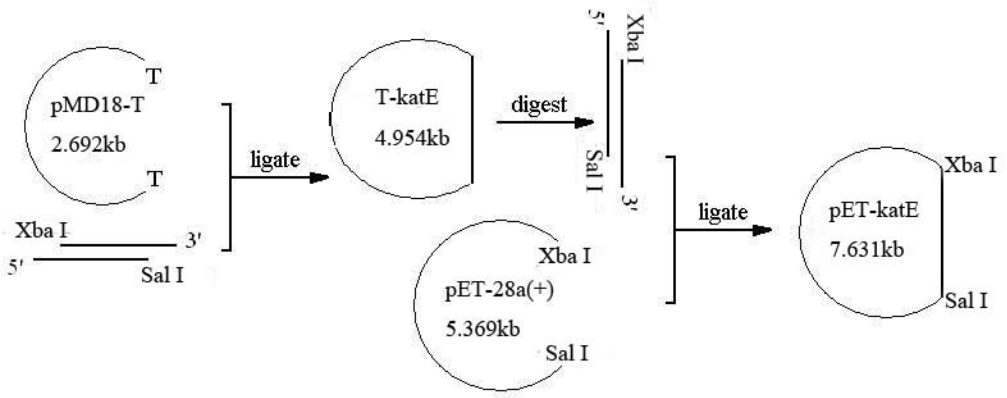


Fig. 1:Construction of recombinant plasmid pET-katE

E. coli was transformed by the method of electroporation (Powell I Bet al.1988) 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.

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’-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℃ for 3 min, then 30 cycles of 94℃ for 0.5 min, 50℃ for 50 sec, 72℃ for 1 min, followed by a final extension of 5 min at 72℃. The amplified fragment was purified using the QIA quick PCR purifikation Kit (QIAGEN Inc), and

digested with EcoR I and Not I. The resulting fragment was respectively ligated into the contained a T7 promotor 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. And katE was Also inserted into Escherichia coli - L. lactis shuttle vector pMG36e and electrotransformed 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 ℃. 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 werecultured in 100 mL of MG17 broth overnight at 30 degree Celsius and induced by adding compound Nisin for 2-3 h. *E.coli* DH5α cultured in 100 mL of LB broth overnight at 37 degree Celsius. The count of bacteria was obtained 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. The samples were prepared for oral of the 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 analysis of the kat gene were performed by DNAstar software. A number of kat homologs have been identified and characterized in many different bacteria, such as Bacillus subtilis subsp, Escherichia coli O157:H7 str, Escherichia coli str. K-12 substr, Pseudomonas syringae , Salmonella enterica subsp, Yersinia pestis, Staphylococcus carnosus subsp, hay bacillus . Alignment of sequences from different clones and analysis of the sequence for ORFs were carried out with DNAstar package. Sequence similarities were analyzed with the BLAST and FASTA programs.

The mice fed with the samples

Female BALB/c and C57BL/50 mice (7–8 weeks old) were gifted by Center of experimental animals in Sunyat University (China) and 5 groups were divided for 10 each one group. This work was performed according to the international guidelines

for animal laboratory care. 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 blood of the experimental mice 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 H. pylori antigens.

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°C 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 (Sigma Chemical Co.,St. Louis, Missouri, USA). The percentages of CD4_ (helper), CD4/ CD8_ (suppressor) T and B cellswere determined by the kit (Becton Dickinson) using Cell Quest software. 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 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.

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°C for 8h, plating serial dilutions at 2h intervals.

Statistical Analysis:

The results were presented as median percentage values and analyzed by the two-tailed Mann-Whitney T test. 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

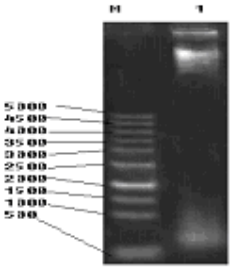


Fig 2 . DNA electrophoresis from E. coli

M:Marker, 1 Genome DNA

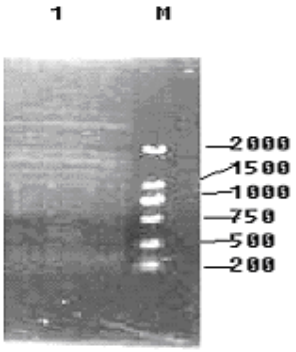


Fig 3 . Electrophoresis from E. coli total genomic DNA partially digested with Sau3AI

M:Marker;1:genomic DNA partially digested with Sau3AI

2)

Positive clone selection

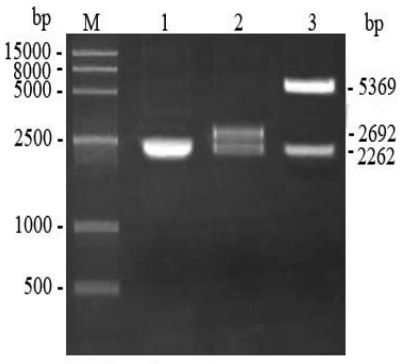
The positive clones more than 1500bp molecular weight with catalase activity on brain heart infusion (BHI) containing tannic acid and ampicillin for screening for tannase activity after transformation were selected (see Fig 4,5).

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.col by electrophoration. The results from the Figure 7 and 8 showed that the kat gene isolated in the E. coli was correct.



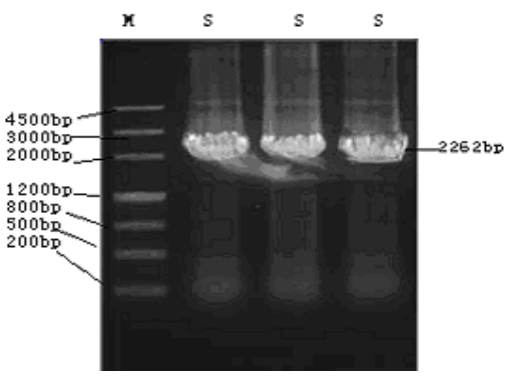


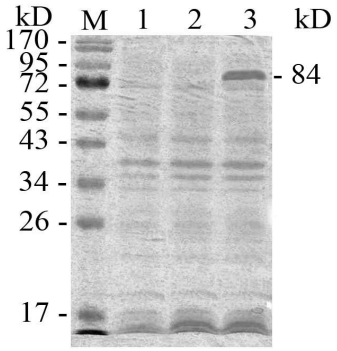
Fig.6 Identifikation of PCR product of *katE*, recombinant plasmid T-katE and pET-katE
M: DNA marker; 1: PCR product; 2: T-katE digested with *Xba*I / *Sal* I; 3: pET- katE digested with *Xba*I / *Sal* I.

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 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 (Figure8,9).



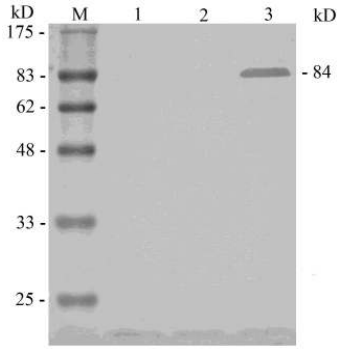


Fig. 8 SDS- PAGE analysis of KATE expressed in *E. coli* BL21（DE3）
M: protein marker; 1: BL21(DE3) with IPTG induction; 2: pET-katE clones transformed *E.coli* BL21(DE3) without IPTG induction; 3: pET-katE clones transformed *E.coli* BL21(DE3) with IPTG induction

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

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 recombinant L lactis exhibited greater salt sensitivity as compared to the parent culture NZ9000 surviving after 8 h. 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℃）. It suggests that recombinant L.lactis has ability against environment salt stress.

Sequence analysis

The open reading fram of pET-katE containg 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 as followed (see Fig 10). The protein sequence was translated as seeing fig 11.

atgtcgcaakataacgaaaagaacccakatcagcaccagtcaccactacacgattccagc… …60

gaagcgaaaccggggatggactcactggcacctgaagacggatctkatcgtccagcggct… ..120

gaaccaacaccgcctggtgcacaacctaccgccccagggagcctgaaagcccctgatacg.....180

cgtaacgaaaaacttaattctctggaagacgtacgcaaaggcagtgaaaattatgcgctg.....240

accactaatcagggcgtgcgkatcgccgacgatcaaaactcactgcgtgccggtagccgt…… 300

ggtccaactctgctggaagattttattctgcgcgagaaaatcactcactttgackatgag.....360

cgkatcccggaacgtattgttkatgcacgcggatcagcagctcacggttatttccagcca.....420

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ccagtatttgttaggttctctaccgttcagggcggcgctggctctgccgataccgtacgt.....540

gatatccgtggctttgccaccaagttctatactgaagagggtatttttgacctcgttggc.....600

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gtaaaaccagaaccgcactgggcaattccacaagggcaaagcgcccacgatactttctgg.... ..720

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gatttcaccaacgacccgttattgcaggggcgtttgttctcttataccgatacacaaatc..... ...1260

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kataatttccagcgtgacggkatgkatcgtatggggatcgacactaacccggcgaattac…. .1380

gaaccgaactcgatcaacgataactggccgcgcgaaacaccgccggggccgaaacgcggc..1440

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cgcgtggtagcaattttgcttaatgatgaagtgagatcggcagaccttctggckattctc..... .1860

aaggcgctgaaggccaaaggcgttkatgccaaactgctctactcccgaatgggggaagtg. ...1920

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gcgcgcaagtttaaagcaacaatcaaggtcgctgaccagggtgaagaagggattgcggaa… 2160

gctgacagcgccgatggtagttttatggatgaactgttaacgctgatgacagcacaccgc..... ...2220

gtgtggtcacgkattcctaagattgacaaaatcccggcgtaa.....2262

Fig.10 Determination of nucleotide sequence of kat katE gene (n1 sequence)

MSQHNEKNPHQHQSPLHDSSEAKPGMDSLAPEDGSHRPAAEPTPPGAQPTAPGSLKAPDT
RNEKLNSLEDVRKGSENYALTTNQGVRIADDQNSLRAGSRGPTLLEDFILREKITHFDHERIPER
IVHARGSAAHGYFQPYKSLSDITKADFLSDTNKITPVFVRFSTVQGGAGSADTVRDIRGFATKF
YTEEGIFDLVGNNTPIFFIQDAHKFPDFVHAVKPEPHWAIPQGQSAHDTFWDYVSLQPETLHNV
MWAMSDRGIPRSYRTMEGFGIHTFRLINAEGKATFVRFHWKPLAGKASLVWDEAQKLTGRDP
DFHRRELWEAIEAGDFPEYELGFQLIPEEDEFKFDFDLLDPTKLIPEELVPVQRVGKMVLNRNP
DNFFAENEQVAFHPGHIVPGLDFTNDPLLQGRLFSYTDTQISRLGGPNFHEIPINRPTCPYHNFQ
RDGMHRMGIDTNPANYEPNSINDNWPRETPPGPKRGGFESYQERVEGNKVRERSPSFGEYYSH
PRLFWLSQTPFEQRHIVDGFSFELSKVVRPYIRERVVDQLAHIDLTLAQAVAKNLGIELTDDQLN
ITPPPDVNGLKKDPSLSLYSIPDGDVKGRVVAILLNDEVRSADLLAILKALKAKGVHAKLLYSR
MGEVTADDGTVLPIAATFAGAPSLTVDAVIVPCGNIADIADNGDANYYLMEAYKHLKPIALAG
DARKFKATIKVADQGEEGIAEADSADGSFMDELLTLMTAHRVWSRIPKIDKIPA.

Fig 11. Sequence of amino acids from KAT protein

It is known that the kat gene is katE because the sequence is homology to katE genes

from Escherichia coli O157:H7 str and Escherichia coli str. K-12 substr, 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). 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

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	E.coli katE(cloned).seq
		97.9	24.8	26.0	26.1	25.7	25.1	25.6	2	E.coli katE Seq #2.seq
			24.9	26.2	26.5	25.5	25.0	25.1	3	E.coliGK12 katE Seq #4.seq
				24.4	26.5	27.1	24.4	26.7	4	bacillus subtilis kat E Seq #5.seq
					26.4	25.9	58.3	27.5	5	hay bacillus cat(ORF) Seq.seq
						29.0	26.1	27.3	6	Pseudomonas katE Seq #8.seq
							24.6	27.0	7	Salmonella katE Seq #6.seq
								26.5	8	Staphylococcus carnosuscat(ORF) Seq.seq
									9	yersinia kat E Seq #7.seq

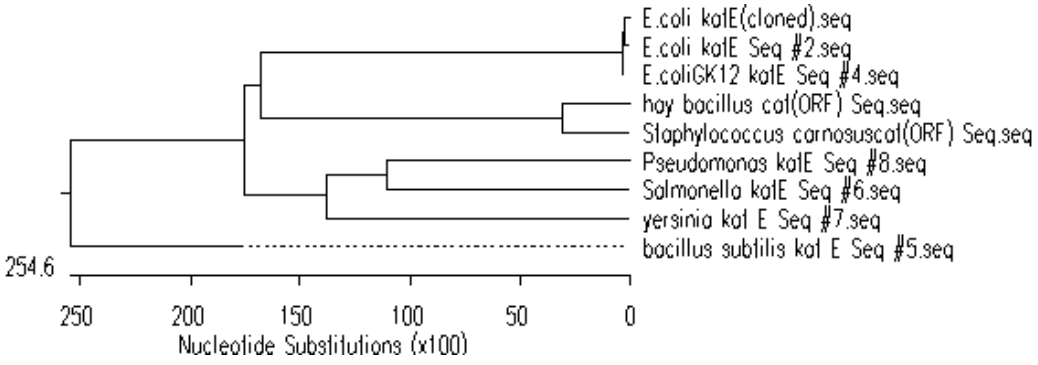


Fig 12. Phylogenetic tree analysis from kat genes of bacteria strains

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* DH5α is 7.2×10^{6}cfu/ml.

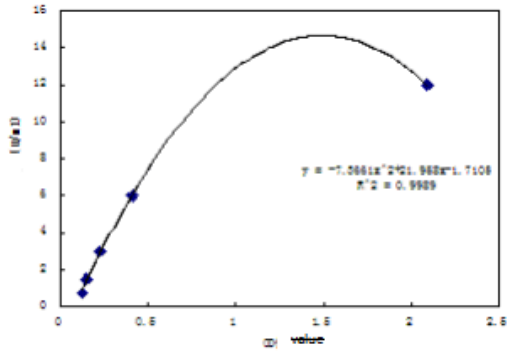


Fig 13. Standard curve of IgG

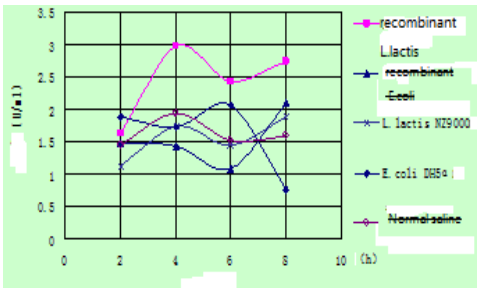


Fig 14. Comparison of IgG among the different treatments

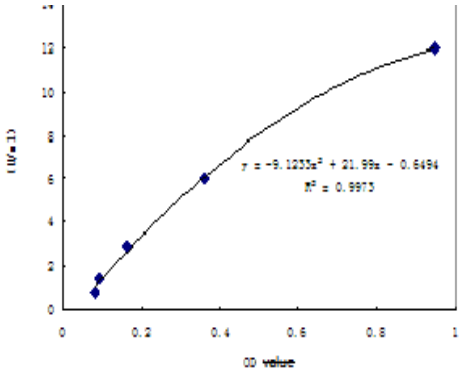


Fig 15. Standard curve of IgE

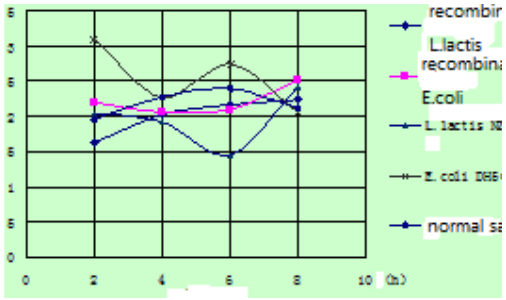


Fig 16. Comparison of IgE among the different treatments

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 Immunoglobulin IgG of the sera from mice with recombinant L.lactis NZ9000 was higher than that of other treatments, and difference is significant ($p<0.05$ see Fig 13,14 and Tab3). However, the level of IgE was no difference between them (see Fig15, 16 and Table 4).

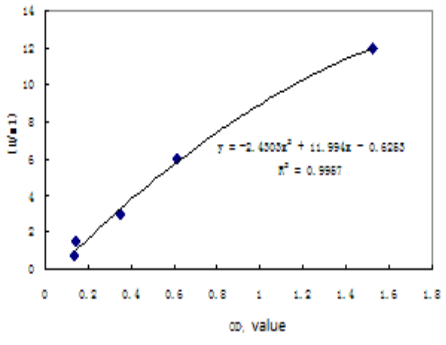
Table 3. Comparison of IgG among different treatment groups (u/ml)

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

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 difference between them (P>0.05,see Tab 5,6 and Fig 18,20).



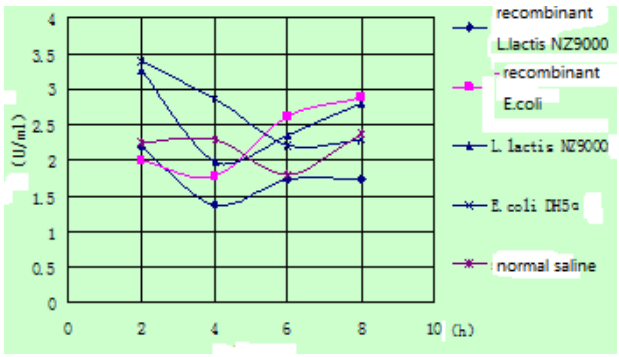


Fig. 17 Standard curve of CD4

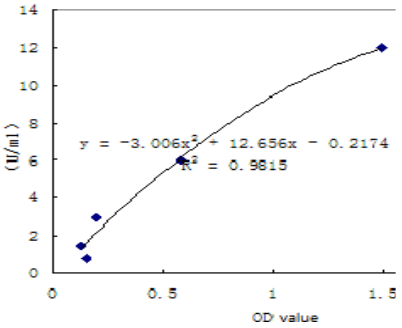


Fig. 18 Variation of CD4 concentration

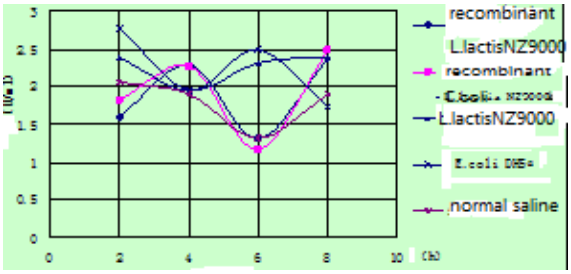


Fig. 19 Standard curve of CD8

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 showed in figure 21.

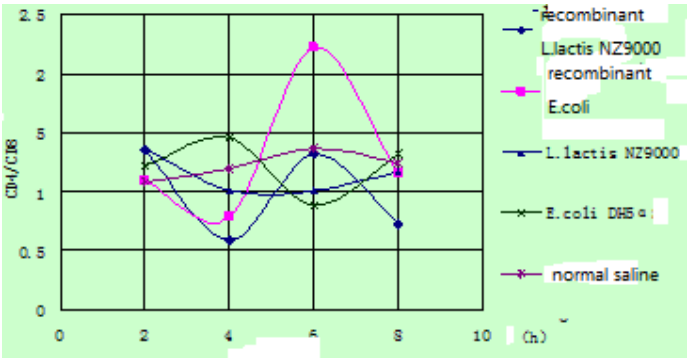


Fig. 21 Variation of CD4/CD8

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

Groups	n	2h	4h	6h	8h
Recombinant *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

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

Groups		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

Tab 7. comparison ofCD4/ CD8 among groups(u/ml)

Groups		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

Survival of the mice

Five days after the treatment, the mice with L.lactis NZ9000 and recombinant L.lactis NZ9000 were more actively 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. A critical factor is that the resistors used in electroporation should be in parallel to shorten the pulse decay time, which should stay between 20 to 40 ms for the best result. 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 (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 got 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. 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 Lactococcus 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 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, 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 is proved in this study.

The genetic improvement of Lactococcus 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 (MartirosyanA.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, it is 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 only 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.

CD4+CD8 cells were considered as T regulatory (TReg) cells. Some animals from selected time points were additionally tested for an intracellular antibody against Foxp3.The relative number of CD4 cells was no 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 is proved that no influence of recombinant L.lactis NZ9000 containing catalase gene for cellular immunity of animal could be detected in the blood.

The result in genetic performance of L. lactis reveals another industrial advantage for producing new protein and genetic engineering. It is testified by the test of animals survival. Not only it is important in dairy production, it also has potential of use as oral vaccine, foreign protein production and metabolite through genetic engineering to manipulate L. lactis in the researchers' favor.

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