

## Genetic Engineering of Bacteria that can Produce Urate Oxidase

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### Abstract

The urate oxidase gene was cloned into *Lactobacillus bulgaria* to produce urate oxidase to decompose uric acid and treat hyperuricemia. Using the *Candida utilis* urate oxidase gene sequences (uricase, E12709) on GenBank, PCR-amplified urate oxidase gene fragments were inserted into the plasmid pMG36e to construct the recombinant plasmid PMG36e-U, which was then electrotransformed into *Lactobacillus bulgaria*. We used SDS-PAGE to identify urate oxidase in the cell lysates of the genetically engineered bacteria and to measure urate oxidase activity. The urate oxidase gene was PCR-amplified from the *Candida utilis* genome. The recombinant plasmid PMG36e-U containing the urate oxidase gene was successfully electrotransformed into *Lactobacillus bulgaria*. The molecular weight of the urate oxidase subunit synthesized by the genetically engineered bacteria was approximately 34 KD based on SDS-PAGE, and the *in vitro* enzymatic activity from the bacteria preparation was up to 0.33 u/mL. Conclusion: The urate oxidase gene was cloned into *Lactobacillus bulgaria* and successfully decomposed uric acid.

**Keywords:** Gene cloning; Genetic engineering of bacteria; Urate oxidase; Lactobacillus bacteria

### Introduction

Hyperuricemia is not only a direct cause of gout and related diseases but also an independent risk factor [1-3] for certain kidney and cardiovascular diseases. Therefore, it is essential to reduce the level of uric acid in blood and tissues to prevent and treat many uric acid-related diseases.

Because the human body cannot synthesize urate oxidase by itself, reducing the level of uric acid requires long-term or even lifelong treatment. However, currently various uric acid-lowering drugs cause different degrees of damage to the human body and thus are not suitable for long-term treatment. A probiotic that can yield a large amount of urate oxidase and be transplanted into the intestines to constantly produce urate oxidase to catalyze uric acid would have important economic and ecological value. Thus, we cloned the urate oxidase gene into *Lactobacillus bulgaria* to assess urate oxidase production and activity as an alternative drug therapy for hyperuricemia.

### Materials

#### Strains and plasmids

*Candida utilis* yeast (*Candida utilis*, AS2.120) strains were provided by the China General Microbiological Culture Center. The PGM36e *Lactobacillus* expression vector was used for this experiment. The pMD-18T vector was provided by Takara. *E.coli* DH5 $\alpha$  was used in our laboratory.

#### Enzymes and reagents

The restriction enzymes XbaI and HindIII, pyrobest Taq DNA polymerase, DNA ligation kit and low-weight protein marker were provided by Takara. SDS and analytically pure uric acid were provided by Sigma. The primers were synthesized by Shanghai Biological Engineering Technology Co., Ltd.

### Methods

We used a DNA extraction kit to extract the *Candida utilis* genome. Primer-1 and Primer-2 were designed according to the *Candida utilis* urate oxidase gene (GenBank serial number E12709, nt1-nt912) reported in the literature. The upstream Primer-1 was 5' AAT CTA

GAA TGT CAA CAA CGC TCT CAT CAT CAT 3', with the restriction site Xba I added to the 5' end. The downstream Primer-2 was 5' AGA AGC TTT TAC AAC TTG GTC TTC TCC TTA 3', with the Hind III restriction site added to the 5' end. For PCR amplification, the reaction conditions were as follows: 95°C for 30 seconds, 60°C for 30 seconds, and then 72°C for 60 seconds, for a total of 25 cycles.

#### Construction of the plasmid pMG36e-U containing the urate oxidase gene

The PCR amplification products were ligated into the pMD18-T vector to construct the recombinant plasmid pMD18-T-U, which was transformed into *E. coli* DH5 $\alpha$ . Positive clones were selected for sequencing and identified by performing an enzyme digestion with Xba I and Hind III. The urate oxidase gene construct was recycled using a double digestion. A DNA Ligation Kit was used to ligate the urate oxidase gene collected by the double digestion into the plasmid pMG36e, which had been digested with the same restriction enzymes. The molar ratio of the urate oxidase gene fragments to pMG36e was approximately 3:1 to construct the recombinant plasmid pMG36e-U, which was transformed into DH5 $\alpha$  for enzyme screening and identification. The recombinant plasmid was extracted to be electrotransformed into *Lactobacillus* bacteria competent cells to screen for positive strains [1].

#### Measurement of urate oxidase enzyme activity in the recombinant bacteria

First, 2.5 mL of 0.001% uric acid solution that was mixed with 0.1 mol/L boric acid buffer solution (pH 8.5) was placed into a 3-mL cuvette. Then, 0.5 mL of diluted enzyme solution was added and mixed

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Received March 02, 2012; Accepted August 28, 2012; Published September 04, 2012

Citation: Cheng X, Yang B, Liu D, He LJ, Chen G (2012) Genetic Engineering of Bacteria that can Produce Urate Oxidase. Intern Med 2:114. doi:10.4172/2165-8048.1000114

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appropriately. Then, the absorbance changes at 293 nm and 25°C were continuously measured. The following formula was used: Activity (u/ml) =  $(\Delta OD_{293nm} \times df \times 0.5) / t$ . Note that df refers to the enzyme dilution factor,  $OD_{293nm}$  refers to the absorbance change, and t refers to time (min). An enzyme activity unit was defined as the amount of enzyme required to catalyze 1  $\mu$ mol of uric acid oxidation per minute at 25°C and pH 8.5. The amount of enzyme was measured using the Bradford dye-binding assay.

## Results

### PCR amplification of the uricase gene

We used Primer-1 and Primer-2 to amplify the uricase gene from *Candida utilis* genome through PCR. Then, 0.9 Kb of the fragment was electrophoresed through a 1% agarose gel, as shown in figure 1. The vertical axis represents the molecular weight of DNA Marker DL2000, and the abscissas indicate the lanes. All of the gene figures in this paper use the same axes. In lane 1, an arrow indicates that the size of the amplified fragment is the same as the expected gene fragment. Double distilled water used for PCR amplification of the template control did not yield the gene fragment (Figure 1).

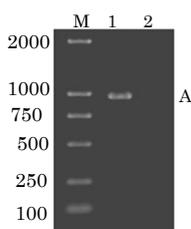
The purified PCR products were ligated into the pMD18-T vector to construct the recombinant plasmid MD18-T-U, which was transformed into *E. coli* and screened. Then, the gene fragment was extracted from the recombinant plasmid using Xba I and Hind III to sequence the recombinant pMD18-T-U and identify the double restriction enzyme digestion. The sequencing results are identical to those for the GenBank urate oxidase gene. The arrow in lanes 1, 2, and 3 of figure 2 identifies gene fragments with a size of approximately 0.9 Kb, which were identified by Xba I and Hind III digestion of pMD18-T-U. In lane 4, a gene fragment of approximately 0.9 Kb was not obtained using Xba I and Hind III digestion of pMD18-T.

### Identification of recombinant *Lactobacilli* containing the urate oxidase gene

The PCR-amplified fragment was ligated into the plasmid PMG36e and transformed into *E. coli* DH5 $\alpha$ . The recombinant *Escherichia coli* containing urate oxidase was identified by PCR. The extracted recombinant plasmid was electrotransformed into lactic acid bacteria. The recombinant plasmid was extracted from the screened recombinant *Lactobacilli*. The extracted plasmid was analyzed by restriction enzyme digestion and PCR. As shown in lanes 1, 2, and 3 in figure 3, the arrow points to the PCR-amplified fragments with a size of approximately 0.9 Kb. Lane 4 used the original *Lactobacillus* as a template control, and no gene fragment was amplified.

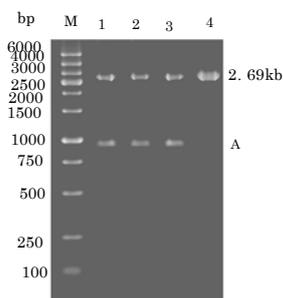
### Determination of creatinine hydrolase activity

The Bradford method was used to determine the protein content



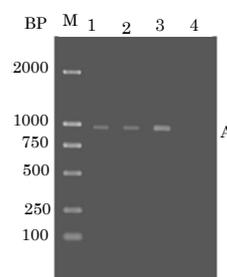
M: DNA Marker DL2000; Lane 1 is the PCR-amplified uric acid oxidase gene; Lane 2 is the PCR amplification using the water template.

Figure 1: Uric acid oxidase gene electrophoresis of the PCR amplification.



M represents the Wide Range DNA Marker. Lanes 1, 2 and 3 represent the gene fragments obtained from pMD18-T-U digested with Xba I and Hind III. In lane 4, a gene fragment of approximately 0.9 Kb was not obtained using Xba I and Hind III digestion of pMD18-T.

Figure 2: Recombinant *E. coli* pMD18-T-C extraction using enzyme digestion.



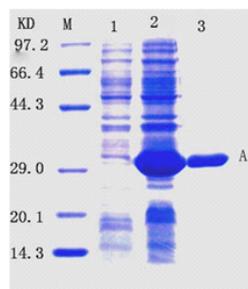
M: DNA Marker DL2000. Lanes 1, 2 and 3 represent gene fragments with a size of approximately 0.9 Kb amplified using the recombinant *Lactobacillus* Lb-PMG36e-U template. Lane 4 shows the PCR product amplified using the Lb template.

Figure 3: PCR identification of recombinant *Lactobacilli*.

in each specimen. Then, the uric acid oxidase activity in each specimen was calculated using the table 1. Both strains are listed in the table 1 (*Candida utilis* and the recombinant lactic acid bacteria Lb-PMG36e-U). The crude enzyme solution and fiber column-purified protein concentration were used to measure the enzyme activity and enzyme-specific activity, respectively. The values are shown in the table 1, which shows that the original *Candida utilis* strain had a relatively high crude enzyme activity, which is 0.51 u/mL. The activity of the crude recombinant lactic acid bacteria with urate oxidase was slightly lower. However, after purification using the fiber column, the activity reached 0.39 u/mL, and the specific activity was as high as 0.48 u/mL.

### Electrophoresis analysis of the crude enzyme and purified products from SDS-PAGE (12%) from the recombinant lactic acid bacteria Lb-PMG36e-U

SDS-PAGE analysis of the Lb-PMG36e-U, which contains urate oxidase, revealed that the recombinant protein molecular weight was approximately 34 KD, which is consistent with the theoretical molecular weight of 303 amino acids deduced from the gene sequence of urate oxidase and which is the same subunit molecular weight as that of the original urate oxidase strain (lane 2). The expression of lactic acid bacteria containing pMG36e but without specific activity is shown in lane 1. Lane 3 shows the results for purified uric acid oxidase. SDS-PAGE analysis revealed that the molecular weight of the protein in the crude enzyme solution from the recombinant lactic acid bacteria Lb-PMG36e-U, containing urate oxidase, was approximately 34 KD (arrow A in Figure 4).



M is the low molecular weight marker. Lane 1 is the crude enzyme solution from the recombinant lactobacillus Lb-pMG36e. Lane 2 is the crude enzyme solution from the recombinant lactobacillus Lb-PMG36e-U. Lane 3 is the uricase purified using a DEAE DE52 column.

**Figure 4:** SDS-PAGE (12%) analysis of recombinant urate oxidase and the purified products of the SDS-PAGE.

Strain	Composition	Protein concentration mg/mL	Enzymatic activity u/ mL	Specific enzymatic activity u/mg
<i>Candida utilis</i>	Crude enzyme	1.18	0.51	0.43
	DEAE fiber column purified	0.84	0.62	0.74
Lb-PMG36e-U	Crude enzyme	1.12	0.33	0.29
	DEAE fiber column purified	0.81	0.39	0.48

**Table 1:** The activity in the different strains.

## Discussion

Urate oxidase (uricase) [4-7] is an enzyme that metabolizes urate acid into allantoin through purine metabolism. Urate oxidase synthesis is regulated by the uricase gene and is the critical enzyme for uric acid metabolism. A study of the urate oxidase cDNA homology sequences in humans, apes and other primates found that uricase is composed of four exons. There are two nonsense mutations (CGA/AGA- + TGA) at 33 and 187, which lead to an early termination of the coding region. Therefore, humans and other primates cannot produce uric acid oxidase, and uric acid becomes the end product of purine metabolism [8,9]. Approximately 70% of uric acid is excreted in the urine, and 30% is excreted from the gut. Excessive uric acid production [10] or insufficient uric acid excretion leads to hyperuricemia. When uric acid reaches a maximum concentration of saturation, crystal deposition occurs on the vascular wall, causing vascular endothelial cell injury and possible expression of platelet growth factor, leading to primary and vascular smooth muscle cell proliferation and the hardening of arteries and hypertension.

This process in the kidneys leads to glomerular and tubulointerstitial lesions, resulting in uric acid stones. In the joints, this same process leads to gout arthritis. The key to disease control is safely reducing serum uric acid. The current uric acid-lowering drugs mainly function in two ways: by promoting uric acid excretion or by reducing uric acid synthesis. Usually uric acid decreases during treatment, but once the drug is discontinued, the uric acid level will rebound. Therefore, controlling uric acid requires a long-term medication. However, many patients cannot tolerate the toxicity of these drugs, and their disease worsens. Thus, in recent years, there have been many studies on the metabolism of uric acid by enzymes, including enzyme purification and artificial synthesis of uricase [11-16]. There are some domestic reports about cloning the uricase gene into *E. coli* to obtain strains that can yield a large amount of uricase. In the United States, PEG-uricase

has been synthesized [17], and it has been shown to significantly lower uric acid, but it can only be used for a short period of time and has side effects that are common for biological agents.

Because of confusion over the degradation of uric acid, this project aimed to build a strain of high-yield urate oxidase and to implant this strain in the intestinal mucosa to continuously decompose uric acid. There are approximately 30 genera and 500 types of bacteria that inhabit the human intestines [18]. The number of these bacteria exceeds the total number of human cells. These bacteria colonize in the intestine and continually proliferate, renew and metabolize throughout the life of a human. *Lactobacillus* is an important member of the normal intestinal microbial flora and exists throughout the entire life of the host. *Lactobacillus* is a probiotic [19-24] of the human intestinal tract that lives in the intestinal lining; it can enhance immunity and suppress pathogens, and it may synthesize vitamins and lower cholesterol [25,26]. This experiment used *Lactobacillus* because it is the most frequently used bacteria for yogurt fermentation and because it is most beneficial for human health. Scholars in China and worldwide have adopted *Lactobacillus* to express heterologous protein [27-34]. Therefore, *Lactobacillus bulgaricus* was chosen as the target bacteria for cloning the urate oxidase gene.

Some research has shown that the yeast *Candida utilis* has a high-yielding urate oxidase gene [35,36]. In this experiment, based on the known *Candida utilis* urate oxidase gene sequence and the PCR-amplified urate oxidase gene's size of 0.9 kb, the amplified fragment was ligated into the sequencing plasmid pMD18-T. After sequence identification, the size of the amplified gene fragment was identical to that of the urate oxidase gene (sequence number E12709) in GenBank, and pMD18-T-U double digestion also confirmed the identity of the uric acid oxidase gene fragment. The pMG36e plasmid is a shuttle expression plasmid that can be copied in *Escherichia coli* and *Lactobacillus*. In addition, this plasmid contains an erythromycin resistance gene, so it is easy to use for screening and identification.

In this study, pMG36e was successfully ligated with the enzyme-digested, PCR-amplified urate oxidase gene fragment to construct recombinant pMG36e-U containing urate oxidase, which was electrotransformed into *Lactobacillus* bacteria [37,38]. Screening for the recombinant lactobacilli containing the recombinant plasmid was performed by using MRS Lactobacillus induction medium containing a final concentration of 200 µg/ml erythromycin and 0.01% uric acid. Specific primers were created to identify by PCR amplification. The amplified gene fragment was the same size as the urate oxidase gene, thus proving that recombinant lactic acid bacteria containing the urate oxidase gene can be successfully constructed. The urate oxidase expressed by the recombinant *Lactobacillus* after induction exhibited uric acid oxidase activity, with a crude enzyme activity was 0.33 u/ml. The molecular weight of the enzyme was approximately 34 KD, as revealed by SDS-PAGE analysis, which is consistent with the theoretical molecular weight of the 303-amino acid protein deduced from its gene sequence, thus proving that bacteria can be genetically engineered to produce urate oxidase. However, the activity of the urate oxidase expressed by the recombinant lactic acid bacteria was lower than the activity of the original *Candida utilis*. This result might have been due to the low copy number of pMG36e, and gene modification may help to obtain higher enzyme activity from the recombinant lactic acid bacteria. Regardless, the success of this study has laid the foundation for further development of orally ingested, genetically engineered bacteria that can metabolize uric acid by gut colonization.

## Acknowledgements

This work is supported by a research grant (30871168) from the National Natural Science Foundation of China. I want to express my thanks to all of my colleagues who helped me complete the research project design, the experiment and the composition of this manuscript.

## Conflicts of Interest

The author has no conflicts of interest that are directly relevant to the content of the review.

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