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Cloning and Expression of a Full-Length Glutamate Decarboxylase Gene from *Lactobacillus plantarum*

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Abstract

In order to investigate the molecular mechanism of γ-aminobutyric acid (GABA) production in lactic acid bacteria, we cloned a glutamate decarboxylase (GAD) gene from *Lactobacillus plantarum* using polymerase chain reaction (PCR). One PCR product DNA was obtained and inserted into a TA cloning vector with a T7 promoter. The recombinant plasmid was used to transform *E. coli*. The insertion of the product was confirmed by *Eco*RI digestion of the plasmid purified from the transformed *E. coli*. Nucleotide sequence analysis showed that the insert is a full-length *Lactobacillus plantarum* GAD and that the sequence is 100% and 72% identical to the regions of *Lactobacillus plantarum* GAD and *Lactococcus lactis* GAD sequences deposited in GenBank, accession nos: NP786643 and NP267446, respectively. The amino acid sequence deduced from the cloned *Lactobacillus plantarum* GAD gene showed 100% and 68% identities to the GAD sequences deduced from the genes of the NP786643 and NP267446, respectively. To express the GAD protein in *E. coli*, an expression vector with the GAD gene (pkk/GAD) was constructed and used to transform the UT481 *E. coli* strain and the expression was confirmed by analyzing the enzyme activity. The *Lactobacillus plantarum* GAD gene obtained may facilitate the study of the molecular mechanisms regulating GABA metabolism in lactic acid bacteria.

Key words: glutamate decarboxylase, gene, cloning, expression, *Lactobacillus plantarum*

INTRODUCTION

γ-Aminobutyric acid (GABA) is a nonprotein amino acid known to function as a major inhibitory neurotransmitter of the central nervous system (1,2). GABA is produced by the irreversible decarboxylation of glutamic acid by glutamate decarboxylase. GAD and GABA occur ubiquitously among plants and animals ranging from microbes to higher class organisms (3).

In animals, GABA activates the cerebral blood flow, increases the amount of air supply, is known to accelerate metabolic function, and is clinically as a medicine to ameliorate sequela of stroke and cerebral artery sclerosis (4). Decreased cerebral GABA concentrations and GAD activity induce epilepsy, parkinsonism, schizophrenia. Furthermore, it is reported that the blood GABA concentration is lower in alcoholics compared to non-alcoholics (1,5).

In plants, GAD is known to be a cytoplasmic enzyme that is primarily induced by increased concentrations of hydrogen ion, Ca^{2+}, and by calmodulin (CaM) and related factors (6). Several environmental factors can impact plants to increase the intracellular calcium concentrations, and as the hydrogen ion concentration increases, the calcium that is joined with CaM activates GAD thereby increasing the intracellular GABA concentration (7,8).

In microbes, the roles of GABA and GAD are not clearly elucidated, but there are reports that GABA has a role in the spore budding of *Bacillus megaterium* (9), and that it is also required for maintaining resistance to acid pH. When *Bacillus megaterium* undergoes the process of spore budding, the activity of the GAD enzyme suddenly increases resulting in a marked increase in GABA concentrations (10). When GABA is produced in *Lactococcus lactis* and *E. coil*, it has been reported that the glutamate inside the cell uses one molecule of H^+ in the process of decarboxylation, and that the utilization of H^+ can help control the intracellular pH (10,11). Therefore, even though the environment around the cell becomes acidic, the intracellular pH remains stable.

Lactic acid bacteria exists not only in dairy products such as yogurt, cheese, butter and in meat products such as fermented sausage, but is also present in Korean traditional fermented products such as kimchi products, salted preserves, and sauces, and exists as a useful strain

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in the intestines of humans and animals. Some species of *Bifidobacterium* and *Lactobacillus* are recognized as probiotics, and are widely used in yogurt products. The beneficial effects of increasing lactic acid bacteria in the intestines by ingesting these strains include: bowel decontamination, amelioration of undesirable intestinal flora, decreased serum cholesterol, acceleration of lactose digestion, and reduced incidence of colon cancer (12,13). Probiotics can only be effective if they have resistance to acid and bile, permitting them to remain viable as they pass through the stomach and colonize the intestine where they can compete with and inhibit the growth of harmful microbes. Currently, functions of GABA and GAD in lactic acid bacteria are not well understood, and insufficient data exist to clearly describe their roles in the bacteria and their impacts on human health in probiotic strains of bacteria; therefore, more research is need to elucidate the roles of GABA and GAD in probiotic microorganims (14).

This study was performed to confirm the existence of a GAD gene in *Lactobacillus plantarum*, to determine the base sequence of the gene, predict the amino acid sequence, and to obtain and characterize the functional protein. This information will facilitate the further study of the mechanisms involved in the increased production of GABA in lactic acid bacteria, and the basis to create a high GABA-containing lactic acid bacteria.

MATERIALS AND METHODS

Bacterial strain and materials

The lactic acid bacteria used was *Lactobacillus plantarum* (KCTC3015) which was stored in a cryogenic freezer (-70°C), in the laboratory. To obtain the template DNA for the PCR, the genomic DNA was purified using the Promega (Madison, Wisconsin, USA) Wizard DNA purification kit according to the method described in the product manual. Biometra PCR machine (Tampa, Florida, USA) was used for PCR amplification, a Promega TA cloning kit was used, the DNA polymerase was purchased from Takara Biochemicals (Japan), and Difco media (Detroit, MI, USA) was used for cell culture. All other chemicals were of high quality analytical grade.

Conditions for PCR and cloning

The primers used to clone the *Lactobacillus plantarum* GAD gene are shown in Table 1. The PCR reaction was tested by using the reacting liquid, 10 mM Tris-HCl (pH 9.0) which contained DNA 100 ng, each primer 200 ng, dNTPs 0.2 mM, $MgCl_2$ 2.0 mM, KCl 50 mM, 0.1% Triton X-100, and reacting it for 5 minutes at 95°C, then, 95°C 30 sec, 60°C 30 sec, 72°C 1 min 30 sec for 30 cycles of denaturation, annealing, and extension. The formation of PCR product was checked by performing a 1% agarose gel electrophoresis, and the amplified DNA fragment was ligated into the TA cloning vector of pGEM T-Easy vector by T4 ligase (Takara).

Transformation of *E. coli* by the recombinant DNA and confirmation

The agent, used for the transformation of the competent *E. coli* cells by the TA cloning vector was the F' One Shot™ kit (Invitrogen, Carlsbad, California, USA). The recombinant DNA from screened was used to transform *E. coli* cultured in the LB medium that contained 50 μg/mL ampicillin and 25 μg of X-Gal (10 mg/mL stock in dimethylformamide), and the transformed *E. coli* were chosen, and further cultured in liquid LB culture medium containing 50 μg/mL ampicillin. The ratio of purified cultured *E. coli* cells to plasmid DNA, was determined using a Promega Wizard DNA purification kit (Madison, Wisconsin, USA), following the specific procedures for isolation of purified plasmid DNA from transformed *E. coli* as described in the product manual. The refined plasmid DNA was confirmed in the PCR product by reacting the limiting enzyme at 37°C for 2 hours, then separating using 1% agarose electrophoresis.

DNA sequencing

The analysis of DNA base sequence was operated by the dideoxynucleotide termination procedures that use synthesis of oligonucleotide primers and a dsDNA Cycle System (Perkin Elmer, USA). Amino acid sequences that were different from those predicted from base sequences were analyzed by using the DNASIS program (Hitachi Software Engineering Co., USA) and Clustal W (1.81).

Enzyme assay

E. coli (UT481) that were transformed by the pKK/GAD were inoculated into 5 mL LB/amp liquid culture medium, cultured overnight at 37°C, and then 100 mL of LB/amp liquid culture medium was inoculated with 2 mL of the culture liquid and cultured until its OD_{550} was 0.8. After centrifuging at 4,000 rpm for 15 minutes, the cell pellet was harvested and floated in the cell lysis

Table 1. Primers used in the PCR amplification of glutamate decarboxylase gene from genomic DNA of *Lactobacillus plantarum*

Primer	Sequence	%GC
Forward (27 mer)	CATATGGCAATGTTATACGGTAAACA	34.6
Reverse (30 mer)	GGAATTCCTCAGTGTGTGAATCCGTATTTC	43.3

buffer (50 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 2 mM β-mercaptoethanol, 10% glycerol and 1 mM PMSF). After decomposing the cells with lysozyme and sonication treatment, the lysed cells were centrifuged to obtain the equal liquid (15). After adding PMSF (final concentration = 1 mM) to the equal liquid, GAD activity was assayed and the protein concentration measured. The remainder of the solution was preserved and stored at -20°C. GAD activity was estimated by the procedure of Ling and Snedden (16,17), which measures the production of $^{14}CO_2$ from L-[1-^{14}C]Glu substrate. In this procedure, to the reaction mixture consisting of 100 mM bis-Tris, pH 7.0, 0.5 mM pyridoxal-5'-phosphate, 1 mM DTT, 10% (v/v) glycerol, 5 mM $CaCl_2$, 2.5 mM L-glutamate, a certain amount of crude enzyme extracts is added and reacted in a water bath at 30°C for 40 minutes. The reaction proceeds in a flask equipped with a CO_2 trap (Kontes, USA), after 40 minutes, is halted by adding a stop solution (18 N H_2SO_4:DW = 1:1), ending the reaction and the enzyme activity is calculated from the amount of $^{14}CO_2$ captured by the CO_2 trap (0.1 N NaOH 0.4 mL) in a liquid scintillation counter. The protein content was measured by the Bradford method (18).

RESULTS AND DISCUSSION

This study was performed to obtain the GAD gene from lactic acid bacteria for studying the regulation of GABA production with the ultimate goal of developing a lactic acid bacteria capable of producing high quantities of GABA. *Lactobacillus plantarum* GAD gene was isolated from the genomic DNA, purified and then amplified by PCR. The primers that were used to amplify the GAD gene from *Lactobacillus plantarum* were synthesized in the direction of sense and antisense from the *Lactobacillus plantarum* GAD gene from the 1~27 bp and 1380~1410 bp segments shown in the Gene Bank (Table 1). By using the synthesized primers and refined genomic DNA, the amplified PCR product was found in a single band (Fig. 1), and recombinant DNA was prepared by inserting this into the pGEM T-Easy vector (Promega). The existence of the DNA fragment was confirmed in the plasmid DNA purified from the transformed *E. coli* by the recombinant DNA, which was cut by the restriction enzyme *Eco*RI (Fig. 2). Also, it was analysed by the dideoxynucleotide termination sequencing procedure using the refined plasmid DNA for comparing the base sequence of the fragment of PCR DNA to the synthesized oligonucleotide primers and dsDNA from the Cycle Sequencing System (Perkin Elmer, USA). By the results of the analysis, the cloned *Lactobacillus plantarum* GAD has a full length of 1,410 bp (Fig. 3), and

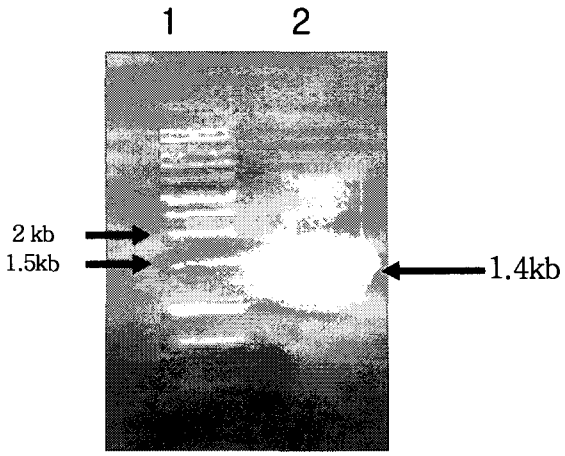

Fig. 1. PCR product of glutamate decarboxylase gene from *Lactobacillus plantarum*.
PCR product was analyzed by 1% agarose gel electrophoresis and the gel was stained with ethdium bromide. Lane 1, DNA molecular weight marker; lane 2, DNA fragment amplified from DNA template with the primers. The arrow indicates the position of the PCR product.

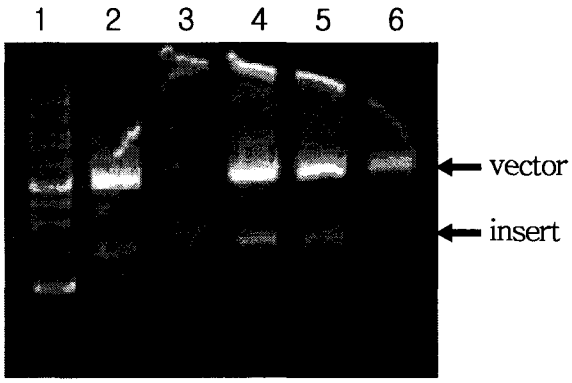

Fig. 2. Restriction digestion of the inserted *Lactobacillus plantarum* PCR product into vector.
The PCR product was ligated into pGEM T-Easy vector and the recombinant pGEMT – Easy vector was used to tranform competent *E. coli* cells. Lane 1, DNA molecular weight marker; lane 3,6, undigested plasmids from selected colonies; lane 2,4,5, *Eco*RI digested plasmids from the selected colonies.

this was the full-length GAD open reading frame. Comparing the base sequence of the cloned *Lactobacillus plantarum* GAD and the reported base sequence of GAD, the *Lactobacillus plantarum* GAD (NP 786643) exhibited a 100% homology, and *Lactococcus lactis* GAD (NP267446) exhibited 74% homology. However, in the comparison with *Clostridium perfringens* GAD (NP562974), *Escherichia coli* GAD (NP753818) and *Bacteroides thetaiotaomicron* GAD (NP811483), a clear homology could not be found (Fig. 3). Also the amino acid sequence analogized from the base sequence and reported for GAD, when compared to the *Lactobacillus plantarum* GAD, exhibited 100% homology, and for *Lactococcus lactis* GAD exhibited 66% homology. But with *Clostridium*

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M A M L Y G K H N H E A E E Y L E P V F  20
ATGGCAATGTTATACGGTAAACACAATCATGAAGCTGAAGAATACTTGGAACCAGTCTTT  60

G A P S E Q H D L P K Y R L P K H S L S  40
GGTGCGCCTTCTGAACAACATGATCTTCCTAAGTATCGGTTACCAAAGCATTCATTATCC  120

P R E A D R L V R D E L L D E G N S R L  60
CCTCGAGAAGCCGATCGCTTAGTTCGTGATGAATTATTAGATGAAGGCAATTCACGACTG  180

N L A T F C Q T Y M E P E A V E L M K D  80
AACCTGGCAACTTTTTGTCAGACCTATATGGAACCCGAAGCCGTTGAATTGATGAAGGAT  240

T L A K N A I D K S E Y P R T A E I E N  100
ACGCTGGCTAAGAATGCCATCGACAAATCTGAGTACCCCCGCACGGCCGAGATTGAAAAT  300

R C V N I I A N L W H A P D D E H F T G  120
CGGTGTGTGAACATTATTGCCAATCTGTGGCACGCACCTGATGACGAACACTTTACGGGT  360

T S T I G S S E A C M L G G L A M K F A  140
ACCTCTACGATTGGCTCCTCTGAAGCTTGTATGTTAGGCGGTTTAGCAATGAAATTCGCC  420

W R K R A Q A A G L D L N A H R P N L V  160
TGGCGTAAACGCGCTCAAGCGGCAGGTTTAGATCTGAATGCCCATCGACCTAACCTCGTT  480

I S A G Y Q V C W E K F C V Y W D V D M  180
ATTTCGGCTGGCTATCAAGTTTGCTGGGAAAAGTTTTGTGTCTACTGGGACGTTGACATG  540

H V V P M D E Q H M A L D V N H V L D Y  200
CACGTGGTCCCAATGGATGAGCAACACATGGCCCTTGACGTTAACCACGTCTTAGACTAC  600

V D E Y T I G I V G I M G I T Y T G Q Y  220
GTGGACGAATACACAATTGGTATCGTCGGTATCATGGGCATCACTTATACCGGTCAATAT  660

D D L A A L D K V V T H Y N H Q H P K L  240
GACGACCTAGCCGCACTCGATAAGGTCGTTACTCACTACAATCATCAGCATCCCAAATTA  720

P V Y I H V D A A S G G F Y T P F I E P  260
CCAGTCTACATTCACGTCGACGCAGCGTCAGGTGGCTTCTATACCCCATTTATTGAGCCG  780

Q L I W D F R L A N V V S I N A S G H K  280
CAACTCATCTGGGACTTCCGGTTGGCTAACGTCGTTTCGATCAACGCCTCCGGGCACAAG  840

Y G L V Y P G V G W V V W R D R Q F L P  300
TACGGTTTAGTTTATCCCGGGGTCGGCTGGGTCGTTTGGCGTGATCGTCAGTTTTTACCG  900

P E L V F K V S Y L G G E L P T M A I N  320
CCAGAATTAGTCTTCAAAGTTAGTTATTTAGGTGGGGAGTTGCCGACAATGGCGATCAAC  960

F S H S A A Q L I G Q Y Y N F I R F G M  340
TTCTCACATAGTGCAGCCCAGCTCATTGGACAATACTATAATTTCATTCGCTTTGGTATG 1020

D G Y R E I Q T K T H D V A R Y L A A A  360
GACGGTTACCGCGAGATTCAAACAAAGACTCACGATGTTGCCCGCTACCTGGCAGCCGCT 1080

L D K V G E F K M I N N G H Q L P L I C  380
CTGGATAAAGTTGGTGAGTTTAAGATGATCAATAACGGACACCAACTCCCCCTGATTTGT 1140

Y Q L A S R E D R E W T L Y D L S D R L  400
TACCAACTAGCCTCGCGCGAAGATCGTGAATGGACCCTTTATGATTTATCGGATCGCCTA 1200

L M N G W Q V P T Y P L P A N L E Q Q V  420
TTAATGAACGGTTGGCAAGTACCAACGTATCCTTTACCTGCTAATCTGGAACAACAAGTC 1260

I Q R I V V R A D F G M N M A H D F M D  440
ATCCAACGAATCGTCGTTCGGGCTGACTTTGGCATGAATATGGCCCACGATTTCATGGAT 1320

D L T K A V H D L N H A H I V Y H H D A  460
GACCTGACCAAGGCTGTCCATGACTTAAACCACGCCCACATTGTCTATCATCATGACGCG 1380

A P K K Y G F T H *  469
GCACCTAAGAAATACGGATTCACACACTGA 1410
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Fig. 3. Nucleotide and deduced amino acid sequence of the cloned *Lactobacillus plantarum* GAD gene.
The predicted amino acids sequence (single-letter abbreviation) is shown above the nucleotide sequence.

perfringens GAD, *Escherichia coli* GAD and *Bacteroides thetaiotaomicron* GAD, the sequence homology was less than for *Lactococcus lactis* GAD.

Among the reported cases of GAD in plants, animals, and microbes, there was a case in which a pyridoxal 5′-phosphate (PLP)-binding domain was discovered, and the domain included a lysine residue that was essential for binding, so it is designated PLP-lysine (15). In this study, the cloned *Lactobacillus plantarum* GAD contained a similar HKY sequence including lysine (K)280 (Fig. 4B).

To confirm the presence of an active GAD gene product in the *E. coli* cells transformed with the recombinant vector (pKK/GAD) containing the GAD gene, GAD enzyme activation detection was attempted. As in Fig. 5, the *Lactobacillus plantarum* GAD manifested in the *E. coli* cell, and it showed a high enzyme activation compared to the accommodation cell. Since we used non-refined crude enzyme extracts, the difference was not big; however, it will be possible to obtain much more data on the enzyme if there are specific studies performed after obtaining a purified enzyme for further characterization of the enzyme.

There are reports of microbial GABA contributing to the spore budding of *Bacillus megaterium*. In the process of budding, the GAD enzyme activation suddenly increases, which is further manifest by the observation of increases in the concentration of GABA. It has also been reported that GABA contributes to the resistance to acid pH in *Lactobacillus lactis* and *E. coli* (10,11). During GABA production, GAD must use a single molecule of H^+, and it has been postulated that even in acidic conditions, this can help maintain a stable pH inside the cell, making cell survival possible. Considering its role in pH resistance, bacteria with a high GAD activity can be expected to be characteristically high resistant to acid, which is requirement for probiotics to be fully functional since they must pass through the stomach and survive in the intestines. Further studies are needed to test the resistance to acid and bile of lactic acid bacteria having high contents of GABA and GAD.

In animals, GABA is well known to be the main inhibitory neurotransmitter of the central nervous system. GABA is related to the control of many physiological mechanisms. In animals, GABA increases cerebral blood flow, increases the amount of air supply to the lungs, and is known to accelerate the metabolic function. Also GABA is known to decrease blood pressure, so it is a substance that is pharmacologically active. It is reported that gabaron tea and red rice yeast are GABA-containing products, and also have hypotensive activity (19). It has been reported that germinated brown rice extracts that are rich in GABA compared to non-germinated extracts are clearly for increasing immune response, and for blocking the proliferation of some cancer cells, and even they promotes the killing of cancer cells (20,21). Now *Lactobacillus* and other GABA-producing lactic acid bacteria seem to show promise for applications in dairy products and health products that can exploit the functional properties of GABA, for producing highly effective functional food products.

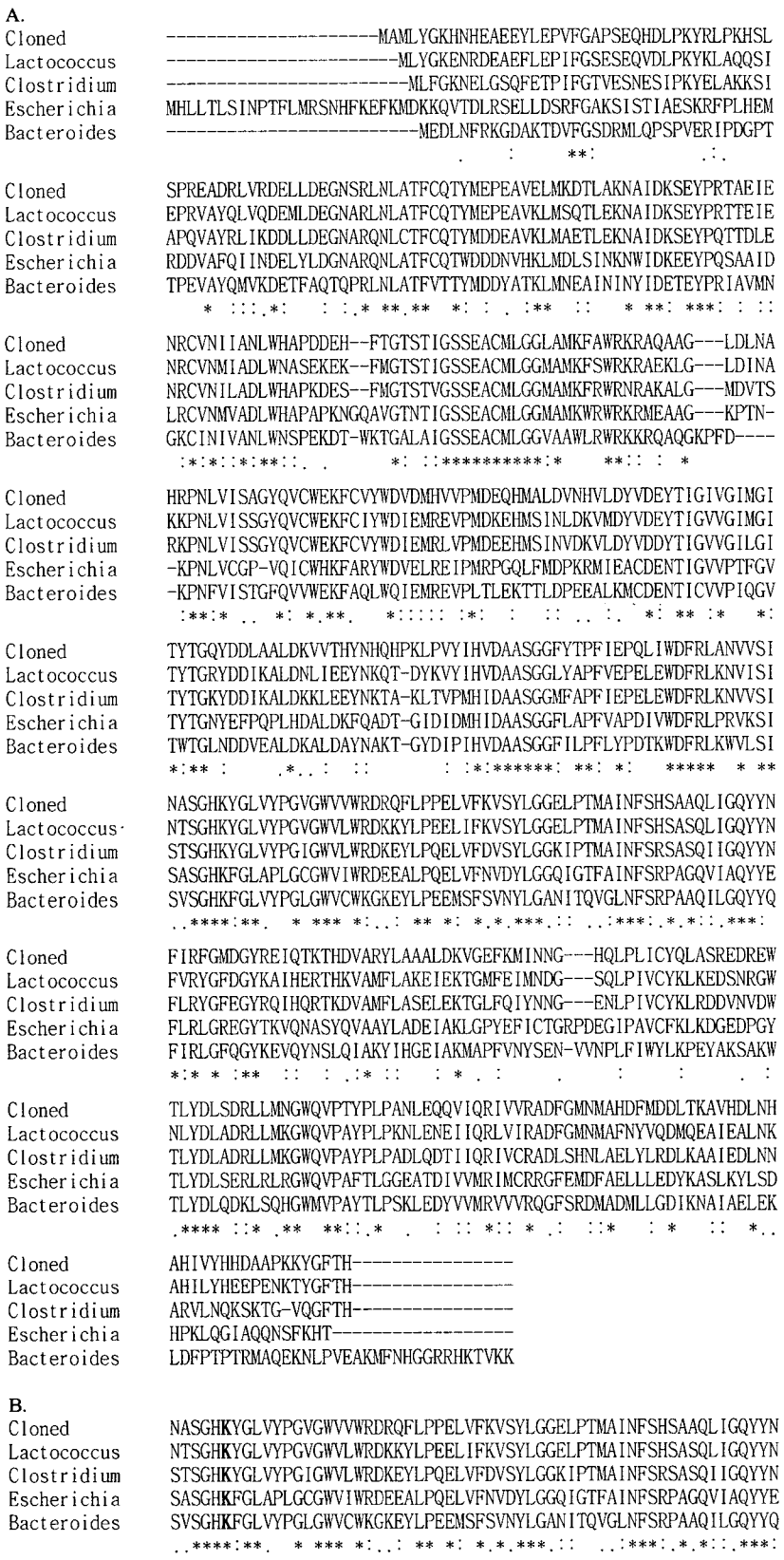

Fig. 4. Comparison of the amino acid sequences of GADs.
A. Alignments of the full-length GAD sequences. Cloned, *Lactobacillus plantarum* GAD; lactococcus, *Lactococcus lactis* GAD; clostridium, *Clostridium perfringens* GAD; escherichia, *Escherichia coli* GAD; bacteroides, *Bacteroides thetaiotaomicron* GAD. B. Alignments of the amino acids in the regions of active sites in GADs. The lysine residues in the active site regions to which pyridoxal phosphate bind are in bold cases.

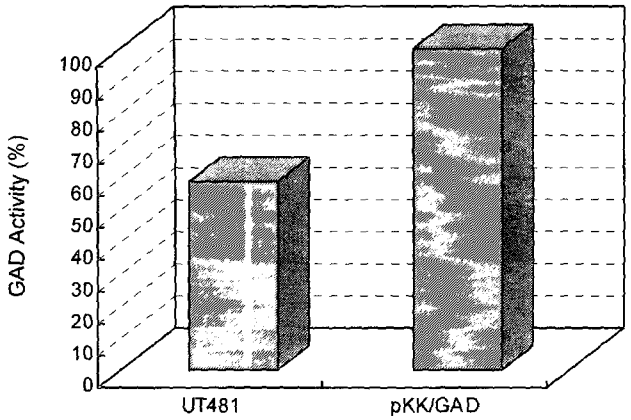

Fig. 5. Comparison of GAD activity between untransformed and transformed *E. coli*. UT481, untransformed *E. coli* UT481; pKK/GAD, transformed *E. coli* UT481 with the recombinant pKK/GAD.

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