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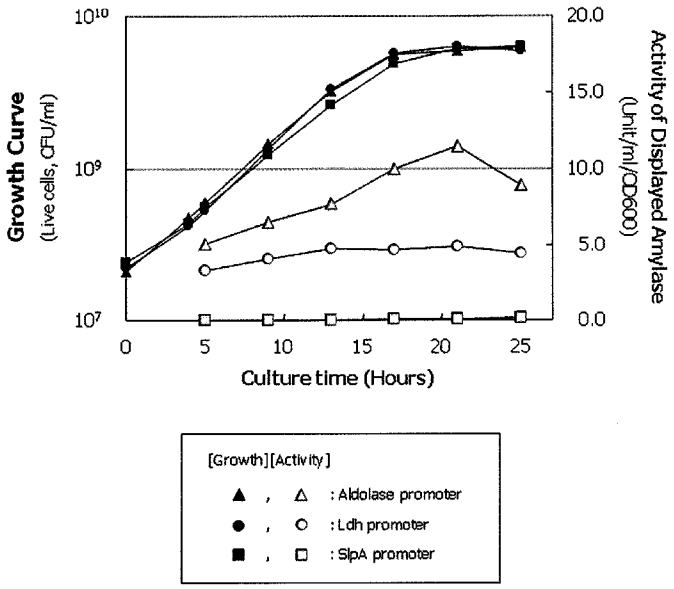
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[Continued on next page]

(54) Title: NOVEL CONSTITUTIVE STRONG PROMOTER AND USE THEREOF

FIG. 3



(57) Abstract: Disclosed herein are an aldolase gene promoter and the use thereof, more particularly, disclosed are a promoter of aldolase gene derived from Lactobacillus casei, having a base sequence of SEQ ID NO: I5 an expression vector containing said promoter, and a recombinant microorganism transformed with said expression vector. The recombinant microorganism transformed with the expression vector containing the disclosed promoter can effectively express a target protein on the surface thereof, and thus will be useful as vaccine vehicles and the like.

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NOVEL CONSTITUTIVE STRONG PROMOTER AND USE THEREOF

TECHNICAL FIELD

The present invention relates to an aldolase gene promoter derived from *Lactobacillus casei* and the use thereof, and more particularly to a promoter of aldolase gene derived from *Lactobacillus casei* represented by SEQ ID NO: 1, an expression vector containing said promoter, and a recombinant microorganism transformed with said expression vector.

BACKGROUND ART

Lactic acid bacteria, which are the most important microorganisms among food microorganisms, have acquired the GRAS (generally recognized as safe) status, and thus have been used in various foods. These lactic acid bacteria have plasmids, bacteriophages, transposons and the like, thus making it possible to develop vectors for introducing genes therein. Also, these lactic acid bacteria are easily transformed according to conventional methods known in the art, and are considered to be most suitable for edible purposes, because edible selectable marker genes have been established. In addition, lactic acid bacteria have the effects of inhibiting harmful intestinal bacteria, cleaning intestines, lowering blood cholesterol levels, increasing the nutritional value, inhibiting infection with pathogens and alleviating liver cirrhosis, as well as an anticancer effect and the effect of boosting the immune system through macrophage activation.

In order to produce useful heterologous proteins in lactic acid bacteria, highly efficient promoters (van der Vossen *et al.*, *Appl. Environ. Microbiol.*, 53:2452,

1987; Koivula *et al.*, *Appl. Environ. Microbiol.*, 57:333, 1991; Pascalle *et al.*, *Appl. Environ. Microbiol.*, 62:3662, 1996) are required, but studies on the genomes of lactic acid bacteria are still very insufficient. Among the genomes of lactic acid bacteria, only constitutive the genomes of *Bifidobacterium longum* NCC 2705, *Enterococcus faecalis* V583 and *Lactobacillus plantarum* WFCS1 have been sequenced to date, and studies on the genome sequencing of a variety of lactic acid bacteria are currently in progress. In addition, as promoters isolated from lactic acid bacteria, only promoters derived from the genomes of *Streptococcus thermophilus* A054, *Lactococcus lactis* MG1614 and *Lactococcus cremoris Wg2* (Slos *et al.*, *Appl. Environ. Microbiol.*, 57:1333,1991; Koivula *et al.*, *Appl. Environ. Microbiol.*, 57:333, 1991; van der Vossen *et al.*, *Appl. Environ. Microbiol.*, 53:2452, 1987) are known to date.

Recently, in USA and Europe, studies on the development of live vaccines using lactic acid bacteria, studies on vehicles for delivering useful hormone drugs into the intestines, and studies on the establishment of efficient genetic resources therefor and the development of insertion vectors for lactic acid bacteria, are being conducted. Particularly, the utility of lactic acid bacteria as vaccine vehicles has been highly evaluated, because unmethylated CpG DNA, lipoteichoic acid, peptidoglycan and the like, which are contained in lactic acid bacteria in large amounts, are known to function as adjuvants. In addition, lactic acid bacteria have many advantages in that they can induce intestinal mucosal immunity, because they show resistance to bile acid and gastric acid to make it possible to deliver antigens to the intestines (Seegers, *Trends Biotechnol.*, 20:508, 2002).

However, in order for lactic acid bacteria to be used as vaccine vehicles, it is required to develop a technology of presenting antigen proteins for the production of disease-preventing antibodies to the inside or outside of bacterial cells so as to allow antigen-antibody reactions to occur smoothly. In fact, various study results, which indicate that lactic acid bacteria are suitable as vaccine vehicles, have been

published. Examples of these studies include the examination of the antibody-inducing capacity of lactic acid bacteria, in which the L1 protein of human papilloma virus (HPV) is expressed (Aires *et al.*, *Appl. Environ. Microbiol.*, 72:745, 2006), and the examination of the disease-treating effects of a lactic acid bacterial strain which secrets and expresses IL-2 (interleukin-2) (Steidler *et al.*, *Nat. Biotechnol.*, 21:785, 2003).

As described above, the development of various applications of lactic acid bacteria expressing target proteins, and scientific studies on the lactic acid bacteria, have been actively conducted, there are problems in that the expression levels of the target proteins are insufficient and that, when inducible promoters are used, the continued expression of the target proteins *in vivo* can also be impossible.

Previously, the present inventors developed a novel vector for effectively expressing exogenous protein on the surface of microorganisms, using a poly-gamma-glutamic acid synthetase complex A (pgsBCA) gene, derived from *Bacillus subtilis* var. Chungkookjang, as a novel surface anchoring motif, and a method for expressing a large amount of target proteins on the surface of microorganisms transformed with said vector (Korean Patent Registration No. 10-0469800).

Accordingly, the present inventors have understood that, if a vector capable of stably expressing an antigen or an antigen determinant at a high level in lactic acid bacteria using the surface anchoring motif described in said patent is developed, it will be possible to develop a vaccine, which is compatible with the human body and can efficiently induce an immune response, because the antigen is exposed on the surface of lactic acid bacteria. Based on this understanding, the present inventors have conducted a process of screening a promoter capable of highly expressing a target protein in lactic acid bacteria and, as a result, have found that, when an aldolase gene promoter is used, the expression of target genes is increased by at least 2.5 times compared to when a conventional promoter is used, thereby

completing the present invention.

SUMMARY OF INVENTION

It is a main object of the present invention to provide a promoter of aldolase gene derived from *Lactobacillus casei,* which induces an increase in the expression of target genes.

Another object of the present invention is to provide an expression vector, in which said promoter and a gene encoding a target protein are linked to each other.

Still another object of the present invention is to provide a recombinant microorganism transformed with said expression vector, and a method for preparing a target protein using said recombinant microorganism.

Yet another object of the present invention is to provide a method for preparing a microbial vaccine using said recombinant microorganism.

To achieve the above objects, in one aspect, the present invention provides a promoter of aldolase gene derived from *Lactobacillus casei,* which induces an increase in the expression of target genes.

In another aspect, the present invention provides an expression vector, in which a target gene is linked to the terminus of said promoter, and a recombinant microorganism transformed with said expression vector.

In still another aspect, the present invention provides a microbial surface expression vector, in which said promoter, a poly-gamma glutamic acid synthetase complex gene and a gene encoding a target protein are linked to each other, and a

recombinant microorganism transformed with said microbial surface expression vector.

In still another aspect, the present invention provides a method for expressing a target protein on the surface of a microorganism, the method comprising culturing said recombinant microorganism.

In still another aspect, the present invention provides a method for preparing a microbial vaccine, the method comprising culturing the recombinant microorganism transformed with said surface expression vector.

Other features and aspects of the present invention will be apparent from the following detailed description and the appended claims.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 shows a method of obtaining an aldolase gene promoter from the genomic DNA of *Lactobacillus casei*.

FIG. 2 is a map of a vector for expressing amylase on the surface of lactic acid bacteria, in which the vector is constructed using the aldolase gene promoter.

FIG. 3 shows comparison of gene expression activities between promoters during the growth period of the lactic acid bacteria, in which the gene expression activities were determined through the measurement of amylase enzyme activity (unit/ml/OD600).

DETAILED DESCRIPTION OF THE INVENTION,

AND PREFERRED EMBODIMENTS

In one aspect, the present invention relates to a promoter of aldolase gene derived from *Lactobacillus casei.*

In the present invention, in order to construct an aldolase gene promoter, a *Lactobacillus casei*-derived aldolase gene promoter was amplified by PCR, and a 400-bp promoter (SEQ ID NO: 1) derived from *Lactobacillus casei* was isolated using a gene cloning technique. The aldolase gene promoter of the present invention is a promoter inducing the expression of aldolase gene present in *Lactobacillus casei.* Generally, promoters include a region to which RNA polymerase binds to induce the initiation of transcription, and the degree of RNA synthesis is determined depending on the base sequences of promoters. For this reason, the expression level of a gene can vary depending on the kind of promoter.

In order to measure the expression inducion capacity of the promoter of the present invention, an alpha-amylase gene was inserted into each of an expression vector containing said promoter and an expression vector containing an *ldh* or *slpA* promoter, and the constructed vectors were used to transform *Lactobacillus casei.* Then, the enzyme activity of amylase, the expression of which has been induced by each of the promoters, was measured. As a result, the amylase enzyme activity was shown to be the highest in transformants containing the aldolase gene promoter. This suggests that the expression-inducing activity of the promoter of the present invention was stronger than those of the two conventional promoters.

In another aspect, the present invention relates to an expression vector containing the aldolase gene promoter and a gene encoding target protein; and a recombinant microorganism transformed with said expression vector.

An expression vector minimally requires a promoter enabling transcription, a gene

expressing a target protein downstream of the promoter, a gene which can be amplified by self-replication in microorganisms, and an antibiotic selection marker gene for selecting a target vector, and said genes except for the target gene can vary depending on the backbone of the vector and a selected host cell. The genes minimally required in vector construction are widely known to those skilled in the art and can be easily selected depending on the expression conditions and intended use of a target gene. Generally, the backbone of the vector may have a replication origin of pWV01 or pAMβ1, but the scope of the present invention is not limited thereto.

Various methods and means may be used to introduce a vector or DNA sequence for expressing not only a target protein, but also a gene containing a regulatory region, into an appropriate host cell. For example, biochemical methods, such as transformation, transfection, conjugation, protoplast fusion and calcium phosphate precipitation, or physical methods, DEAE (diethylaminoethyl) dextran and electroporation, may be used.

After the expression vector is introduced into an appropriate host cell, only transformants can be screened using conventional techniques known in the art. In other words, transformants containing the vector capable of expressing a target gene can be screened using a selection medium suitable for the growth of host cells containing antibiotic substances.

As used herein, the term "target protein" or "heterologous protein" means a protein which is not normally present in the transformed host cells expressing the protein. For example, when a virus-derived or tumor-derived protein is manipulated to be artificially expressed in lactic acid bacteria, the protein will be referred to as "heterologous protein" or "target protein".

In still another aspect, the present invention relates to a surface expression vector for microorganisms, which has the aldolase gene promoter, a poly-gamma glutamic acid synthetase complex gene and a gene encoding a target protein linked to each other, and a recombinant microorganism transformed with said microbial surface expression vector.

The downstream of the promoter contains a poly-gamma glutamic acid synthetase complex gene, which is a surface expression motif, located between the promoter and the target protein in the DNA sequence of the vector. The gene of the surface anchoring motif plays a decisive role in the surface expression of the target gene, because it is linked to the initial portion of the target protein so as to induce the expressed protein to bind to lipid of the cell membrane, after it is encoded into amino acids. A method of linking the gene of the surface anchoring motif with the promoter and the target gene can be performed by conventional techniques which can be easily practiced by those skilled in the art, including PCR, restriction enzyme digestion and ligation.

The target proteins, which are expressed by the promoter of the present invention and displayed on the surface of host cells, may be enzymes, antibodies, antigens, adsorption proteins or adhesion proteins, and preferably antigens.

Said target proteins or antigens may include, but are not limited to, infectious microorganisms, immune disease-derived antigens or tumor-derived antigens, for example, fungal pathogens, bacteria, parasites, helminths, viruses or allergy-causing substances. More specifically, the antigens include tetanus toxoid, hemagglutinin molecules or nuclera protein of influenza virus, diphtheria toxoid, HIV gp120 or its fragments, HIV gag protein, IgA protease, insulin peptide B, *Spongospora* subterranea antigen, vibriose antigens, *Salmonella* antigens, pneumococcus antigens, respiratory syncytial virus antigens, *Haemophilus influenza* outer membrane protein, *Streptococcus pneumoniae* antigen,

Helicobacter pylori urease, *Neisseria meningitidis* pilins, *N. gonorrhoeae* pilins, melanoma-associated antigens (TRP-2, MAGE-1, MAGE-3, gp-100, tyrosinase, MART-1, HSP-70, beta-HCG), human papilloma virus antigens E1, E2, E6 and E7 derived from HPV type 16, 18, 31, 33, 35 or 45, tumor antigen CEA, normal or mutant ras protein, normal or mutant p53 protein, Muc1, pSA, antigens well known in the art, which is derived from the followings: cholera, diphtheria, *Haemophilus*, hepatitis A, hepatitis B, influenza, measles, meningitis, mumps, pertussis, small pox, pneumococcal pneumonia, polio, rabies, rubella, tetanus, tuberculosis, Addison's disease, immunogens, allergen, cancer including solid and blood borne tumors, acquired immune deficiency syndrome, and factor involved in transplant rejection, such as kidney, heart, pancreas, lung, bone, and liver transplant rejections, and antigens inducing autoimmunity.

As used herein, the term "host cells" or "microorganisms" means probiotic gram-positive lactic acid bacteria. Common criteria used for selecting probiotic microorganisms include: (i) microorganisms of human origin; (ii) being stable in bile, acid, enzyme and oxygen; (iii) capability to adhere to the intestinal mucosal membrane; (iv) capability to form colonies in the digestive organs; (v) capability to produce antibacterial substances; and (vi) unequivocal demonstration of efficacy or stability. Based on the above conditions, it is evident that lactic acid bacteria are biocompatible and harmless to the human body during the growth thereof in the human body. Thus, when transformants containing lactic acid bacteria as hosts are applied to the human body so as to deliver genes or proteins for preventing or treating diseases, a step of detoxifying bacterial strains is not required, unlike conventional methods of preparing vaccines using bacterial strains.

In the present invention, the microorganisms may include *Lactobacillus* sp., *Streptococcus* sp. and *Bifidobacterium* sp. Typically, *Lactobacillus* sp. includes *L. acidophilus*, *L. casei*, *L. plantarum*, *L. ferementum*, *L. delbrueckii*, *L. johnsonii LJI*, *L. reuteri* and *L. bulgaricus*; *Streptococcus* sp. includes *S. thermophilus*; and

Bifidobacterium sp. includes *B. infantis*, *B. bifidum*, *B. longum*, *B. psuedolongum*, *B. breve*, *B. lactis* Bb-12 and *B. adolescentis*. Preferred is *Lactobacillus* sp.

In the present invention, an expression vector (pDT1-PgsAL-Amylase), which contains a base sequence having the promoter linked to a surface anchoring motif *pgsA* and can express an alpha-amylase gene as a target gene, was constructed, and the expression vector was inserted into *L. casei*, thus preparing transformants expressing amylase.

The target protein, which is expressed by the inventive promoter having improved gene expression capacity, is expressed on the surface of microorganisms, and thus the transformed microorganisms of the present invention can be used as vaccines.

In still another aspect, the present invention relates to a method for preparing a microbial vaccine, the method comprising culturing the recombinant microorganism transformed with said surface expression vector.

Vaccines are drugs which are used to stimulate the immune system using bio-organisms in order to prevent diseases. As used herein, the term "immune activation" means a process of efficiently removing an antigen by the production of antibodies in organisms, stimulation of T-lymphocytes or the stimulation of other immune cells (e.g. macrophages). An introduction to immunology of the immune activation will be readily understood by those skilled in the art (Barrett, J.T., *Textbook of Immunology*, 1983).

The vaccine comprising a transformed microorganism, which expresses an antigen, may be administered to mammals, and preferably human beings.

The preparation of the vaccine composition can be carried out using a standard technique, and the amount required will vary with the antigenicity of the gene

product and need only be an amount sufficient to induce an immune response typical of existing vaccines. Routine experimentation will easily establish the required amount. Typical initial dosages of vaccine could be 0.001-1 mg antigen/kg body weight, with increasing amounts or multiple dosages used as needed to provide the desired level of protection. The required amount can be determined by those skilled in the art and may vary depending on various factors, such as formulation method, administration mode, age, weight and sex of patients, pathological conditions, diet, administration time, administration route, excretion rate and response sensitivity.

In order for a vaccine to be effective in producing antibodies, the antigenic material must be released in such a way that the antibody-producing mechanism of the vaccinated animal can come into play. Therefore, the microbe carrier of the gene product must be introduced into the body. In order to stimulate a preferred response by an antigen presented by the transformants of the present invention, introduction of the microbe or gene product directly into the gut or bronchus is preferred, such as by oral administration, gastric intubation or intranasally in the form of aerosols, although other methods of administering the vaccine, such as intravenous, intramuscular or subcutaneous injection, are possible.

The vaccine is advantageously presented in a lyophilized form, for example in a capsular form, for oral administration to a patient. Such capsules may be provided with an enteric coating comprising for example Eudragate S, Eudragate L, cellulose acetate, cellulose phthalate or hydroxy propylmethyl cellulose. These capsules may be used as such, or alternatively, the lyophilised material may be reconstituted prior to administration, e.g. as a suspension. Reconstitution is advantageously effected in a buffer at a suitable pH to ensure the viability of the organisms. In order to protect the attenuated bacteria and the vaccine from gastric acidity, a sodium bicarbonate preparation is advantageously administered before each administration of the vaccine. Alternatively the vaccine may be prepared for

parenteral administration, intranasal administration or intramammary administration.

The transformed lactic acid bacteria, which include the inventive promoter and contain a gene expressing a target protein capable of acting as an antigen, can show the desired efficacy while forming colonies in mucosa in the digestive organs, can be simultaneously administered together with selective antibiotics in the vector for active colony formation while maintaining the desired transformation properties, and can control the development of undesired lactic acid bacteria having no vector, which can be developed during cell division in transformants. The selection method can be easily performed using conventional techniques known in the art, and the selective antibiotics, which can be used in the above process, may vary depending on antibiotic genes contained in the expression vector.

Examples

Hereinafter, the present invention will be described in further detail with reference to examples. It is to be understood, however, that these examples are for illustrative purposes only and are not to be construed to limit the scope of the present invention.

Particularly, although only an amylase gene was exemplified as a target gene in the following examples, those skilled in the art will appreciate that the scope of the present invention is not limited thereto.

Example 1: Construction of *Lactobacillus casei*-derived aldolase gene promoter

A 400-bp DNA fragment corresponding to the promoter region of the aldolase gene was obtained by PCR from *Lactobacillus casei* ATCC 393.

For this purpose, *Lactobacillus casei* was cultured in basal MRS medium (containing 1% casein hydrolysate, 1.5% yeast extract, 2% dextrose, 0.2% ammonium citrate, 0.5% sodium acetate, 0.01% magnesium sulfate, 0.05% manganese sulfate and 0.2% dipotassium phosphate; Acumedia Manufacturers, Inc.), and 10^9 cultured cells were disrupted to obtain a solution. The solution was used as a template for PCR. In order to facilitate insertion into a vector in gene cloning, *Hin*dIII and *Nae*I restriction sites were located at the ends of each of primers (SEQ ID NO: 2 and SEQ ID NO: 3). PCR was performed using the primers and, as a result, an amplified product (SEQ ID NO: 1) having a total length of 415 bp was obtained. The PCR-amplified product was cloned into a pGEM-Teasy vector (Promega Co., USA), and the base sequence thereof was analyzed (FIG. 1).

SEQ ID NO: 2: 5'-aag ctt aat acc cac tta ttg-3'

SEQ ID NO: 3: 5'-gcc ggc cat gta gat atc ctc-3'

Example 2: Construction of amylase surface expression vector using aldolase gene promoter

In order to prepare an amylase expression vector whose expression is induced by an aldolase gene promoter, the *Lactobacillus casei*-derived aldolase gene promoter was inserted into a vector having RepA, as a replication origin, which is replicable in *E. coli* and *Lactobacillus casei*, and then a surface anchoring motif *pgsA* derived from *Bacillus subtilis* var. Chungkookjang was introduced downstream of the promoter, and *Bam*HI and *Xba*I restriction enzyme sites, allowing a target gene to be inserted into the carboxy end of the *pgsA* gene, was added thereto, thus constructing a pDT1-PgsA vector containing the aldolase gene promoter. As the *pgsA* gene, the one disclosed in Korean Patent 0469800 was used. Also, as a selection marker for maintaining the vector, an erythromycin-resistant gene was selected (FIG. 2).

In order to obtain an α-amylase gene, PCR was performed using *Streptococcus bovis* (ATCC 700410)-derived genome as a template with primers of SEQ ID NO: 4 and SEQ ID NO: 5.

SEQ ID NO: 4: 5'-tct gga tcc gat gaa caa gtg tca atg-3'
SEQ ID NO: 5: 5'-cag tta tct aga tta ttt tag ccc atc-3'

As a result, a 2,130-bp DNA gene fragment, which had a sequence encoding 703 amino acids except for 39 N-terminal amino acid residues, as the secretion signal of extracellular α-amylase, and contained *Bam*HI and *Xba*I restriction enzyme sites at both ends, respectively, was obtained.

The DNA fragment containing the α-amylase gene was transformed into lactic acid bacterial host cells by digesting the DNA fragment with *Bam*HI and *Xba*I and ligating the digested fragment into the C terminal end of PgsA of the pJT1-PgsA vector. As a result, an expression vector pDT1-PgsA-amylase, which could express a PgsA-α-amylase fusion protein on the surface of bacterial cells, was constructed (FIG. 2).

Example 3: Preparation of transformants using alpha-amylase surface expression vector

Lactobacillus casei was transformed with an alpha-amylase surface expression vector pDT1-PgsA-amylase, and PCR was performed in the following manner in order to examine whether the alpha-amylase gene-containing vector would be present in the transformant. The transformed strain was recovered and washed with water, and PCR was performed using the washed strain as a template with primers (SEQ ID NO: 6 and SEQ ID NO: 7) which were annealed in the erythromycin-resistant gene contained in the plasmid. Thus, it was confirmed by

detecting a 1,156-bp PCR product, indicating that the target vector was present in the transformant.

SEQ ID NO: 6: 5'-gtg tgt tga tag tgc agt atc-3'

SEQ ID NO: 7: 5'-ccg tag gcg cta ggg acc tct tta gc-3'

In addition, in order to analyze whether the gene was normally expressed in the vector inserted into the transformant, the measurement of alpha-amylase enzyme activity was carried out as described in Example 4.

Example 4: Comparison of promoter activities through measurement of amylase activity

The expression-inducing activity of the novel aldolase gene promoter was compared with those of the existing *ldh* promoter and *slpA* promoter. An amylase gene as a reporter gene was linked to each of the promoters, and the expression-inducing activities of the promoters were compared through the measurement of amylase enzyme activity proportional to the expression level of amylase.

The existing vectors used to compare the promoter activities were pJT1-PgsAL-Amylase (Korean Patent 0578395) expressing amylase using the *ldh* promoter, and pBT-PgsAL-Amylase (Korean Patent Application No. 10-2006-0088995) expressing amylase using the *slpA* promoter. The two vectors have the same structure as that of pDT1-PgsAL-Amylase, but are different from each other with respect to the promoter region.

The enzyme activity of amylase expressed on the surface of the lactic acid bacteria was measured using an Enzyme-Activity Assay Kit (Kikkoman Co., Tokyo, Japan) and N3-G5-β-CNP (2-chloro-4-nitrophenyl 6^5-azido-6^5-deoxy-β-maltopentaoside) as a substrate.

The bacterial cells recovered by centrifuging culture broth were washed twice with PBS solution and suspended in 100 ㎕ of the same buffer solution. The cell suspension was mixed with 400 ㎕ of a substrate solution, and the mixture was incubated at 37℃ for 10 minutes to induce an enzymatic reaction. 800 ㎕ of a reaction stop solution was added thereto to stop the reaction, and the absorbance at 400 nm was measured with a spectrophotometer. 1 unit of enzyme was defined as the amount of enzyme that produces 1 μmole of CNP (2-chloro-4-nitrophenol) showing absorbance at 400 nm, from N3-G5-β-CNP at 37℃ for 1 minute.

To culture *Lactobacillus casei* transformed with each of the vectors, MRS medium containing erythromycin (SIGMA-ALDRICH Co.) at a final concentration of 16 ㎍/ml was used, and the culture process was performed in a 3-L fermenter containing 2.0 L of the medium. Culture broth was recovered at 4-hr intervals starting 5 hours after the initiation of the culture process and was measured for the number of living bacteria and amylase activity. For pH adjustment in the culture process, NaOH was used.

As a result, it could be observed that the use of the aldolase gene promoter showed amylase activity, which was 2.0~2.5 fold higher than that of the *ldh* promoter and 150 to 180 fold higher than that of the *slpA* promoter. This suggests that the activity of the aldolase gene promoter was stronger than those of the *ldh* and *slpA* promoters (FIG. 3).

INDUSTRIAL APPLICABILITY

As described in detail above, the present invention provides a *Lactobacillus casei*-derived aldolase promoter, which can highly express a target protein in lactic acid bacteria, and an expression vector containing said promoter. Because the vector

contains a gene expressing a target protein on the surface of microorganisms, lactic acid bacteria transformed with the vector can effectively express a target protein on the surface thereof, and thus the transformed lactic acid bacteria can be used as vaccine vehicles.

Although the present invention has been described in detail with reference to the specific features, it will be apparent to those skilled in the art that this description is only for a preferred embodiment and does not limit the scope of the present invention. Thus, the substantial scope of the present invention will be defined by the appended claims and equivalents thereof.

THE CLAIMS

What is Claimed is:

1. A promoter of aldolase gene derived from *Lactobacillus casei.*

2. The promoter according to claim 1, which has a base sequence of SEQ ID NO: 1.

3. An expression vector, which contains the promoter of claim 1 and a gene encoding a target protein.

4. A recombinant microorganism transformed with the expression vector of claim 3.

5. A microbial surface expression vector, which has the promoter of claim 1, a poly-gamma glutamic acid synthetase complex gene, and a gene encoding a target protein linked to each other.

6. The microbial surface expression vector according to claim 5, wherein the target protein is an antigen.

7. The microbial surface expression vector according to claim 5, wherein the poly-gamma glutamic acid synthetase complex gene is *pgsA*.

8. A recombinant microorganism transformed with the microbial surface expression vector of any one claim among claims 5-7.

9. The recombinant microorganism according to claim 8, wherein said microorganism is a lactic acid bacterium.

10. A method for expressing a target protein on the surface of a microorganism, the method comprises culturing the recombinant microorganism of claim 8.

11. A method for preparing a microbial vaccine, the method comprising:

expressing an antigen on the surface of a microorganism by culturing the recombinant microorganism transformed with the microbial surface expression vector of claim 6; and

recovering the microorganism having said antigen expressed on the surface thereof.

12. The method for preparing a microbial vaccine according to claim 11, wherein said microorganism is a lactic acid bacterium.

DRAWINGS

1/3

FIG. 1

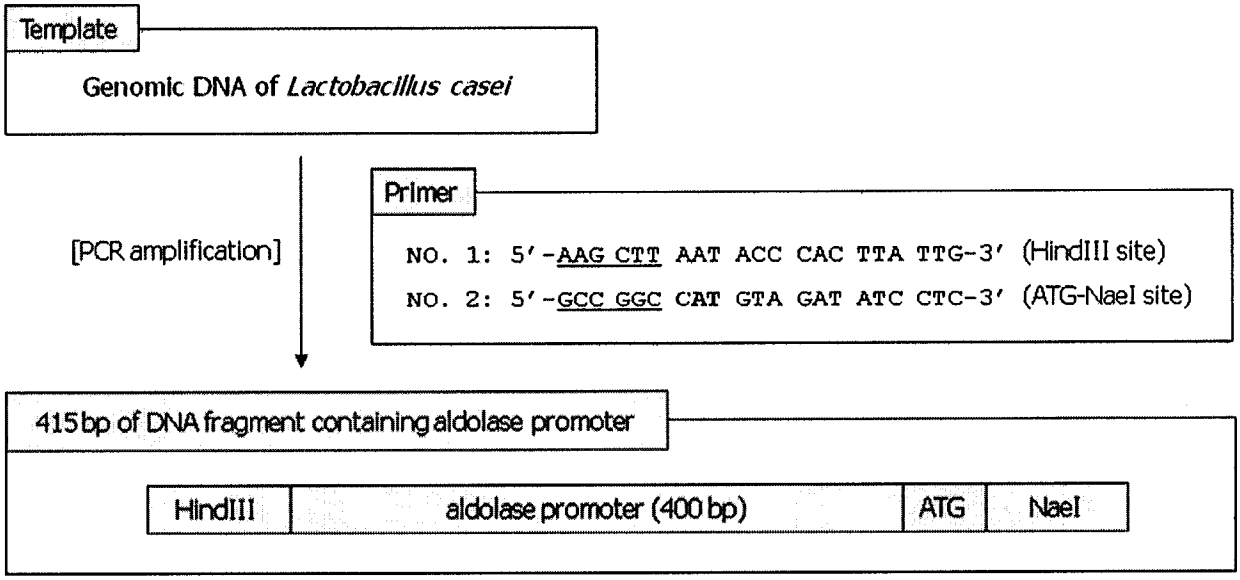


FIG. 2

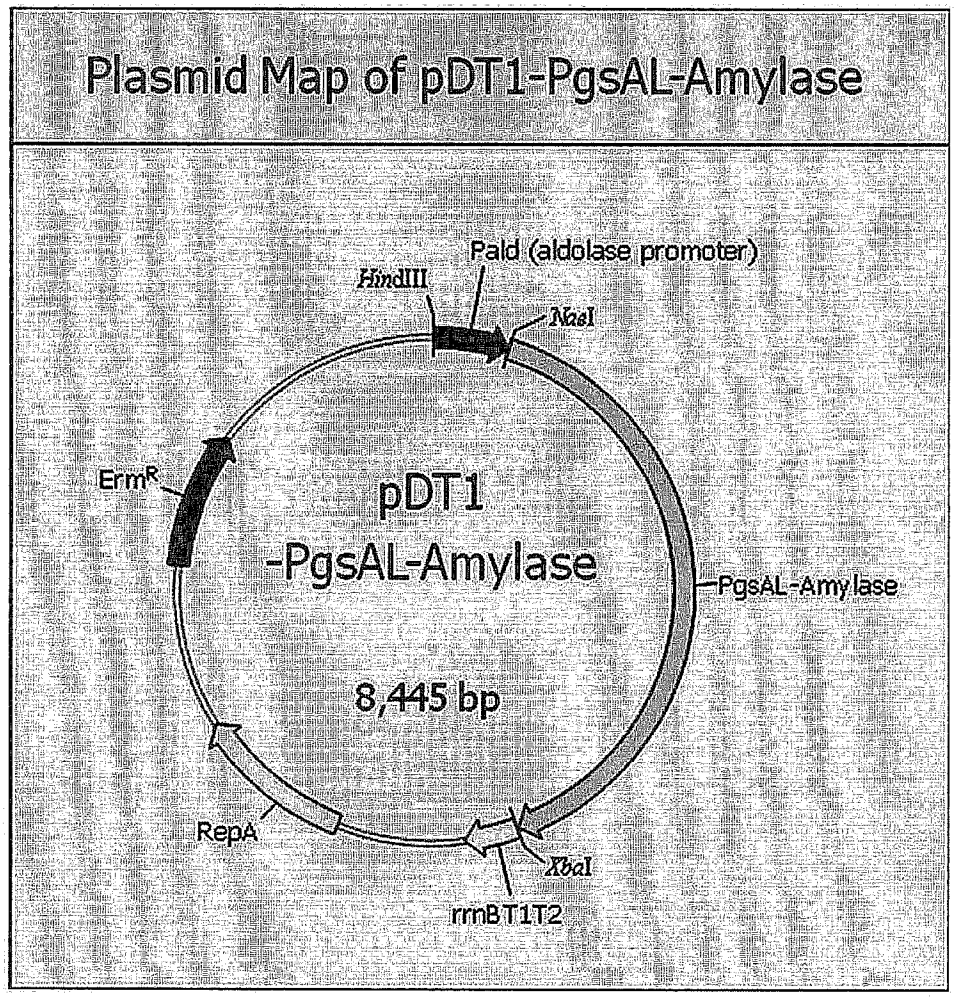
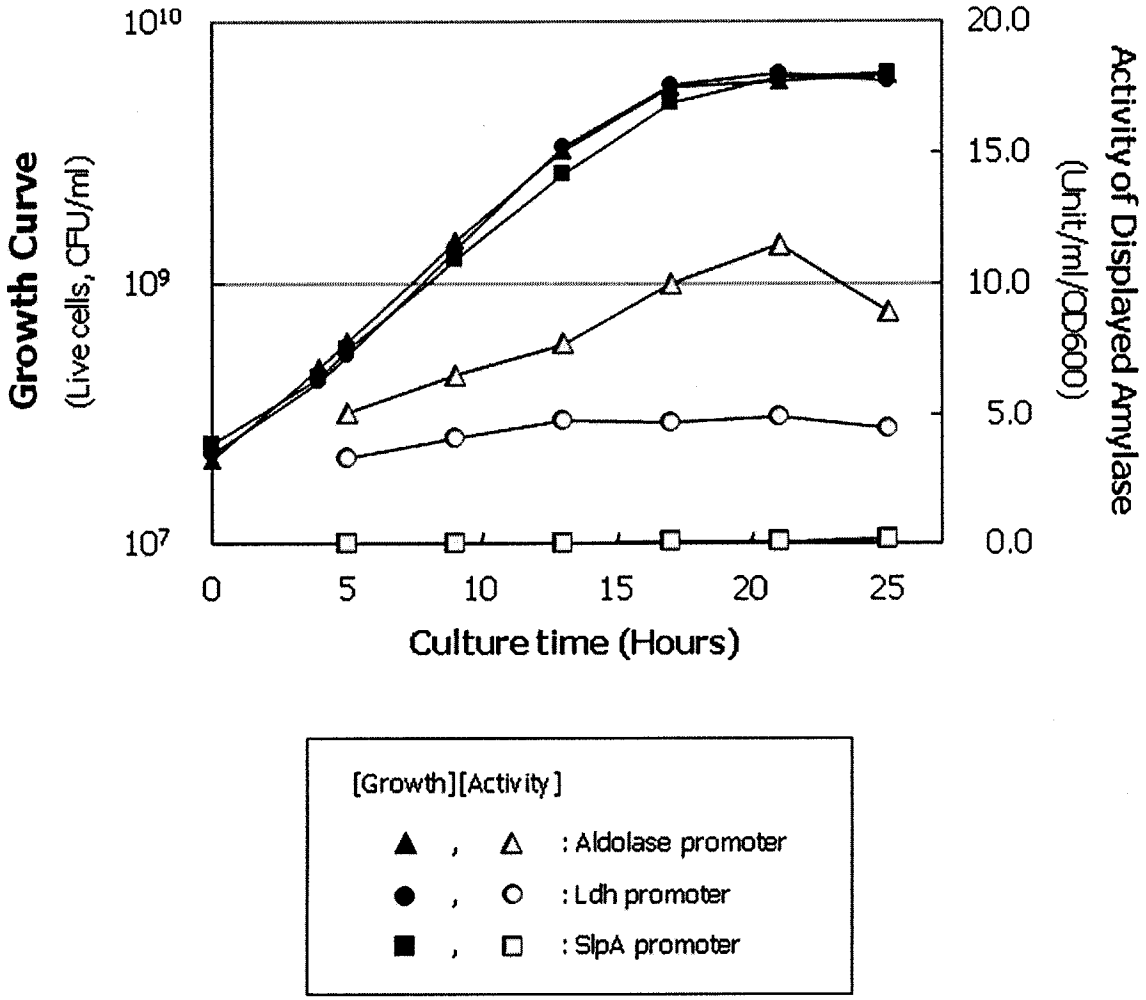

Plasmid Map of pDT1-PgsAL-Amylase
Pald (aldolase promoter)
HindIII
NdeI
ErmR
pDT1
-PgsAL-Amylase
8,445 bp
PgsAL-Amylase
RepA
XbaI
rrnBT1T2

FIG. 3


Growth Curve
(Live cells, CFU/ml)
10^{10}
10^{9}
10^{7}
20.0
15.0
10.0
5.0
0.0
Activity of Displayed Amylase
(Unit/ml/OD600)
0
5
10
15
20
25
Culture time (Hours)
[Growth][Activity]
▲ , △ : Aldolase promoter
● , ○ : Ldh promoter
■ , □ : SlpA promoter

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2008/001579

A. CLASSIFICATION OF SUBJECT MATTER

C12N 9/88(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 8 C12N 1/21, C12P 19/00, A61K 39/02, C07K 14/165, C12N 15/74

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean Utility Models and Applications for Utility Models since 1975
Japanese Utility Models and Applications for Utility Models since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKIPASS, WPI, USPTO, PAJ, NCBI, CAPLUS(STN), INSPECT "Lactobacillus casei, aldolase gene, promoter, target protein, expression vector, pgsA, antigen, etc."

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FEMS MIcrobio. Lett., Vol.122(1): 123-127 (2003) "Optimization of the green fluorescent protein (GFP) expression from a lactose-inducible promoter in Lactobacillus casei" - see the whole document	2
A	Appl. Environ. Microbiol., Vol.71(3): 1356-1363 (2005) "Differential activities of four Lactobacillus casei promoters during bacterial transit through the gastrointestinal tracts of human-microbiota-associated mice" - see the whole document	2
A	Vaccine, Vol.23: 1335-1342 (2005) "Intragastric administration of Lactobacillus casei expressing transmissible gastroentritis coronavirus spike glycoprotein induced specific antibody production" - see the whole document	2
A	Arch. Biochem. Biophy., Vol.140(2): 334-340 (1970) "Adaptive alterations in Lactobacillus casei.II. variation in the levels of aldolase in cells grown on glucose and ribose" - see the whole docuemnt	2

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 JUNE 2008 (18.06.2008)	**18 JUNE 2008 (18.06.2008)**

Name and mailing address of the ISA/KR
Korean Intellectual Property Office
Government Complex-Daejeon, 139 Seonsa-ro, Seo-gu, Daejeon 302-701, Republic of Korea
Facsimile No. 82-42-472-7140

Authorized officer
KIM Kee-Yeun
Telephone No. 82-42-481-8387

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2008/001579

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of :

   a. type of material
   - [x] a sequence listing
   - [ ] table(s) related to the sequence listing

   b. format of material
   - [ ] on paper
   - [x] in electronic form

   c. time of filing/furnishing
   - [ ] contained in the international application as filed
   - [x] filed together with the international application in electronic form
   - [ ] furnished subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2008/001579

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1, 3-12
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

- Claims 1 and 3-12 are directed at or refer to promoter of the aldolase gene without limiting the nucleic acid and are considered too broad and so inadequately supported by the description that no meaningful search could be made.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2008/001579

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Arch. Biochem. Biophys., Vol.160(1): 52-57 (1974) "Fructose-1,6-diphosphate aldolase form Lactobacillus casei.II. multiple forms and their characteristics" - see the whole document	2
A	The Journal of Biological Chemistry, Vol.249(24): 7977-7983 (1974) "Variations in the quaternary structure of three lactic acid bacteria aldolases" - see the whole document	2
A	Bacteriological Reviews, Vol.37(4): 453-478 (1973) "Aldolase of lactiv acid bacteria: a case history in the use of an enzyme as an evolutionary marker" - see the whole document	2