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(54) Title: A METHOD FOR PRODUCING AN L-AMINO ACID USING A BACTERIUM OF THE ENTEROBACTERIACEAE FAMILY HAVING EXPRESSION OF THE *sanA* GENE ATTENUATED

(57) Abstract: The present invention provides a method for producing an L-amino acid using a bacterium of the Enterobacteriaceae family, particularly a bacterium belonging to the genus *Escherichia* or *Pantoea*, which has been modified to attenuate expression of the *sanA* gene.



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DESCRIPTION

A METHOD FOR PRODUCING AN L-AMINO ACID USING A BACTERIUM OF THE *ENTEROBACTERIACEAE* FAMILY HAVING EXPRESSION OF THE *sanA* GENE ATTENUATEDTechnical Field

The present invention relates to the microbiological industry, and specifically to a method for producing an L-amino acid using a bacterium of the *Enterobacteriaceae* family which has been modified to attenuate expression of the *sanA* gene.

Background Art

It is well known that constitutive expression of the histidine operon (His^C) disturbs the process of septation and results in filamentation of the culture. Two loci involved in the pleiotropic response of His^C strains of *S. typhimurium* have been characterized as homologs of two *E. coli* genes, the *sanA* and *rfe* genes (Mouslim, C. et al, Mol. Gen. Genet., 259(1), 46-53 (1998)).

In *E. coli*, it has been shown that the protein encoded by the *sanA* gene is required for vancomycin resistance at high temperatures. The protein is partially anchored in the inner membrane by the amino-terminal region, while the rest of it is probably periplasmic (Rida, S. et al, J. Bacteriol., 178(1), 94-102 (1996)).

But currently, there have been no reports of inactivating the *sanA* gene for the purpose of producing L-amino acids.

Disclosure of the Invention

Objects of the present invention include enhancing the productivity of L-amino acid producing strains, and providing a method for producing an L-amino acid using these strains.

The above objects were achieved by finding that attenuating expression of the *sanA* gene can enhance production of L-amino acids, such as L-threonine, L-lysine, L-cysteine, L-methionine, L-leucine, L-isoleucine, L-valine, L-histidine, glycine, L-serine, L-alanine, L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, L-proline, L-arginine, L-phenylalanine, L-tyrosine, and L-tryptophan.

The present invention provides a bacterium of the *Enterobacteriaceae* family having an increased ability to produce amino acids, such as L-threonine, L-lysine, L-cysteine, L-methionine, L-leucine, L-isoleucine, L-valine, L-histidine, glycine, L-serine, L-alanine, L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, L-proline, L-arginine, L-phenylalanine, L-tyrosine, and L-tryptophan.

It is an object of the present invention to provide an L-amino acid producing bacterium of the *Enterobacteriaceae* family, wherein the bacterium has been modified to attenuate expression of the *sanA* gene.

It is a further object of the present invention to provide the bacterium as described above, wherein the expression of the *sanA* gene is attenuated by inactivation of the *sanA* gene.

It is a further object of the present invention to provide the bacterium as described above, wherein the bacterium belongs to the genus *Escherichia*.

It is a further object of the present invention to provide the bacterium as described above, wherein the bacterium belongs to the genus *Pantoea*.

It is a further object of the present invention to provide the bacterium as described above, wherein said L-amino acid is selected from the group consisting of an aromatic L-amino acid and a non-aromatic L-amino acid.

It is a further object of the present invention to provide the bacterium as described above, wherein said aromatic L-amino acid is selected from the group consisting of L-phenylalanine, L-tyrosine, and L-tryptophan.

It is a further object of the present invention to provide the bacterium as described above, wherein said non-aromatic L-amino acid is selected from the group consisting of L-threonine, L-lysine, L-cysteine, L-methionine, L-leucine, L-isoleucine, L-valine, L-histidine, glycine, L-serine, L-alanine, L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, L-proline, and L-arginine.

It is a further object of the present invention to provide a method for producing an L-amino acid comprising:

- cultivating the bacterium as described above in a medium to produce and excrete said L-amino acid into the medium, and
- collecting said L-amino acid from the medium.

It is a further object of the present invention to provide the method as described above, wherein said L-amino acid is selected from the group consisting of an aromatic L-amino acid and a non-aromatic L-amino acid.

It is a further object of the present invention to provide the method as described above, wherein said aromatic L-amino acid is selected from the group consisting of L-phenylalanine, L-tyrosine, and L-tryptophan.

It is a further object of the present invention to provide the method as described above, wherein said non-aromatic L-amino acid is selected from the group consisting of L-threonine, L-lysine, L-cysteine, L-methionine, L-leucine, L-isoleucine, L-valine, L-histidine, glycine, L-serine, L-alanine, L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, L-proline, and L-arginine.

The present invention is described in detail below.

Detailed Description of the Preferred Embodiments

1. Bacterium of the present invention

The bacterium of the present invention is an L-amino acid producing bacterium of the *Enterobacteriaceae* family, wherein the bacterium has been modified to attenuate expression of the *sanA* gene.

In the present invention, "L-amino acid producing bacterium" means a bacterium which has an ability to produce and excrete an L-amino acid into a medium, when the bacterium is cultured in the medium.

The phrase "L-amino acid-producing bacterium" as used herein also means a bacterium which is able to produce and cause accumulation of an L-amino acid in a culture medium in an amount larger than a wild-type or parental strain of *E. coli*, such as *E. coli* K-12, and preferably means that the microorganism is able to cause accumulation in a medium of an amount not less than 0.5 g/L, more preferably not less than 1.0 g/L of the target L-amino acid. The term "L-amino acids" includes L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamic acid, L-glutamine, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, and L-valine.

The term "aromatic L-amino acid" includes L-phenylalanine, L-tyrosine, and L-tryptophan. The term "non-aromatic L-amino acid" includes L-threonine, L-lysine, L-cysteine, L-methionine, L-leucine, L-isoleucine, L-valine, L-histidine, glycine, L-serine, L-alanine, L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, L-proline, and L-arginine. L-threonine, L-lysine, L-cysteine, L-leucine, L-histidine, L-glutamic acid, L-phenylalanine, L-tryptophan, L-proline, and L-arginine are particularly preferred.

The *Enterobacteriaceae* family includes bacteria belonging to the genera *Escherichia*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Pantoea*, *Photobacterium*, *Providencia*, *Salmonella*, *Serratia*, *Shigella*, *Morganella*, *Yersinia*, etc.. Specifically, those classified into the *Enterobacteriaceae* according to the taxonomy used in the NCBI (National Center for Biotechnology Information) database (<http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=91347>) can be used. A bacterium belonging to the genus of *Escherichia* or *Pantoea* is preferred.

The phrase “a bacterium belonging to the genus *Escherichia*” means that the bacterium is classified into the genus *Escherichia* according to the classification known to a person skilled in the art of microbiology. Examples of a bacterium belonging to the genus *Escherichia* as used in the present invention include, but are not limited to, *Escherichia coli* (*E. coli*).

The bacterium belonging to the genus *Escherichia* that can be used in the present invention is not particularly limited, however for example, bacteria described by Neidhardt, F.C. et al. (*Escherichia coli* and *Salmonella typhimurium*, American Society for Microbiology, Washington D.C., 1208, Table 1) are encompassed by the present invention.

The phrase “a bacterium belonging to the genus *Pantoea*” means that the bacterium is classified as the genus *Pantoea* according to the classification known to a person skilled in the art of microbiology. Some species of *Enterobacter agglomerans* have been recently reclassified into *Pantoea agglomerans*, *Pantoea ananatis*, *Pantoea stewartii* or the like, based on nucleotide sequence analysis of 16S rRNA, etc. (Int. J. Syst. Bacteriol., 43, 162-173 (1993)).

The phrase “bacterium has been modified to attenuate expression of the *sanA* gene” means that the bacterium has been modified in such a way that the modified bacterium contains a reduced amount of the SanA protein as compared with an unmodified bacterium, or the modified bacterium is unable to synthesize the SanA protein. The phrase “bacterium has been modified to attenuate expression of the *sanA* gene” also means that the target gene is modified in such a way that the modified gene encodes a mutant SanA protein which has a decreased activity.

The phrase “inactivation of the *sanA* gene” means that the modified gene encodes a completely inactive protein. It is also possible that the modified DNA region is unable to naturally express the gene due to the deletion of a part of the gene, the shifting of the reading frame of the gene, the introduction of missense/nonsense mutation(s), or the modification of

an adjacent region of the gene, including sequences controlling gene expression, such as a promoter, enhancer, attenuator, ribosome-binding site, etc.

The *sanA* gene encodes the SanA protein (synonyms – B2144, YeiF). The *sanA* gene of *E. coli* (nucleotides 2230900 to 2231619 in the GenBank accession number NC_000913.2; gi:49175990; SEQ ID NO: 1) is located between the *cdd* gene and the *yeiS* ORF on the chromosome of *E. coli* K-12. The nucleotide sequence of the *sanA* gene and the amino acid sequence of SanA encoded by the *sanA* gene are shown in SEQ ID NO: 1 and SEQ ID NO: 2, respectively.

Since there may be some differences in DNA sequences between the genera or strains of the *Enterobacteriaceae* family, the *sanA* gene to be inactivated on the chromosome is not limited to the gene shown in SEQ ID No:1, but may include genes homologous to SEQ ID No:1 encoding a variant protein of the SanA protein. The phrase “variant protein” as used in the present invention means a protein which has changes in the sequence, whether they are deletions, insertions, additions, or substitutions of amino acids, but still maintains the activity of the product as the SanA protein which is involved in vancomycin resistance. The number of changes in the variant protein depends on the position or the type of amino acid residues in the three dimensional structure of the protein. It may be 2 to 30, preferably 2 to 15, and more preferably 2 to 5 in SEQ ID NO: 2. These changes in the variants can occur in regions of the protein which are not critical for the function of the protein. This is because some amino acids have high homology to one another so the three dimensional structure or activity is not affected by such a change. These changes in the variant protein can occur in regions of the protein which are not critical for the function of the protein. Therefore, the protein variant encoded by the *sanA* gene may be one which has a homology of not less than 80%, preferably not less than 90%, and most preferably not less than 95%, with respect to the entire amino acid sequence shown in SEQ ID NO. 2, as long as the native activity of the SanA protein prior to inactivation is maintained.

Homology between two amino acid sequences can be determined using the well-known methods, for example, the computer program BLAST 2.0, which calculates three parameters: score, identity and similarity.

Moreover, the *sanA* gene may be a variant which hybridizes under stringent conditions with the nucleotide sequence shown in SEQ ID NO: 1, or a probe which can be prepared from the nucleotide sequence, provided that it encodes a functional SanA protein prior to inactivation. “Stringent conditions” include those under which a specific hybrid, for

example, a hybrid having homology of not less than 60%, preferably not less than 70%, more preferably not less than 80%, still more preferably not less than 90%, and most preferably not less than 95%, is formed and a non-specific hybrid, for example, a hybrid having homology lower than the above, is not formed. For example, stringent conditions are exemplified by washing one time or more, preferably two or three times at a salt concentration of $1 \times \text{SSC}$, 0.1% SDS, preferably $0.1 \times \text{SSC}$, 0.1% SDS at 60°C. Duration of washing depends on the type of membrane used for blotting and, as a rule, should be what is recommended by the manufacturer. For example, the recommended duration of washing for the Hybond™ N+ nylon membrane (Amersham) under stringent conditions is 15 minutes. Preferably, washing may be performed 2 to 3 times. The length of the probe may be suitably selected depending on the hybridization conditions, and is usually 100 bp to 1 kbp.

Expression of the *sanA* gene can be attenuated by introducing a mutation into the gene on the chromosome so that intracellular activity of the protein encoded by the gene is decreased as compared with an unmodified strain. Such a mutation on the gene can be replacement of one base or more to cause amino acid substitution in the protein encoded by the gene (missense mutation), introduction of a stop codon (nonsense mutation), deletion of one or two bases to cause a frame shift, insertion of a drug-resistance gene, or deletion of a part of the gene or the entire gene (J. Biol. Chem., 1997, 272 (13): 8611-8617, J. Antimicrobial Chemotherapy, 2000, 46: 793-79). Expression of the *sanA* gene can also be attenuated by modifying an expression regulating sequence such as the promoter, the Shine-Dalgarno (SD) sequence, etc. (WO95/34672, Biotechnol. Prog. 1999, 15, 58-64).

For example, the following methods may be employed to introduce a mutation by gene recombination. A mutant gene encoding a mutant protein having a decreased activity is prepared, and a bacterium to be modified is transformed with a DNA fragment containing the mutant gene. Then the native gene on the chromosome is replaced with the mutant gene by homologous recombination, and the resulting strain is selected. Such gene replacement using homologous recombination can be conducted by the method employing a linear DNA, which is known as "Red-driven integration" (Proc. Natl. Acad. Sci. USA, 2000, 97 (12): 6640-6645, WO2005/010175), or by the method employing a plasmid containing a temperature-sensitive replication control region (Proc. Natl. Acad. Sci. USA, 2000, 97 (12): 6640-6645, U.S. Patent Nos. 6,303,383 and 5,616,480). Furthermore, introduction of a site-specific mutation by gene replacement using homologous recombination as set forth above can also be performed by using a plasmid lacking the ability to replicate in the host.

Expression of the gene can also be attenuated by insertion of a transposon or an IS factor into the coding region of the gene (U.S. Patent No. 5,175,107), or by conventional methods, such as mutagenesis treatment using UV irradiation or nitrosoguanidine (N-methyl-N'-nitro-N-nitrosoguanidine) treatment.

The presence of activity of the SanA protein can be detected by, for example, complementation of a *sanA*⁻ mutations by the method described by, for example, Rida, S. et al (J. Bacteriol., 178(1), 94-102 (1996)). So, the reduced or absent activity of the SanA protein in the bacterium according to the present invention can be determined when compared to the parent unmodified bacterium.

The presence or absence of the *sanA* gene on the chromosome of a bacterium can be detected by well-known methods, including PCR, Southern blotting and the like. In addition, the level of gene expression can be estimated by measuring the amount of mRNA transcribed from the gene using various well-known methods, including Northern blotting, quantitative RT-PCR, and the like. Amount or molecular weight of the protein encoded by the gene can be measured by well-known methods, including SDS-PAGE followed by immunoblotting assay (Western blotting analysis), and the like.

Methods for preparation of plasmid DNA, digestion and ligation of DNA, transformation, selection of an oligonucleotide as a primer, and the like may be ordinary methods well known to one skilled in the art. These methods are described, for instance, in Sambrook, J., Fritsch, E.F., and Maniatis, T., "Molecular Cloning A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press (1989).

L-amino acid producing bacteria

As a bacterium of the present invention which is modified to attenuate expression of the *sanA* gene, bacteria which are able to produce either an aromatic or a non-aromatic L-amino acid may be used.

The bacterium of the present invention can be obtained by attenuating expression of the *sanA* gene in a bacterium which inherently has the ability to produce an L-amino acid. Alternatively, the bacterium of present invention can be obtained by imparting the ability to produce an L-amino acid to a bacterium already having attenuated expression of the *sanA* gene.

L-threonine-producing bacteria

Examples of parent strains for deriving the L-threonine-producing bacteria of the present invention include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* TDH-6/pVIC40 (VKPM B-3996) (U.S. Patent No. 5, 175, 107, U.S. Patent No. 5,705,371), *E. coli* 472T23/pYN7 (ATCC 98081) (U.S. Patent No.5,631,157), *E. coli* NRRL-21593 (U.S. Patent No. 5,939,307), *E. coli* FERM BP-3756 (U.S. Patent No. 5,474,918), *E. coli* FERM BP-3519 and FERM BP-3520 (U.S. Patent No. 5,376,538), *E. coli* MG442 (Gusyatiner et al., Genetika (in Russian), 14, 947-956 (1978)), *E. coli* VL643 and VL2055 (EP 1149911 A), and the like.

The strain TDH-6 is deficient in the *thrC* gene, as well as being sucrose-assimilative, and the *ilvA* gene has a leaky mutation. This strain also has a mutation in the *rhtA* gene, which imparts resistance to high concentrations of threonine or homoserine. The strain B-3996 contains the plasmid pVIC40 which was obtained by inserting a *thrA*BC* operon which includes a mutant *thrA* gene into a RSF1010-derived vector. This mutant *thrA* gene encodes aspartokinase homoserine dehydrogenase I which has substantially desensitized feedback inhibition by threonine. The strain B-3996 was deposited on November 19, 1987 in the All-Union Scientific Center of Antibiotics (Nagatinskaya Street 3-A, 117105 Moscow, Russian Federation) under the accession number RIA 1867. The strain was also deposited in the Russian National Collection of Industrial Microorganisms (VKPM) (Russia, 117545 Moscow 1, Dorozhny proezd. 1) on April 7, 1987 under the accession number B-3996.

E. coli VKPM B-5318 (EP 0593792B) may also be used as a parent strain for deriving L-threonine-producing bacteria of the present invention. The strain B-5318 is prototrophic with regard to isoleucine, and a temperature-sensitive lambda-phage C1 repressor and PR promoter replaces the regulatory region of the threonine operon in plasmid pVIC40. The strain VKPM B-5318 was deposited in the Russian National Collection of Industrial Microorganisms (VKPM) on May 3, 1990 under accession number of VKPM B-5318.

Preferably, the bacterium of the present invention is additionally modified to enhance expression of one or more of the following genes:

- the mutant *thrA* gene which codes for aspartokinase homoserine dehydrogenase I resistant to feed back inhibition by threonine;
- the *thrB* gene which codes for homoserine kinase;
- the *thrC* gene which codes for threonine synthase;
- the *rhtA* gene which codes for a putative transmembrane protein;
- the *asd* gene which codes for aspartate- β -semialdehyde dehydrogenase; and

- the *aspC* gene which codes for aspartate aminotransferase (aspartate transaminase);

The *thrA* gene which encodes aspartokinase homoserine dehydrogenase I of *Escherichia coli* has been elucidated (nucleotide positions 337 to 2799, GenBank accession NC_000913.2, gi: 49175990). The *thrA* gene is located between the *thrL* and *thrB* genes on the chromosome of *E. coli* K-12. The *thrB* gene which encodes homoserine kinase of *Escherichia coli* has been elucidated (nucleotide positions 2801 to 3733, GenBank accession NC_000913.2, gi: 49175990). The *thrB* gene is located between the *thrA* and *thrC* genes on the chromosome of *E. coli* K-12. The *thrC* gene which encodes threonine synthase of *Escherichia coli* has been elucidated (nucleotide positions 3734 to 5020, GenBank accession NC_000913.2, gi: 49175990). The *thrC* gene is located between the *thrB* gene and the *yaaX* open reading frame on the chromosome of *E. coli* K-12. All three genes functions as a single threonine operon. To enhance expression of the threonine operon, the attenuator region which affects the transcription is desirably removed from the operon (WO2005/049808, WO2003/097839).

A mutant *thrA* gene which codes for aspartokinase homoserine dehydrogenase I resistant to feed back inhibition by threonine, as well as, the *thrB* and *thrC* genes can be obtained as one operon from well-known plasmid pVIC40 which is presented in the threonine producing *E. coli* strain VKPM B-3996. Plasmid pVIC40 is described in detail in U.S. Patent No. 5,705,371.

The *rhtA* gene exists at 18 min on the *E. coli* chromosome close to the *glnHPQ* operon, which encodes components of the glutamine transport system. The *rhtA* gene is identical to ORF1 (*ybiF* gene, nucleotide positions 764 to 1651, GenBank accession number AAA218541, gi:440181) and located between the *pexB* and *ompX* genes. The unit expressing a protein encoded by the ORF1 has been designated the *rhtA* gene (rht: resistance to homoserine and threonine). Also, it was revealed that the rhtA23 mutation is an A-for-G substitution at position -1 with respect to the ATG start codon (ABSTRACTS of the 17th International Congress of Biochemistry and Molecular Biology in conjunction with Annual Meeting of the American Society for Biochemistry and Molecular Biology, San Francisco, California August 24-29, 1997, abstract No. 457, EP 1013765 A).

The *asd* gene of *E. coli* has already been elucidated (nucleotide positions 3572511 to 3571408, GenBank accession NC_000913.1, gi:16131307), and can be obtained by PCR (polymerase chain reaction; refer to White, T.J. et al., Trends Genet., 5, 185 (1989)) utilizing

primers prepared based on the nucleotide sequence of the gene. The *asd* genes of other microorganisms can be obtained in a similar manner.

Also, the *aspC* gene of *E. coli* has already been elucidated (nucleotide positions 983742 to 984932, GenBank accession NC_000913.1, gi:16128895), and can be obtained by PCR. The *aspC* genes of other microorganisms can be obtained in a similar manner.

L-lysine-producing bacteria

Examples of L-lysine-producing bacteria belonging to the genus *Escherichia* include mutants having resistance to an L-lysine analogue. The L-lysine analogue inhibits growth of bacteria belonging to the genus *Escherichia*, but this inhibition is fully or partially desensitized when L-lysine coexists in a medium. Examples of the L-lysine analogue include, but are not limited to, oxalysine, lysine hydroxamate, S-(2-aminoethyl)-L-cysteine (AEC), γ -methyllysine, α -chlorocaprolactam and so forth. Mutants having resistance to these lysine analogues can be obtained by subjecting bacteria belonging to the genus *Escherichia* to a conventional artificial mutagenesis treatment. Specific examples of bacterial strains useful for producing L-lysine include *Escherichia coli* AJ11442 (FERM BP-1543, NRRL B-12185; see U.S. Patent No. 4,346,170) and *Escherichia coli* VL611. In these microorganisms, feedback inhibition of aspartokinase by L-lysine is desensitized.

The strain WC196 may be used as an L-lysine producing bacterium of *Escherichia coli*. This bacterial strain was bred by conferring AEC resistance to the strain W3110, which was derived from *Escherichia coli* K-12. The resulting strain was designated *Escherichia coli* AJ13069 strain and was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (currently National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary, Tsukuba Central 6, 1-1, Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, 305-8566, Japan) on December 6, 1994 and received an accession number of FERM P-14690. Then, it was converted to an international deposit under the provisions of the Budapest Treaty on September 29, 1995, and received an accession number of FERM BP-5252 (U.S. Patent No. 5,827,698).

Examples of parent strains for deriving L-lysine-producing bacteria of the present invention also include strains in which expression of one or more genes encoding an L-lysine biosynthetic enzyme are enhanced. Examples of such genes include, but are not limited to, genes encoding dihydrodipicolinate synthase (*dapA*), aspartokinase (*lysC*),

dihydrodipicolinate reductase (*dapB*), diaminopimelate decarboxylase (*lysA*), diaminopimelate dehydrogenase (*ddh*) (U.S. Patent No. 6,040,160), phosphoenolpyruvate carboxylase (*ppc*), aspartate semialdehyde dehydrogenase (*asd*), and aspartase (*aspA*) (EP 1253195 A). In addition, the parent strains may have an increased level of expression of the gene involved in energy efficiency (*cyo*) (EP 1170376 A), the gene encoding nicotinamide nucleotide transhydrogenase (*pntAB*) (U.S. Patent No. 5,830,716), the *ybjE* gene (WO2005/073390), or combinations thereof.

Examples of parent strains for deriving L-lysine-producing bacteria of the present invention also include strains having decreased or eliminated activity of an enzyme that catalyzes a reaction for generating a compound other than L-lysine by branching off from the biosynthetic pathway of L-lysine. Examples of the enzymes that catalyze a reaction for generating a compound other than L-lysine by branching off from the biosynthetic pathway of L-lysine include homoserine dehydrogenase, lysine decarboxylase (U.S. Patent No. 5,827,698), and the malic enzyme (WO2005/010175).

L-cysteine-producing bacteria

Examples of parent strains for deriving L-cysteine-producing bacteria of the present invention include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* JM15 which is transformed with different *cysE* alleles coding for feedback-resistant serine acetyltransferases (U.S. Patent No. 6,218,168, Russian patent application 2003121601); *E. coli* W3110 having over-expressed genes which encode proteins suitable for secreting substances toxic for cells (U.S. Patent No. 5,972,663); *E. coli* strains having lowered cysteine desulfohydase activity (JP11155571A2); *E. coli* W3110 with increased activity of a positive transcriptional regulator for cysteine regulon encoded by the *cysB* gene (WO0127307A1), and the like.

L-leucine-producing bacteria

Examples of parent strains for deriving L-leucine-producing bacteria of the present invention include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* strains resistant to leucine (for example, the strain 57 (VKPM B-7386, U.S. Patent No. 6,124,121)) or leucine analogs including β -2-thienylalanine, 3-hydroxyleucine, 4-azaleucine, 5,5,5-trifluoroleucine (JP 62-34397 B and JP 8-70879 A); *E. coli* strains obtained by the gene engineering method described in WO96/06926; *E. coli* H-9068 (JP 8-70879 A), and the like.

The bacterium of the present invention may be improved by enhancing the expression of one or more genes involved in L-leucine biosynthesis. Examples include genes of the *leuABCD* operon, which are preferably represented by a mutant *leuA* gene coding for isopropylmalate synthase freed from feedback inhibition by L-leucine (US Patent 6,403,342). In addition, the bacterium of the present invention may be improved by enhancing the expression of one or more genes coding for proteins which excrete L-amino acid from the bacterial cell. Examples of such genes include the b2682 and b2683 genes (*ygaZH* genes) (EP 1239041 A2).

L-histidine-producing bacteria

Examples of parent strains for deriving L-histidine-producing bacteria of the present invention include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* strain 24 (VKPM B-5945, RU2003677); *E. coli* strain 80 (VKPM B-7270, RU2119536); *E. coli* NRRL B-12116 – B12121 (U.S. Patent No. 4,388,405); *E. coli* H-9342 (FERM BP-6675) and H-9343 (FERM BP-6676) (U.S. Patent No. 6,344,347); *E. coli* H-9341 (FERM BP-6674) (EP1085087); *E. coli* AI80/pFM201 (U.S. Patent No. 6,258,554) and the like.

Examples of parent strains for deriving L-histidine-producing bacteria of the present invention also include strains in which expression of one or more genes encoding an L-histidine biosynthetic enzyme are enhanced. Examples of such genes include genes encoding ATP phosphoribosyltransferase (*hisG*), phosphoribosyl AMP cyclohydrolase (*hisI*), phosphoribosyl-ATP pyrophosphohydrolase (*hisIE*), phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase (*hisA*), amidotransferase (*hisH*), histidinol phosphate aminotransferase (*hisC*), histidinol phosphatase (*hisB*), histidinol dehydrogenase (*hisD*), and so forth.

It is known that the L-histidine biosynthetic enzymes encoded by *hisG* and *hisBHAFI* are inhibited by L-histidine, and therefore an L-histidine-producing ability can also be efficiently enhanced by introducing a mutation conferring resistance to the feedback inhibition into ATP phosphoribosyltransferase (Russian Patent Nos. 2003677 and 2119536).

Specific examples of strains having an L-histidine-producing ability include *E. coli* FERM-P 5038 and 5048 which have been introduced with a vector carrying a DNA encoding an L-histidine-biosynthetic enzyme (JP 56-005099 A), *E. coli* strains introduced with *rht*, a gene for an amino acid-export (EP1016710A), *E. coli* 80 strain imparted with sulfaguanidine,

DL-1,2,4-triazole-3-alanine, and streptomycin-resistance (VKPM B-7270, Russian Patent No. 2119536), and so forth.

L-glutamic acid-producing bacteria

Examples of parent strains for deriving L-glutamic acid-producing bacteria of the present invention include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* VL334thrC⁺ (EP 1172433). *E. coli* VL334 (VKPM B-1641) is an L-isoleucine and L-threonine auxotrophic strain having mutations in *thrC* and *ilvA* genes (U.S. Patent No. 4,278,765). A wild-type allele of the *thrC* gene was transferred by the method of general transduction using a bacteriophage P1 grown on the wild-type *E. coli* strain K12 (VKPM B-7) cells. As a result, an L-isoleucine auxotrophic strain VL334thrC⁺ (VKPM B-8961), which is able to produce L-glutamic acid, was obtained.

Examples of parent strains for deriving the L-glutamic acid-producing bacteria of the present invention include, but are not limited to, strains in which expression of one or more genes encoding an L-glutamic acid biosynthetic enzyme are enhanced. Examples of such genes include genes encoding glutamate dehydrogenase (*gdhA*), glutamine synthetase (*glnA*), glutamate synthetase (*gltAB*), isocitrate dehydrogenase (*icdA*), aconitate hydratase (*acnA*, *acnB*), citrate synthase (*gltA*), phosphoenolpyruvate carboxylase (*ppc*), pyruvate carboxylase (*pyc*), pyruvate dehydrogenase (*aceEF*, *lpdA*), pyruvate kinase (*pykA*, *pykF*), phosphoenolpyruvate synthase (*ppsA*), enolase (*eno*), phosphoglyceromutase (*pgmA*, *pgmI*), phosphoglycerate kinase (*pgk*), glyceraldehyde-3-phosphate dehydrogenase (*gapA*), triose phosphate isomerase (*tpiA*), fructose biphosphate aldolase (*fbp*), phosphofructokinase (*pfkA*, *pfkB*), and glucose phosphate isomerase (*pgi*).

Examples of strains modified so that expression of the citrate synthetase gene, the phosphoenolpyruvate carboxylase gene, and/or the glutamate dehydrogenase gene is/are enhanced include those disclosed in EP1078989A, EP955368A, and EP952221A.

Examples of strains modified so that expression of the citrate synthetase gene, the phosphoenolpyruvate carboxylase gene, and/or the glutamate dehydrogenase gene is/are enhanced include those disclosed in EP1078989A, EP955368A, and EP952221A.

Examples of parent strains for deriving the L-glutamic acid-producing bacteria of the present invention also include strains having decreased or eliminated activity of an enzyme that catalyzes synthesis of a compound other than L-glutamic acid by branching off from an L-glutamic acid biosynthesis pathway. Examples of such enzymes include isocitrate lyase

(*aceA*), α -ketoglutarate dehydrogenase (*sucA*), phosphotransacetylase (*pta*), acetate kinase (*ack*), acetohydroxy acid synthase (*ilvG*), acetolactate synthase (*ilvI*), formate acetyltransferase (*pfl*), lactate dehydrogenase (*ldh*), and glutamate decarboxylase (*gadAB*). Bacteria belonging to the genus *Escherichia* deficient in the α -ketoglutarate dehydrogenase activity or having a reduced α -ketoglutarate dehydrogenase activity and methods for obtaining them are described in U.S. Patent Nos. 5,378,616 and 5,573,945. Specifically, these strains include the following:

E. coli W3110*sucA::Kmr*

E. coli AJ12624 (FERM BP-3853)

E. coli AJ12628 (FERM BP-3854)

E. coli AJ12949 (FERM BP-4881)

E. coli W3110*sucA::Kmr* is a strain obtained by disrupting the α -ketoglutarate dehydrogenase gene (hereinafter referred to as "*sucA* gene") of *E. coli* W3110. This strain is completely deficient in the α -ketoglutarate dehydrogenase.

Other examples of L-glutamic acid-producing bacterium include those which belong to the genus *Escherichia* and have resistance to an aspartic acid antimetabolite. These strains can also be deficient in the α -ketoglutarate dehydrogenase activity and include, for example, *E. coli* AJ13199 (FERM BP-5807) (U.S. Patent No. 5,908,768), FFRM P-12379, which additionally has a low L-glutamic acid decomposing ability (U.S. Patent No. 5,393,671); AJ13138 (FERM BP-5565) (U.S. Patent No. 6,110,714), and the like.

Examples of L-glutamic acid-producing bacteria, include mutant strains belonging to the genus *Pantoea* which are deficient in the α -ketoglutarate dehydrogenase activity or have a decreased α -ketoglutarate dehydrogenase activity, and can be obtained as described above. Such strains include *Pantoea ananatis* AJ13356. (U.S. Patent No. 6,331,419). *Pantoea ananatis* AJ13356 was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (currently, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary, Central 6, 1-1, Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, 305-8566, Japan) on February 19, 1998 under an accession number of FERM P-16645. It was then converted to an international deposit under the provisions of Budapest Treaty on January 11, 1999 and received an accession number of FERM BP-6615. *Pantoea ananatis* AJ13356 is deficient in the α -ketoglutarate dehydrogenase activity as a result of

disruption of the α KGDH-E1 subunit gene (*sucA*). The above strain was identified as *Enterobacter agglomerans* when it was isolated and deposited as the *Enterobacter agglomerans* AJ13356. However, it was recently re-classified as *Pantoea ananatis* on the basis of nucleotide sequencing of 16S rRNA and so forth. Although AJ13356 was deposited at the aforementioned depository as *Enterobacter agglomerans*, for the purposes of this specification, they are described as *Pantoea ananatis*.

L-phenylalanine-producing bacteria

Examples of parent strains for deriving L-phenylalanine-producing bacteria of the present invention include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* AJ12739 (*tyrA*::Tn10, *tyrR*) (VKPM B-8197); *E. coli* HW1089 (ATCC 55371) harboring the mutant *pheA34* gene (U.S. Patent No. 5,354,672); *E. coli* MWEC101-b (KR8903681); *E. coli* NRRL B-12141, NRRL B-12145, NRRL B-12146 and NRRL B-12147 (U.S. Patent No. 4,407,952). Also, as a parent strain, *E. coli* K-12 [W3110 (*tyrA*)/pPHAB (FERM BP-3566), *E. coli* K-12 [W3110 (*tyrA*)/pPHAD] (FERM BP-12659), *E. coli* K-12 [W3110 (*tyrA*)/pPHATerm] (FERM BP-12662) and *E. coli* K-12 [W3110 (*tyrA*)/pBR-aroG4, pACMAB] named as AJ 12604 (FERM BP-3579) may be used (EP 488424 B1). Furthermore, L-phenylalanine producing bacteria belonging to the genus *Escherichia* with an enhanced activity of the protein encoded by the *yedA* gene or the *yddG* gene may also be used (U.S. patent applications 2003/0148473 A1 and 2003/0157667 A1).

L-tryptophan-producing bacteria

Examples of parent strains for deriving the L-tryptophan-producing bacteria of the present invention include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* JP4735/pMU3028 (DSM10122) and JP6015/pMU91 (DSM10123) deficient in the tryptophanyl-tRNA synthetase encoded by mutant *trpS* gene (U.S. Patent No. 5,756,345); *E. coli* SV164 (pGH5) having a *serA* allele encoding phosphoglycerate dehydrogenase free from feedback inhibition by serine and a *trpE* allele encoding anthranilate synthase free from feedback inhibition by tryptophan (U.S. Patent No. 6,180,373); *E. coli* AGX17 (pGX44) (NRRL B-12263) and AGX6(pGX50)aroP (NRRL B-12264) deficient in the enzyme tryptophanase (U.S. Patent No. 4,371,614); *E. coli* AGX17/pGX50,pACKG4-pps in which a phosphoenolpyruvate-producing ability is enhanced (WO9708333, U.S. Patent No. 6,319,696), and the like may be used. L-tryptophan-

producing bacteria belonging to the genus *Escherichia* with an enhanced activity of the protein encoded by the *yedA* gene or the *yddG* gene may also be used (U.S. patent applications 2003/0148473 A1 and 2003/0157667 A1).

Examples of parent strains for deriving the L-tryptophan-producing bacteria of the present invention also include strains in which one or more activities of the enzymes selected from anthranilate synthase, phosphoglycerate dehydrogenase, and tryptophan synthase are enhanced. The anthranilate synthase and phosphoglycerate dehydrogenase are both subject to feedback inhibition by L-tryptophan and L-serine, so that a mutation desensitizing the feedback inhibition may be introduced into these enzymes. Specific examples of strains having such a mutation include a *E. coli* SV164 which harbors desensitized anthranilate synthase and a transformant strain obtained by introducing into the *E. coli* SV164 the plasmid pGH5 (WO 94/08031), which contains a mutant *serA* gene encoding feedback-desensitized phosphoglycerate dehydrogenase.

Examples of parent strains for deriving the L-tryptophan-producing bacteria of the present invention also include strains into which the tryptophan operon which contains a gene encoding desensitized anthranilate synthase has been introduced (JP 57-71397 A, JP 62-244382 A, U.S. Patent No. 4,371,614). Moreover, L-tryptophan-producing ability may be imparted by enhancing expression of a gene which encodes tryptophan synthase, among tryptophan operons (*trpBA*). The tryptophan synthase consists of α and β subunits which are encoded by the *trpA* and *trpB* genes, respectively. In addition, L-tryptophan-producing ability may be improved by enhancing expression of the isocitrate lyase-malate synthase operon (WO2005/103275).

L-proline-producing bacteria

Examples of parent strains for deriving L-proline-producing bacteria of the present invention include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* 702ilvA (VKPM B-8012) which is deficient in the *ilvA* gene and is able to produce L-proline (EP 1172433). The bacterium of the present invention may be improved by enhancing the expression of one or more genes involved in L-proline biosynthesis. Examples of such genes for L-proline producing bacteria which are preferred include the *proB* gene coding for glutamate kinase of which feedback inhibition by L-proline is desensitized (DE Patent 3127361). In addition, the bacterium of the present invention may be improved by enhancing the expression of one or more genes coding for proteins excreting L-amino acid from

bacterial cell. Such genes are exemplified by b2682 and b2683 genes (*ygaZH* genes) (EP1239041 A2).

Examples of bacteria belonging to the genus *Escherichia*, which have an activity to produce L-proline include the following *E. coli* strains: NRRL B-12403 and NRRL B-12404 (GB Patent 2075056), VKPM B-8012 (Russian patent application 2000124295), plasmid mutants described in DE Patent 3127361, plasmid mutants described by Bloom F.R. et al (The 15th Miami winter symposium, 1983, p.34), and the like.

L-arginine-producing bacteria

Examples of parent strains for deriving L-arginine-producing bacteria of the present invention include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* strain 237 (VKPM B-7925) (U.S. Patent Application 2002/058315 A1) and its derivative strains harboring mutant N-acetylglutamate synthase (Russian Patent Application No. 2001112869), *E. coli* strain 382 (VKPM B-7926) (EP1170358A1), an arginine-producing strain into which *argA* gene encoding N-acetylglutamate synthetase is introduced therein (EP1170361A1), and the like.

Examples of parent strains for deriving L-arginine producing bacteria of the present invention also include strains in which expression of one or more genes encoding an L-arginine biosynthetic enzyme are enhanced. Examples of such genes include genes encoding N-acetylglutamyl phosphate reductase (*argC*), ornithine acetyl transferase (*argJ*), N-acetylglutamate kinase (*argB*), acetylornithine transaminase (*argD*), ornithine carbamoyl transferase (*argF*), argininosuccinic acid synthetase (*argG*), argininosuccinic acid lyase (*argH*), and carbamoyl phosphate synthetase (*carAB*).

L-valine-producing bacteria

Example of parent strains for deriving L-valine-producing bacteria of the present invention include, but are not limited to, strains which have been modified to overexpress the *ilvGMEDA* operon (U.S. Patent No. 5,998,178). It is desirable to remove the region of the *ilvGMEDA* operon which is required for attenuation so that expression of the operon is not attenuated by L-valine that is produced. Furthermore, the *ilvA* gene in the operon is desirably disrupted so that threonine deaminase activity is decreased.

Examples of parent strains for deriving L-valine-producing bacteria of the present invention include also include mutants having a mutation of amino-acyl t-RNA synthetase

(U.S. Patent No. 5,658,766). For example, *E. coli* VL1970, which has a mutation in the *ileS* gene encoding isoleucine tRNA synthetase, can be used. *E. coli* VL1970 has been deposited in the Russian National Collection of Industrial Microorganisms (VKPM) (Russia, 113545 Moscow, 1 Dorozhny Proezd, 1) on June 24, 1988 under accession number VKPM B-4411.

Furthermore, mutants requiring lipoic acid for growth and/or lacking H⁺-ATPase can also be used as parent strains (WO96/06926).

L-isoleucine-producing bacteria

Examples of parent strains for deriving L-isoleucine producing bacteria of the present invention include, but are not limited to, mutants having resistance to 6-dimethylaminopurine (JP 5-304969 A), mutants having resistance to an isoleucine analogue such as thiaisoleucine and isoleucine hydroxamate, and mutants additionally having resistance to DL-ethionine and/or arginine hydroxamate (JP 5-130882 A). In addition, recombinant strains transformed with genes encoding proteins involved in L-isoleucine biosynthesis, such as threonine deaminase and acetohydroxate synthase, can also be used as parent strains (JP 2-458 A, FR 0356739, and U.S. Patent No. 5,998,178).

2. Method of the present invention

The method of the present invention is a method for producing an L-amino acid comprising cultivating the bacterium of the present invention in a culture medium to produce and excrete the L-amino acid into the medium, and collecting the L-amino acid from the medium.

In the present invention, the cultivation, collection, and purification of an L-amino acid from the medium and the like may be performed in a manner similar to conventional fermentation methods wherein an amino acid is produced using a bacterium.

A medium used for culture may be either a synthetic or natural medium, so long as the medium includes a carbon source and a nitrogen source and minerals and, if necessary, appropriate amounts of nutrients which the bacterium requires for growth. The carbon source may include various carbohydrates such as glucose and sucrose, and various organic acids. Depending on the mode of assimilation of the used microorganism, alcohol, including ethanol and glycerol, may be used. As the nitrogen source, various ammonium salts such as ammonia and ammonium sulfate, other nitrogen compounds such as amines, a natural nitrogen source such as peptone, soybean-hydrolysate, and digested fermentative

microorganism can be used. As minerals, potassium monophosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, calcium chloride, and the like can be used. As vitamins, thiamine, yeast extract, and the like, can be used.

The cultivation is preferably performed under aerobic conditions, such as a shaking culture, and a stirring culture with aeration, at a temperature of 20 to 40 °C, preferably 30 to 38 °C. The pH of the culture is usually between 5 and 9, preferably between 6.5 and 7.2. The pH of the culture can be adjusted with ammonia, calcium carbonate, various acids, various bases, and buffers. Usually, a 1 to 5-day cultivation leads to accumulation of the target L-amino acid in the liquid medium.

After cultivation, solids such as cells can be removed from the liquid medium by centrifugation or membrane filtration, and then the L-amino acid can be collected and purified by ion-exchange, concentration, and/or crystallization methods.

Brief Description of Drawings

Figure 1 shows the relative positions of primers sanAL and sanAR on plasmid pACYC184, which is used for amplification of the *cat* gene.

Figure 2 shows the construction of the chromosomal DNA fragment containing the inactivated *sanA* gene.

Examples

The present invention will be more concretely explained below with reference to the following non-limiting Examples.

Example 1. Construction of a strain with an inactivated *sanA* gene

1. Deletion of the *sanA* gene

A strain having deletion of the *sanA* gene was constructed by the method initially developed by Datsenko, K.A. and Wanner, B.L. (Proc. Natl. Acad. Sci. USA, 2000, 97(12), 6640-6645) called "Red-driven integration". According to this procedure, the PCR primers sanAL (SEQ ID NO: 3) and sanAR (SEQ ID NO: 4), which are homologous to both the regions adjacent to the *sanA* gene and the gene conferring antibiotic resistance, respectively, in the template plasmid, were constructed. The plasmid pACYC184 (NBL Gene Sciences Ltd., UK) (GenBank/EMBL accession number X06403) was used as a template in the PCR reaction. Conditions for PCR were as follows: denaturation step: 3 min at 95 °C; profile for

two first cycles: 1 min at 95 °C, 30 sec at 50 °C, 40 sec at 72 °C; profile for the last 25 cycles: 30 sec at 95 °C, 30 sec at 54 °C, 40 sec at 72 °C; final step: 5 min at 72 °C.

A 1152 bp PCR product (Fig. 1) was obtained and purified in agarose gel and used for electroporation of *E. coli* MG1655 (ATCC 700926), which contains the plasmid pKD46 having a temperature-sensitive replication. The plasmid pKD46 (Datsenko, K.A. and Wanner, B.L., Proc. Natl. Acad. Sci. USA, 2000, 97:12:6640-45) includes a 2,154 nucleotide (31088-33241) DNA fragment of phage λ (GenBank accession No. J02459), and contains genes of the λ Red homologous recombination system (γ , β , exo genes) under the control of the arabinose-inducible P_{araB} promoter. The plasmid pKD46 is necessary for integration of the PCR product into the chromosome of strain MG1655.

Electrocompetent cells were prepared as follows: *E. coli* MG1655/pKD46 was grown overnight at 30 °C in LB medium containing ampicillin (100 mg/l), and the culture was diluted 100 times with 5 ml of SOB medium (Sambrook et al, "Molecular Cloning A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press (1989)) containing ampicillin and L-arabinose (1 mM). The cells were grown with aeration at 30 °C to an OD₆₀₀ of ≈ 0.6 and then were made electrocompetent by concentrating 100-fold and washing three times with ice-cold deionized H₂O. Electroporation was performed using 70 μ l of cells and ≈ 100 ng of PCR product. Cells after electroporation were incubated with 1 ml of SOC medium (Sambrook et al, "Molecular Cloning A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press (1989)) at 37 °C for 2.5 hours and then were plated onto L-agar containing chloramphenicol (30 μ g/ml) and grown at 37 °C to select Cm^R recombinants. Then, to eliminate the pKD46 plasmid, 2 passages on L-agar with Cm at 42 °C were performed and the obtained colonies were tested for sensitivity to ampicillin.

2. Verification of the *sanA* gene deletion by PCR.

The mutants, which have the *sanA* gene deleted, marked with the Cm resistance gene, were verified by PCR. Locus-specific primers sanA1 (SEQ ID NO: 5) and sanA2 (SEQ ID NO: 6) were used in PCR for verification. Conditions for PCR verification were as follows: denaturation step: 3 min at 94 °C; profile for the 30 cycles: 30 sec at 94 °C, 30 sec at 54 °C, 1 min at 72 °C; final step: 7 min at 72 °C. The PCR product obtained in the reaction with the cells of the parental *sanA*⁺ strain MG1655 as the template was 839 bp in length. The PCR

product obtained in the reaction with the cells of the mutant strain as the template was 1344 bp in length (Fig.2). The mutant strain was named MG1655 Δ sanA::cat.

Example 2. Production of L-threonine by *E. coli* B-3996- Δ sanA.

To test the effect of inactivation of the *sanA* gene on threonine production, DNA fragments from the chromosome of the above-described *E. coli* MG1655 Δ sanA::cat can be transferred to the threonine-producing *E. coli* strain VKPM B-3996 by P1 transduction (Miller, J.H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Lab. Press, Plainview, NY) to obtain the strain B-3996- Δ sanA.

Both *E. coli* B-3996 and B-3996- Δ sanA can be grown for 18-24 hours at 37 °C on L-agar plates. To obtain a seed culture, the strains can be grown on a rotary shaker (250 rpm) at 32 °C for 18 hours in 20x200 mm test tubes containing 2 ml of L-broth with 4% sucrose. Then, the fermentation medium can be inoculated with 0.21 ml (10%) seed material. The fermentation can be performed in 2 ml of minimal medium for fermentation in 20x200 mm test tubes. Cells can be grown for 48 hours at 32°C with shaking at 250 rpm.

After cultivation, the amount of L-threonine which has accumulated in the medium can be determined by paper chromatography using the following mobile phase: butanol : acetic acid : water = 4 : 1 : 1 (v/v). A solution (2%) of ninhydrin in acetone can be used as a visualizing reagent. A spot containing L-threonine can be cut out, L-threonine can be eluted in 0.5 % water solution of CdCl₂, and the amount of L-threonine can be estimated spectrophotometrically at 540 nm.

The composition of the fermentation medium (g/l) is as follows:

Glucose	80.0
(NH ₄) ₂ SO ₄	22.0
NaCl	0.8
KH ₂ PO ₄	2.0
MgSO ₄ 7H ₂ O	0.8
FeSO ₄ 7H ₂ O	0.02
MnSO ₄ 5H ₂ O	0.02
Thiamine HCl	0.0002
Yeast extract	1.0
CaCO ₃	30.0

Glucose and magnesium sulfate are sterilized separately. CaCO₃ is sterilized by dry-

heat at 180°C for 2 hours. The pH is adjusted to 7.0. Antibiotic is introduced into the medium after sterilization.

Example 3. Production of L-lysine by *E. coli* WC196(pCABD2)- Δ sanA.

To test the effect of inactivation of the *sanA* gene on lysine production, DNA fragments from the chromosome of the above-described *E. coli* MG1655 Δ sanA::cat can be transferred to the lysine-producing *E. coli* strain WC196(pCABD2) by P1 transduction (Miller, J.H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Lab. Press, Plainview, NY) to obtain the strain WC196(pCABD2)- Δ sanA. pCABD2 is a plasmid which includes a *dapA* gene coding for a dihydrodipicolinate synthase having a mutation which desensitizes feedback inhibition by L-lysine, a *lysC* gene coding for aspartokinase III having a mutation which desensitizes feedback inhibition by L-lysine, a *dapB* gene coding for a dihydrodipicolinate reductase gene, a *ddh* gene coding for diaminopimelate dehydrogenase, and a streptomycin resistance gene (U.S. Patent No. 6,040,160).

Both *E. coli* WC196(pCABD2) and WC196(pCABD2)- Δ sanA can be cultured in the L-medium containing 20 mg/l of streptomycin at 37 °C. 0.3 ml of the obtained cultures can each be inoculated into 20 ml of the fermentation medium containing the drug in a 500 ml-flask. The cultivation can be carried out at 37 °C for 16 hours by using a reciprocal shaker at the agitation speed of 115 rpm. After the cultivation, the amounts of L-lysine and residual glucose in the medium can be measured by a known method (Biotech-analyzer AS210, manufactured by Sakura Seiki Co.). Then, the yield of L-lysine relative to consumed glucose can be calculated for each of the strains.

The composition of the fermentation medium (g/l) is as follows:

Glucose	40
(NH ₄) ₂ SO ₄	24
K ₂ HPO ₄	1.0
MgSO ₄ ·7H ₂ O	1.0
FeSO ₄ ·7H ₂ O	0.01
MnSO ₄ ·5H ₂ O	0.01
Yeast extract	2.0

pH is adjusted to 7.0 by KOH and the medium is autoclaved at 115°C for 10 min.

Glucose and MgSO₄·7H₂O are sterilized separately. 30 g/l of CaCO₃, which has been dry-

heat sterilized at 180°C for 2 hours, is added.

Example 4. Production of L-cysteine by *E. coli* JM15(ydeD)- Δ sanA.

To test the effect of inactivation of the *sanA* gene on L-cysteine production, DNA fragments from the chromosome of the above-described *E. coli* MG1655 Δ sanA::cat can be transferred to the *E. coli* L-cysteine producing strain JM15(ydeD) by P1 transduction (Miller, J.H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Lab. Press, Plainview, NY) to obtain the strain JM15(ydeD)- Δ sanA.

E. coli JM15(ydeD) is a derivative of *E. coli* JM15 (U.S. Patent No. 6,218,168) which can be transformed with DNA having the *ydeD* gene, which codes for a membrane protein, and is not involved in a biosynthetic pathway of any L-amino acid (U.S. Patent No. 5,972,663). The strain JM15 (CGSC# 5042) can be obtained from The Coli Genetic Stock Collection at the *E.coli* Genetic Resource Center, MCD Biology Department, Yale University (<http://cgsc.biology.yale.edu/>).

Fermentation conditions for evaluation of L-cysteine production are described in detail in Example 6 of U.S. Patent No. 6,218,168.

Example 5. Production of L-leucine by *E. coli* 57- Δ sanA.

To test the effect of inactivation of the *sanA* gene on L-leucine production, DNA fragments from the chromosome of the above-described *E. coli* MG1655 Δ sanA::cat can be transferred to the *E. coli* L-leucine producing strain 57 (VKPM B-7386, U.S. Patent No. 6,124,121) can be performed by P1 transduction (Miller, J.H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Lab. Press, Plainview, NY) to obtain the strain 57- Δ sanA. The strain 57 has been deposited in the Russian National Collection of Industrial Microorganisms (VKPM) (Russia, 117545 Moscow, 1 Dorozhny proezd, 1) on May 19, 1997 under accession number VKPM B-7386.

Both *E. coli* 57 and 57- Δ sanA can be cultured for 18-24 hours at 37 °C on L-agar plates. To obtain a seed culture, the strains can be grown on a rotary shaker (250 rpm) at 32 °C for 18 hours in 20x200 mm test tubes containing 2 ml of L-broth with 4% sucrose. Then, the fermentation medium can be inoculated with 0.21 ml (10%) seed material. The fermentation can be performed in 2 ml of minimal medium for fermentation in 20x200 mm test tubes. Cells can be grown for 48-72 hours at 32°C with shaking at 250 rpm. The amount of L-

leucine can be measured by paper chromatography (liquid phase composition: butanol - acetic acid - water = 4:1:1)

The composition of the fermentation medium (g/l) is as follows (pH 7.2):

Glucose	60.0
(NH ₄) ₂ SO ₄	25.0
K ₂ HPO ₄	2.0
MgSO ₄ ·7H ₂ O	1.0
Thiamine	0.01
CaCO ₃	25.0

Glucose and CaCO₃ are sterilized separately.

Example 6. Production of L-histidine by *E. coli* 80-ΔsanA.

To test the effect of inactivation of the *sanA* gene on L-histidine production, DNA fragment from the chromosome of the above-described *E. coli* MG1655 ΔsanA::cat was transferred to the histidine-producing *E. coli* strain 80 by P1 transduction (Miller, J.H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Lab. Press, Plainview, NY) to obtain the strain 80-ΔsanA. The strain 80 has been described in Russian patent 2119536 and deposited in the Russian National Collection of Industrial Microorganisms (Russia, 117545 Moscow, 1 Dorozhny proezd, 1) on October 15, 1999 under accession number VRPM B-7270 and then converted to a deposit under the Budapest Treaty on July 12, 2004.

Both *E. coli* 80 and 80-ΔsanA were cultivated in L-broth for 6 hours at 29 °C. Then, 0.1 ml of obtained cultures were each inoculated into 2 ml of fermentation medium in 20x200mm test tube and cultivated for 65 hours at 29 °C with a rotary shaker (350 rpm). After cultivation, the amount of histidine which had accumulated in the medium was determined by paper chromatography. The paper was developed with a mobile phase: n-butanol : acetic acid : water = 4 : 1 : 1 (v/v). A solution of ninhydrin (0.5%) in acetone was used as a visualizing reagent.

The composition of the fermentation medium (g/l) was as follows (pH 6.0):

Glucose	100.0
Mameno (soybean hydrolysate)	0.2 as total nitrogen
L-proline	1.0
(NH ₄) ₂ SO ₄	25.0
KH ₂ PO ₄	2.0

MgSO ₄ ·7H ₂ O	1.0
FeSO ₄ ·7H ₂ O	0.01
MnSO ₄	0.01
Thiamine	0.001
Betaine	2.0
CaCO ₃	60.0

Glucose, proline, betaine and CaCO₃ were sterilized separately. pH was adjusted to 6.0 before sterilization.

Table 1

Strain	Amount of L-histidine, g/l
80	16.8 ± 0.5
80-ΔsanA	18.4 ± 0.8

It can be seen from the Table 1, the strain 80-ΔsanA caused accumulation of a higher amount of L-histidine, as compared with the strain 80.

Example 7. Production of L-glutamate by *E. coli* VL334thrC⁺-ΔsanA.

To test the effect of inactivation of the *sanA* gene on L-glutamate production, DNA fragments from the chromosome of the above-described *E. coli* MG1655 ΔsanA::cat can be transferred to the *E. coli* L-glutamate producing strain VL334thrC⁺ (EP 1172433) by P1 transduction (Miller, J.H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Lab. Press, Plainview, NY) to obtain the strain VL334thrC⁺-ΔsanA. The strain L334thrC⁺ has been deposited in the Russian National Collection of Industrial Microorganisms (VKPM) (Russia, 117545 Moscow, 1 Dorozhny proezd, 1) on December 6, 2004 under the accession number B-8961 and then converted to a deposit under the Budapest Treaty on December 8, 2004.

Both strains, VL334thrC⁺ and VL334thrC⁺-ΔsanA, can be grown for 18-24 hours at 37 °C on L-agar plates. Then, one loop of the cells can be transferred into test tubes containing 2ml of fermentation medium. The fermentation medium contains 60g/l glucose, 25 g/l ammonium sulfate, 2g/l KH₂PO₄, 1 g/l MgSO₄, 0.1 mg/ml thiamine, 70 μg/ml L-isoleucine and 25 g/l CaCO₃ (pH 7.2). Glucose and CaCO₃ are sterilized separately. Cultivation can be

carried out at 30 °C for 3 days with shaking. After the cultivation, the amount of L-glutamic acid produced can be determined by paper chromatography (liquid phase composition: butanol-acetic acid-water=4:1:1) with subsequent staining by ninhydrin (1% solution in acetone) and further elution of the compounds in 50% ethanol with 0.5% CdCl₂.

Example 8. Production of L- phenylalanine by *E. coli* AJ12739-ΔsanA.

To test the effect of inactivation of the *sanA* gene on L-phenylalanine production, DNA fragments from the chromosome of the above-described *E. coli* MG1655 ΔsanA::cat can be transferred to the phenylalanine-producing *E. coli* strain AJ12739 by P1 transduction (Miller, J.H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Lab. Press, Plainview, NY) to obtain the strain AJ12739-ΔsanA. The strain AJ12739 has been deposited in the Russian National Collection of Industrial Microorganisms (VKPM) (Russia, 117545 Moscow, 1 Dorozhny proezd, 1) on November 6, 2001 under accession number VKPM B-8197 and then converted to a deposit under the Budapest Treaty on August 23, 2002.

Both strains, AJ12739-ΔsanA and AJ12739, can be cultivated at 37 °C for 18 hours in a nutrient broth. 0.3 ml of the obtained cultures can each be inoculated into 3 ml of a fermentation medium in a 20 x 200 mm test tube and cultivated at 37 °C for 48 hours with a rotary shaker. After cultivation, the amount of phenylalanine which accumulates in the medium can be determined by TLC. 10 x 15 cm TLC plates coated with 0.11 mm layers of Sorbfil silica gel without fluorescent indicator (Stock Company Sorbpolymer, Krasnodar, Russia) can be used. Sorbfil plates can be developed with a mobile phase: propan-2-ol : ethylacetate : 25% aqueous ammonia : water = 40 : 40 : 7 : 16 (v/v). A solution (2%) of ninhydrin in acetone can be used as a visualizing reagent.

The composition of the fermentation medium (g/l) is as follows:

Glucose	40.0
(NH ₄) ₂ SO ₄	16.0
K ₂ HPO ₄	0.1
MgSO ₄ ·7H ₂ O	1.0
FeSO ₄ ·7H ₂ O	0.01
MnSO ₄ ·5H ₂ O	0.01
Thiamine HCl	0.0002
Yeast extract	2.0
Tyrosine	0.125

CaCO₃ 20.0

Glucose and magnesium sulfate are sterilized separately. CaCO₃ is sterilized by dry-heat at 180 °C for 2 hours. pH is adjusted to 7.0.

Example 9. Production of L- tryptophan by *E. coli* SV164 (pGH5)-ΔsanA.

To test the effect of inactivation of the *sanA* gene on L-tryptophan production, DNA fragments from the chromosome of the above-described *E. coli* MG1655 ΔsanA::cat can be transferred to the tryptophan-producing *E. coli* strain SV164 (pGH5) by P1 transduction (Miller, J.H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Lab. Press, Plainview, NY) to obtain the strain SV164(pGH5)-ΔsanA. The strain SV164 has the *trpE* allele encoding anthranilate synthase free from feedback inhibition by tryptophan. The plasmid pGH5 harbors a mutant *serA* gene encoding phosphoglycerate dehydrogenase free from feedback inhibition by serine. The strain SV164 (pGH5) is described in detail in U.S. Patent No. 6,180,373.

Both strains, SV164(pGH5)-ΔsanA and SV164(pGH5), can be cultivated with shaking at 37 °C for 18 hours in a 3 ml of nutrient broth supplemented with 20 μg/ml of tetracycline (marker of pGH5 plasmid). 0.3 ml of the obtained cultures can be inoculated into 3 ml of a fermentation medium containing tetracycline (20 μg/ml) in 20 x 200 mm test tubes, and cultivated at 37 °C for 48 hours with a rotary shaker at 250 rpm. After cultivation, the amount of tryptophan which accumulates in the medium can be determined by TLC as described in Example 8. The fermentation medium components are set forth in Table 2, but should be sterilized in separate groups A, B, C, D, E, F, and H, as shown, to avoid adverse interactions during sterilization.

Table 2

Groups	Component	Final concentration, g/l
A	KH ₂ PO ₄	1.5
	NaCl	0.5
	(NH ₄) ₂ SO ₄	1.5
	L-Methionine	0.05
	L-Phenylalanine	0.1
	L-Tyrosine	0.1
	Mameno (total N)	0.07
B	Glucose	40.0
	MgSO ₄ ·7H ₂ O	0.3
C	CaCl ₂	0.011
D	FeSO ₄ ·7H ₂ O	0.075
	Sodium citrate	1.0
E	Na ₂ MoO ₄ ·2H ₂ O	0.00015
	H ₃ BO ₃	0.0025
	CoCl ₂ ·6H ₂ O	0.00007
	CuSO ₄ ·5H ₂ O	0.00025
	MnCl ₂ ·4H ₂ O	0.0016
	ZnSO ₄ ·7 H ₂ O	0.0003
F	Thiamine HCl	0.005
G	CaCO ₃	30.0
H	Pyridoxine	0.03

Group A has pH 7.1 adjusted by NH₄OH. Each of groups A, B, C, D, E, F and H is sterilized separately, chilled, and mixed together, and then CaCO₃ sterilized by dry heat is added to the complete fermentation medium.

Example 10. Production of L-proline by *E. coli* 702ilvA-ΔsanA.

To test the effect of inactivation of the *sanA* gene on L-proline production, DNA fragments from the chromosome of the above-described *E. coli* MG1655 Δ*sanA*::cat can be transferred to the proline-producing *E. coli* strain 702ilvA by P1 transduction (Miller, J.H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Lab. Press, Plainview, NY) to obtain the strain 702ilvA-Δ*sanA*. The strain 702ilvA has been deposited in the Russian National Collection of Industrial Microorganisms (VKPM) (Russia, 117545 Moscow, 1 Dorozhny proezd, 1) on July 18, 2000 under accession number VKPM B-8012 and then converted to a deposit under the Budapest Treaty on May 18, 2001.

Both *E. coli* 702ilvA and 702ilvA-Δ*sanA* can be grown for 18-24 hours at 37 °C on L-agar plates. Then, these strains can be cultivated under the same conditions as in Example 7.

Example 11. Production of L-arginine by *E. coli* 382- Δ sanA.

To test the effect of inactivation of the *sanA* gene on L-arginine production, DNA fragments from the chromosome of the above-described *E. coli* MG1655 Δ sanA::cat can be transferred to the arginine-producing *E. coli* strain 382 by P1 transduction (Miller, J.H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Lab. Press, Plainview, NY) to obtain the strain 382- Δ sanA. The strain 382 has been deposited in the Russian National Collection of Industrial Microorganisms (VKPM) (Russia, 117545 Moscow, 1 Dorozhny proezd, 1) on April 10, 2000 under accession number VKPM B-7926 and then converted to a deposit under the Budapest Treaty on May 18, 2001.

Both strains, 382- Δ sanA and 382, can be each cultivated with shaking at 37 °C for 18 hours in a 3 ml of nutrient broth. 0.3 ml of the obtained cultures can be inoculated into 3 ml of a fermentation medium in 20 x 200 mm test tubes, and cultivated at 32 °C for 48 hours on a rotary shaker.

After the cultivation, the amount of L-arginine which accumulates in the medium can be determined by paper chromatography using following mobile phase: butanol : acetic acid : water = 4 : 1 : 1 (v/v). A solution (2%) of ninhydrin in acetone can be used as a visualizing reagent. A spot containing L-arginine can be cut out, L-arginine can be eluted in 0.5 % water solution of CdCl₂, and the amount of L-arginine can be estimated spectrophotometrically at 540 nm.

The composition of the fermentation medium (g/l) is as follows:

Glucose	48.0
(NH ₄) ₂ SO ₄	35.0
KH ₂ PO ₄	2.0
MgSO ₄ ·7H ₂ O	1.0
Thiamine HCl	0.0002
Yeast extract	1.0
L-isoleucine	0.1
CaCO ₃	5.0

Glucose and magnesium sulfate are sterilized separately. CaCO₃ dry-heat sterilized at 180 °C for 2 hours. pH is adjusted to 7.0.

While the invention has been described in detail with reference to preferred embodiments thereof, it will be apparent to one skilled in the art that various changes can be

made, and equivalents employed, without departing from the scope of the invention. All the cited references herein are incorporated as a part of this application by reference.

Industrial Applicability

According to the present invention, production of L-amino acid of a bacterium of the *Enterobacteriaceae* family can be enhanced.

CLAIMS

1. An L-amino acid producing bacterium of the *Enterobacteriaceae* family, wherein said bacterium has been modified to attenuate expression of the *sanA* gene.
2. The bacterium according to claim 1, wherein said expression of the *sanA* gene is attenuated by inactivation of the *sanA* gene.
3. The bacterium according to claim 1, wherein said bacterium belongs to the genus *Escherichia*.
4. The bacterium according to claim 1, wherein said bacterium belongs to the genus *Pantoea*.
5. The L-amino acid producing bacterium according to any of claims 1 to 4, wherein said L-amino acid is selected from the group consisting of an aromatic L-amino acid and a non-aromatic L-amino acid.
6. The L-amino acid producing bacterium according to claim 5, wherein said aromatic L-amino acid is selected from the group consisting of L-phenylalanine, L-tyrosine, and L-tryptophan.
7. The L-amino acid producing bacterium according to claim 5, wherein said non-aromatic L-amino acid is selected from the group consisting of L-threonine, L-lysine, L-cysteine, L-methionine, L-leucine, L-isoleucine, L-valine, L-histidine, glycine, L-serine, L-alanine, L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, L-proline, and L-arginine.
8. A method for producing an L-amino acid comprising:
 - cultivating the bacterium according to any of claims 1 to 7 in a medium to produce and excrete said L-amino acid into the medium, and
 - collecting said L-amino acid from the medium.
9. The method according to claim 8, wherein said L-amino acid is selected from the group consisting of an aromatic L-amino acid and a non-aromatic L-amino acid.
10. The method according to claim 9, wherein said aromatic L-amino acid is selected from the group consisting of L-phenylalanine, L-tyrosine, and L-tryptophan.
11. The method according to claim 9, wherein said non-aromatic L-amino acid is selected from the group consisting of L-threonine, L-lysine, L-cysteine, L-methionine, L-leucine, L-isoleucine, L-valine, L-histidine, glycine, L-serine, L-alanine, L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, L-proline, and L-arginine.

Fig. 1

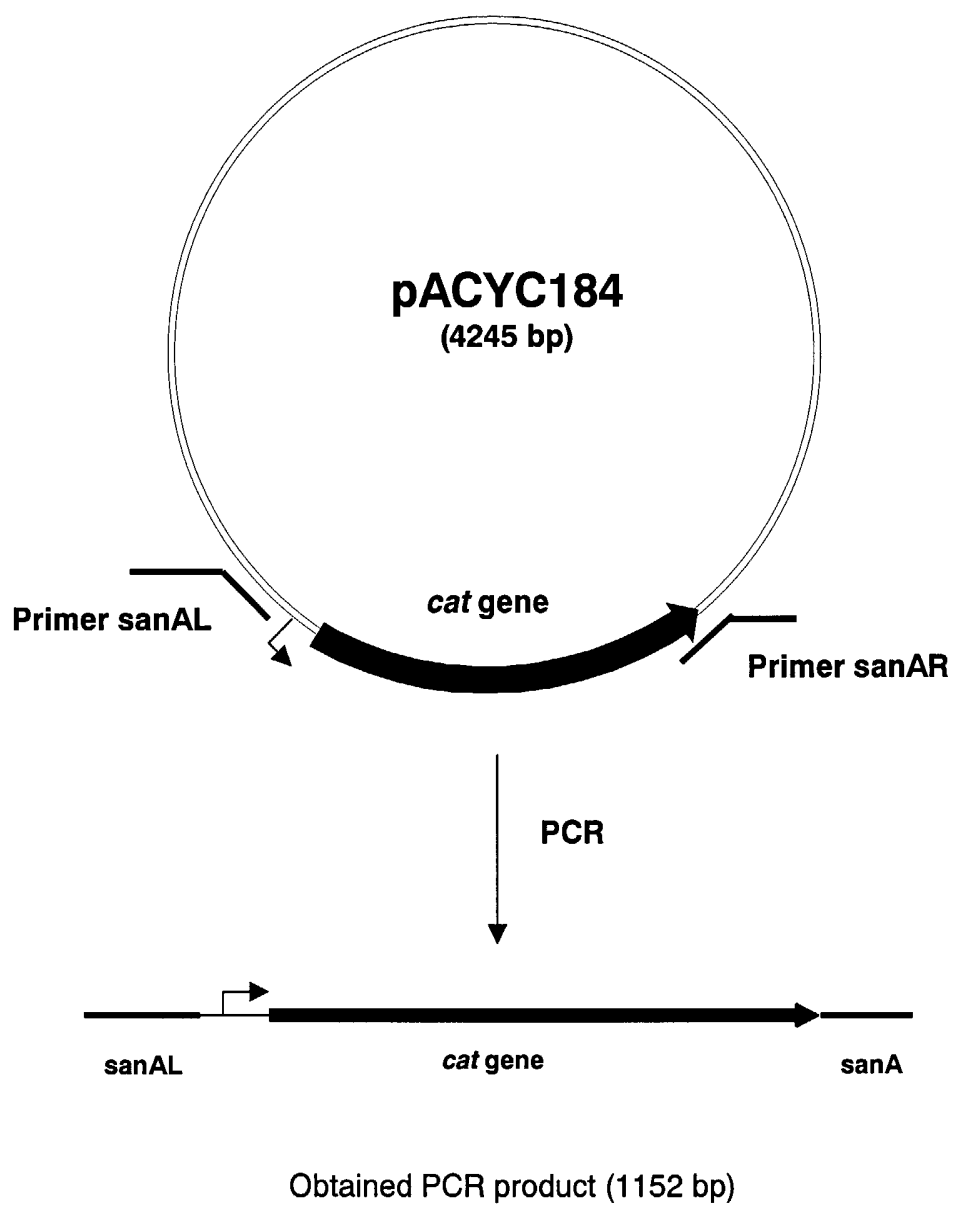
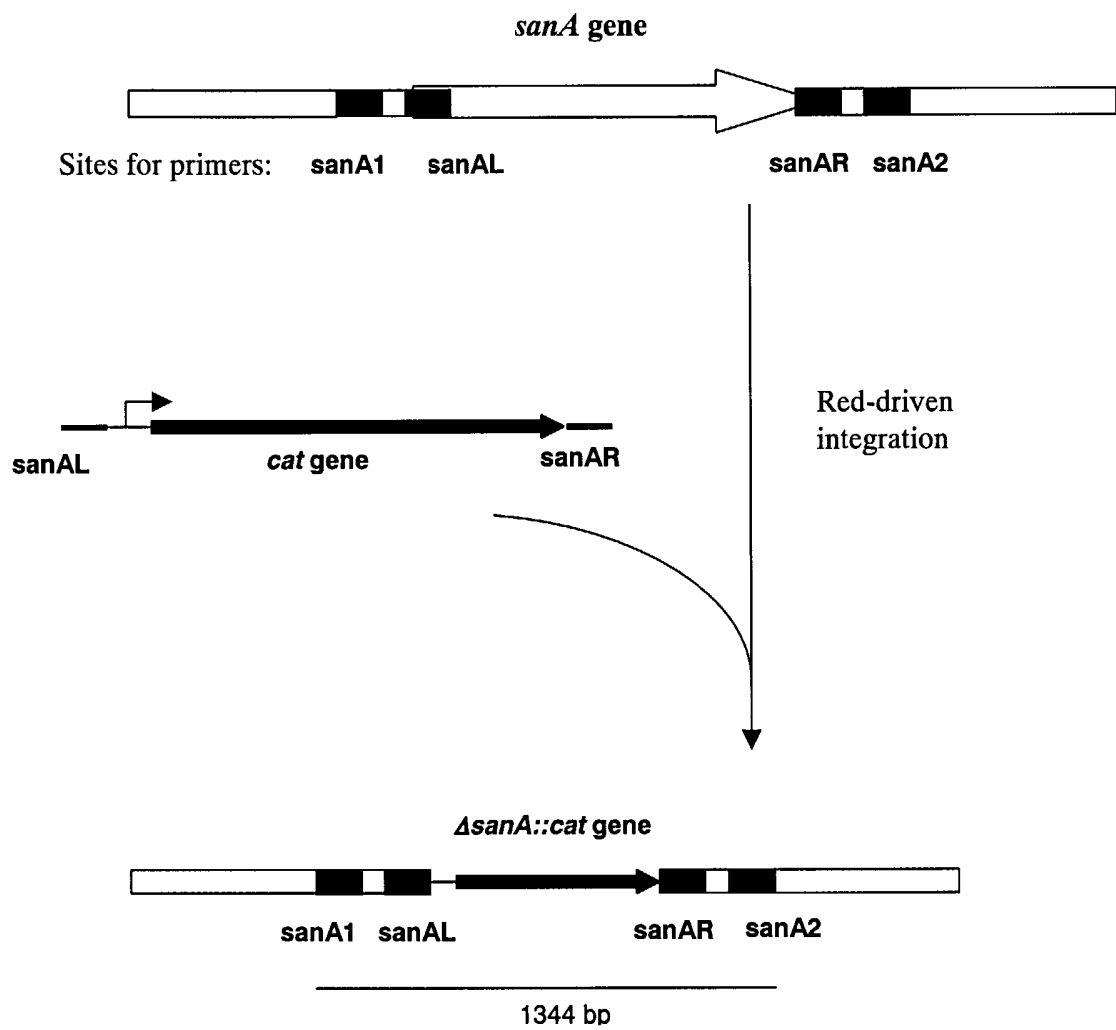


Fig. 2



SEQUENCE LISTING

<110> Ajinomoto Co., Inc.

<120> A METHOD FOR PRODUCING AN L-AMINO ACID USING A BACTERIUM OF THE ENTEROBACTERIACEAE FAMILY HAVING EXPRESSION OF THE *sanA* GENE ATTENUATED

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