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(54) **NUCLEOTIDE SEQUENCES OF
CORYNEFORM BACTERIA CODING FOR
PROTEINS INVOLVED IN L-SERINE
METABOLISM AND METHOD FOR
PRODUCING L-SERINE**

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ABSTRACT

The invention relates to the nucleotide sequences of coryneform bacteria coding for proteins which are involved in L-serine metabolism with reduced and switched off L-serine dehydratase. Said invention also relates to micro-organisms and to methods for producing L-serine.

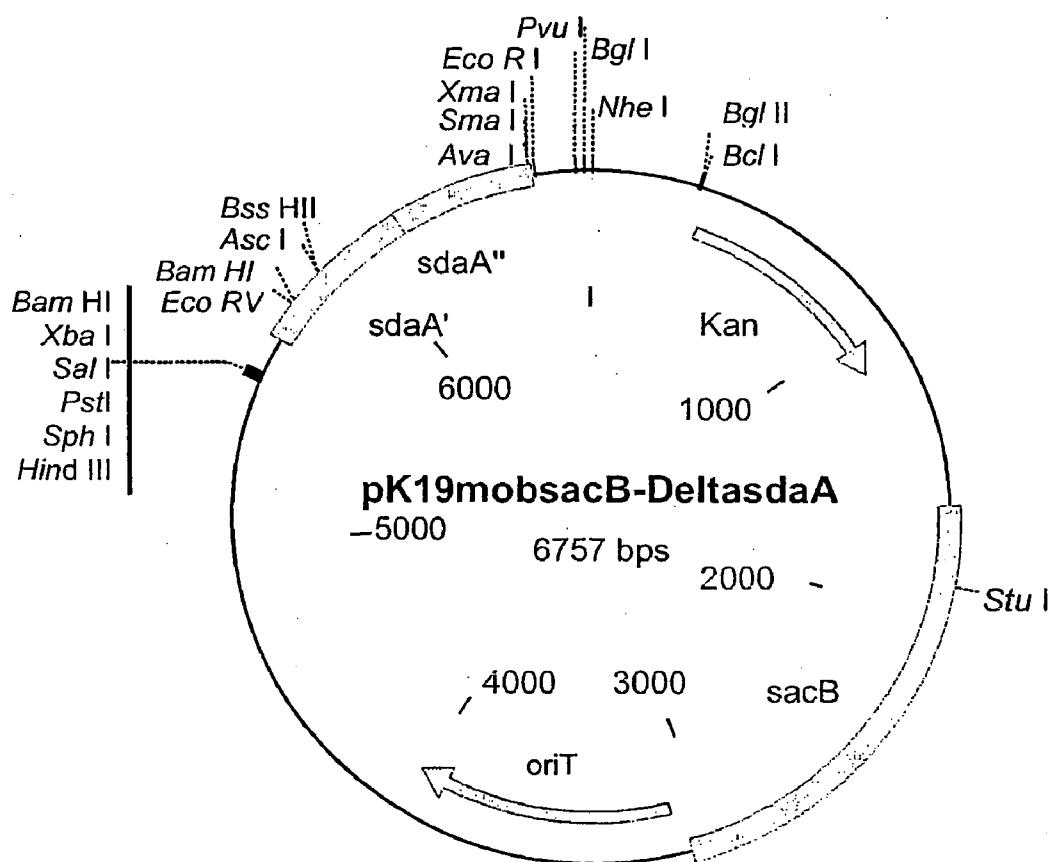


Fig. 1

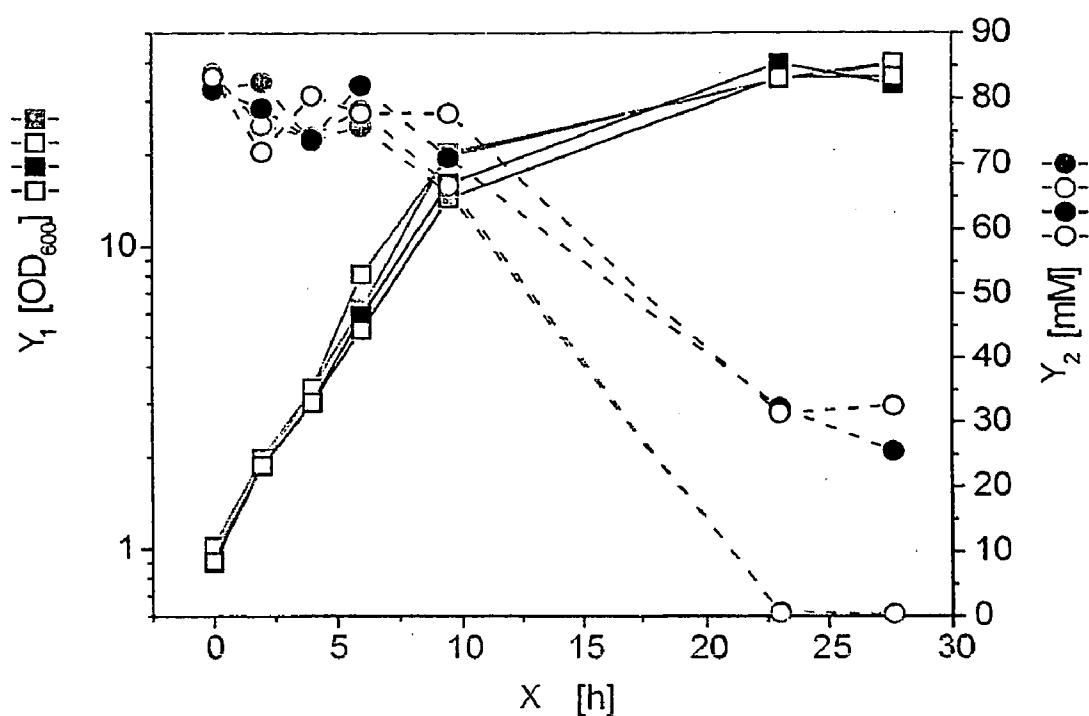


Fig. 2

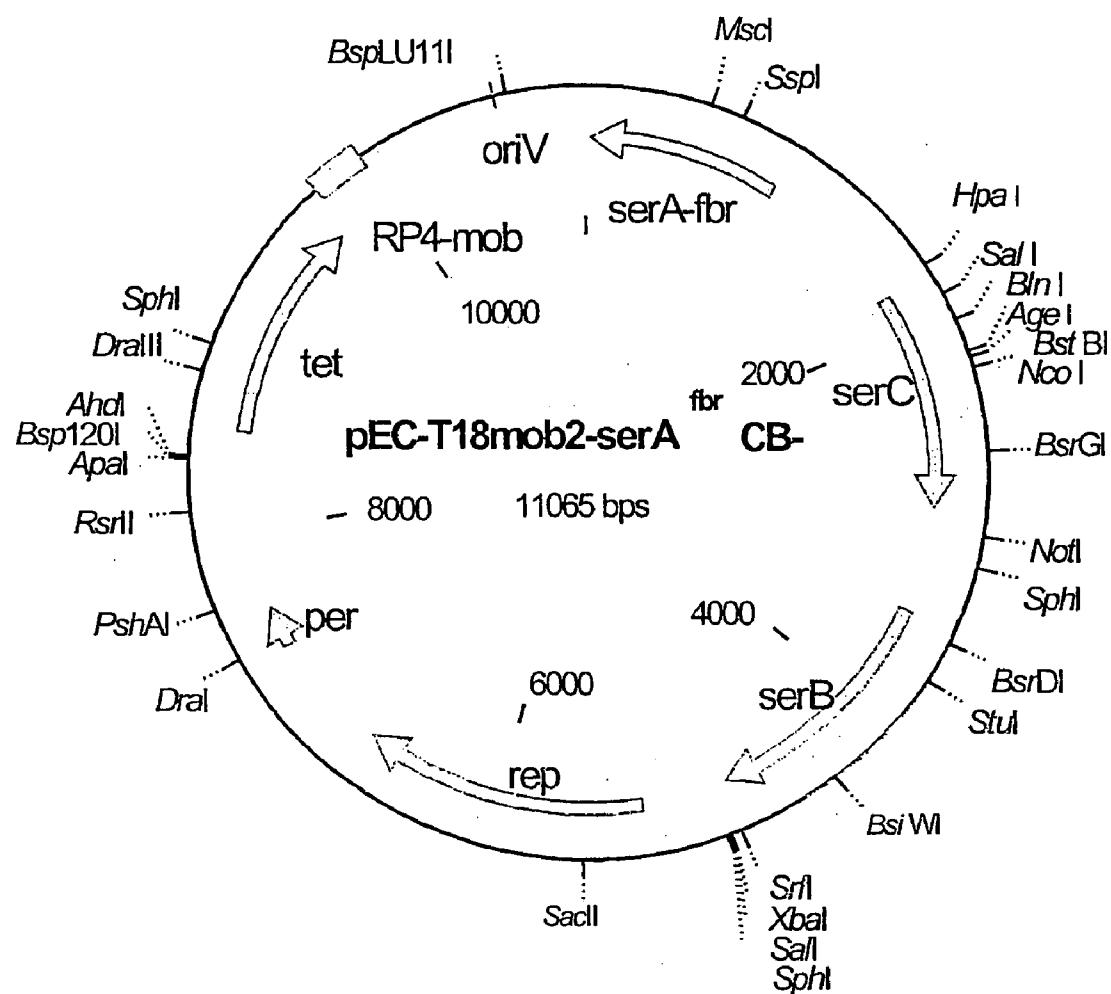


Fig. 3

NUCLEOTIDE SEQUENCES OF CORYNEFORM BACTERIA CODING FOR PROTEINS INVOLVED IN L-SERINE METABOLISM AND METHOD FOR PRODUCING L-SERINE

[0001] The invention relates to nucleotide sequences of coryneform bacteria coded for proteins which participate in L-serine metabolism with reduced or omitted L-serine dehydratase and microorganisms for and method of making L-serine.

[0002] The amino acid L-serine has been found to be useful in the food industry, the animal feed industry and the pharmaceutical industry as well as in human medicine. It serves as a building block for the synthesis of other industrially valuable products like for example L-tryptophan from indole and L-serine.

[0003] It is known that L-serine can be produced by the fermentation of coryneform bacteria strains. Thus for example a strain of *Corynebacterium glycinophilum* is capable of forming L-serine from glycine and carbohydrates (Kubota K, Kageyama K, Shiro T and Okumura S (1971) Journal of General Applications in Microbiology, 17: 167-168; Kubota K, Kageyama K, Maeyashiki I, Yamada K and Okumura S (1972) Journal of General Applications in Microbiology 18: 365). The enzyme L-serine-hydroxymethyltransferase here participates in the conversion of glycine to L-serine. (Kubota K and Yokozeki K (1989) Journal of Fermentation and Bioengineering, 67(6):387-390). These *Corynebacterium glycinophilum* strains have a defective serine dehydratase which produces undirected mutagenesis (Kubota K (1985) Improved production of L-serine by mutants of *Corynebacterium glycinophilum* with less serine dehydratase activity. Agricultural Biological Chemistry, 49:7-12). This enzymatic activity is Pyridoxal 5'-Phosphate dependent and not molecularly characterized. (Kubota K., Yokozeki K, Ozaki H. (1989) Effects of L-serine dehydratase activity on L-serine production by *Corynebacterium glycinophilum* of an examination of the properties of the enzyme. Agric. Biol. Chem. 49:7-12). From U.S. Pat. No. 4,528,273 a method of producing L-serine from glycine is known in which the microorganism serine dehydratase is negative.

[0004] Furthermore, L-serine can be produced fermentatively from methanol and glycine with the aid of methylorophic bacteria like for example *Hyphomicrobium* strains (Izumi Y, Yoshida T, Miyazaki S S, Mitsunaga T, Ohshiro T, Shiamo M, Miyata A and Tanabe T (1993) Applied Microbiology and Biotechnology, 39: 427-432). In both cases the amino acid glycine must be introduced as a precursor for the formation of the amino acid L-serine.

[0005] In addition, coryneform bacteria are known which can produce the L-serine directly from carbohydrates without further addition of precursors.

[0006] This is advantageous for industrial scale economical production of L-serine since the L-serine can be made directly from carbohydrates without the expensive addition of precursors, these strains which belong to the family *Corynebacterium glutamicum* have resistance to the L-serine analog serine hydroxamate and β -chloroalanine and are obtained by undirected mutagenesis (Yoshida H and Nakamaya K (197) NIHON-Nogli-Kagakukaishi 48: 201-208).

[0007] There are also *Brevibacterium flavum* strains known which have because of undirected mutagenesis defects in the breakdown of L-serine, an enhanced activity of the serA coded 3-phosphoglycerate dehydrogenase and an overexpression of serB and serC genes deriving from *Escherichia coli* (EP0931833A2).

[0008] It is the object of the invention to make available features which will permit improved production of L-serine or metabolic products which derive therefrom like for example tryptophan. It is thus also an object of the invention to provide nucleic acids which code for proteins participating in L-serine metabolism and which by comparison with the proteins derived from the wild type organism show a no decomposition of L-serine to pyruvate or a reduced decomposition of L-serine to pyruvate. In this connection it is a further object of the invention to provide an L-serine dehydratase as well as microorganisms with an L-serine dehydratase shown to reduce decomposition of L-serine. Further it is an object of the invention to provide an improved method for the microbial production of L-serine.

[0009] Starting from the preamble of claim 1, the objects are achieved, in accordance with the invention with the features given in the characterizing clause of claim 1. Furthermore, the objects are achieved starting from the preamble of claim 7 with the features given in the characterizing part of claim 7. The objects are also attained starting from the preamble of claim 8 according to the invention with the features given in the characterizing part of claim 8. The objects are also achieved starting with the preamble of claim 9 with the features given in the characterizing part of claim 9. The objects are also achieved starting with the preamble of claim 14, in accordance with the invention, with the features given in the characterizing part of claim 14. Starting with the preamble of claim 20, the objects are also achieved according to the invention by the features given in the characterizing part of claim 20. Furthermore, the objects are attained according to the invention starting from the preamble of claim 21 by the features of the characterizing part of claim 21.

[0010] With the nucleic acids and polypeptides according to the invention it is possible to produce an L-serine dehydratase such that there is a reduced decomposition of L-serine or no longer any decomposition of L-serine. Furthermore, it is possible to provide microorganisms and a method by which L-serine production can be obtained with higher yield by comparison with hitherto known microbial methods.

[0011] Further advantages have been given in the dependent claims.

[0012] According to the invention, in microorganisms of the corynebacterium family, replicatable and optionally recombinant nucleic acid is provided with nucleotide sequence coding for the L-serine dehydratase, hereinafter referred to also as SDA, which is partially or completely deleted or mutated or is expressed to a reduced extent by comparison with the naturally occurring nucleotide sequence or is not expressed at all.

[0013] The subject of the invention is, further, the provision of nucleic acids whose sdaA gene sequence is partially or completely deleted or mutated or has, relative to the naturally available nucleotide sequence reduced expression

or which does not express at all. For example the nucleic acids with a nucleotide sequence according to SEQ ID No 1 can have its nucleotides from position 506 to position 918, partly or completely deleted or mutated or can be allele, homologue or derivative of this nucleotide sequence or a nucleotide sequence which hybridizes therewith have been found to be advantageous. In addition, it has been found to be advantageous for the deletion or mutation of the cysteine-containing sequence required for forming the iron-sulfur clusters (Hofmeister et al., (1994) Iron-sulfur cluster-containing L-serine dehydratase from *Peptostreptococcus asaccharolyticus*: correlation of the cluster type with enzymatic activity. FEBS Letters 351: 416-418) has been found to be advantageous.

[0014] The wild type L-serine-dehydratase (sdaA) gene sequence is generally known and can be obtained by the artisan from the known data bank (NCBI Accession Nr. AP005279) or from the attached sequence protocol according to SEQ ID No. 1.

[0015] The complete deletion of the L-serine dehydratase (sdaA) gene can be achieved for example by directed recombinant DNA techniques. Suitable methods for this purpose are found in Schafer et al. (Gene (1994) 145: 69-73) or also Link et al. (Journal of Bacteriology (1998) 179: 6228-6237). Furthermore, only a part of the gene can be deleted or also mutated fragments of the L-serine dehydratase gene can be formed by replacement. By deletion or replacement it is possible to achieve a loss or a reduction in the L-serine dehydratase activity. An example of such a mutant is the *C. glutamicum* strain ATCC133032ΔsdaA which has a deletion in the sdaA gene.

[0016] To limit the expression of the sdaA gene or achieve reduced expression, for example, the promoter and regulatory regions which are located upstream of the structural gene can be mutated. In a similar manner, expression regulatory cassettes can be built onto the structural gene, upstream thereof. By regulatable promoters it is additionally possible to reduce the expression in the course of fermentative L-serine formation. It is also possible to provide a regulation of the translation in which for the example of stability of the m-RNA is reduced. Furthermore, genes can be used which code for the corresponding enzyme with reduced activity. Alternatively, furthermore, a reduced expression of the L-serine dehydratase gene can be achieved by varying the medium composition and culture conditions. Guides thereto for the artisan can be found among others in Martin et al. (Bio/Technology 5, 137-146 (1987)), by Guererro et al. (Gene 138, 35-41 (1994)), Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)), Eikmanns et al. (Gene 102, 93-98 (1991)), in the European Patent EPS 0 472 869, U.S. Pat. No. 4,601,893, Schwarzer and Puhler (Bio/Technology 9, 84-87 (1991)), Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994), LaBarre et al. (Journal of Bacteriology 175, 1001-1007 (1993)) and in patent application WO 96/15246.

[0017] The nucleic acids according to the invention are characterized that they can be isolated from the coryneform bacteria, preferably of corynebacterium or brevibacterium family and especially preferably from *Corynebacterium glutamicum*. Examples of the coryneform bacteria wild types, from this parental line are for example,

- [0018] *Corynebacterium acetoacidophilum* ATCC 13870;
- [0019] *Corynebacterium acetoglutamicum* ATCC 15806;
- [0020] *Corynebacterium callunae* ATCC 15991;
- [0021] *Corynebacterium glutamicum* ATCC 13032;
- [0022] *Brevibacterium divaricatum* ATCC 14020;
- [0023] *Brevibacterium lactofermentum* ATCC 13869;
- [0024] *Corynebacterium lilium* ATCC 15990;
- [0025] *Brevibacterium flavum* ATCC 14067;
- [0026] *Corynebacterium melassecola* ATCC 17965;
- [0027] *Brevibacterium saccharolyticum* ATCC 14066;
- [0028] *Brevibacterium immariophilum* ATCC 14068;
- [0029] *Brevibacterium roseum* ATCC 13825;
- [0030] *Brevibacterium thiogenitalis* ATCC 19240;
- [0031] *Microbacterium ammoniaphilum* ATCC 15354.

[0032] Examples of the production of mutants or production strains suitable for the production of L-serine are organisms from the group of *Arthrobacter*, *Pseudomonas*, *Nocardia*, *Methylobacterium*, *Hyphomycrobium*, *Alcaligenes* or *Klebsiella*. The present invention is characterized more particularly by the naming of the aforesaid bacterial strains but should not be considered limited thereto.

[0033] By a "nucleic acid" or a "nucleic acid fragment" there is meant, in accordance with the invention, a polymer of RNA or DNA which can be single stranded or double stranded and can have optional natural, chemically synthesized, modified or artificial nucleotides. The term "DNA polymer" includes in this case also genomic DNA, cDNA or mixtures thereof.

[0034] Under "alleles" are to be understood functional equivalents in accordance with the invention, that is substantially similarly effective nucleotide sequences. Functionally equivalent sequences are such sequences which, in spite of different nucleotide sequences, for example because of the degeneration of the genetic code, still retain the desired function. Functional equivalents thus encompass naturally occurring variants of the sequences described therein as well as synthetic nucleotide sequences, for example those obtained by chemical synthesis and optionally nucleotide sequences matched to the codon requirements of the host organism.

[0035] Under a functional equivalent is to be understood especially also natural or synthetic mutations of the original isolated sequence which retain the desired function. Mutations include substitutions, additions, deletions, replacements or insertions of one or more nucleotide residues. Included here are also sense mutations which in the protein plane can result for example from the replacement of conserved amino acids which however do not lead to any basic alteration in the activity of the protein and thus can be considered functionally neutral. This includes modifications of the nucleotide sequence which involve in the protein plane the N-terminus of a protein without however affecting significantly the function of these proteins.

[0036] With the present invention, such nucleotide sequences are encompassed which, by modification of the

nucleotide sequences can result in corresponding derivatives. The target of such modification can, for example, be a restriction of the coding sequence contained therein or for example also the insertion of further restriction enzyme cutting sites.

[0037] In addition, the present invention includes artificial DNA sequences as long as they, as described above, afford the desired characteristics. Such artificial DNA sequences can, for example, be those obtained by reverse translation from proteins established by means of computer supported programming (molecular modeling) or by *in vitro* selection. Especially suitable are coded DNA sequences which, by reverse translation, can produce a polypeptide sequence which has a specific code on utilization for the host organism. The specific code on utilization can be easily determined by molecular genetic methods common in the art using computer evaluations from other previously known genes of the organism to be transformed.

[0038] "Homologous sequences" are to be understood in accordance with the invention to be those sequences which are complementary to the nucleotide sequences according to the invention and/or such sequences which can hybridize with them. The hybridizing sequences include, according to the invention, substantially similar nucleotide sequences from the group of DNA or RNA which under stringent conditions known *per se* undergo a specific interaction (binding) of the aforementioned nucleotide sequences. In this category are to be counted also short nucleotide sequences with a length of for example 10 to 30 and preferably 12 to 15 nucleotides. These include according to the invention among others, also so-called primers or probes.

[0039] Included in the invention are also the coding regions (structure genes) and preceding (5' or upstream) sequence regions and/or following (3' or downstream) sequence regions. Especially in this category are sequence regions with regulatory functions. They can influence the transcription, the RNA stability or RNA processing as well as the translation. Example of regulatory sequences are, among others, promoters, enhancers, operators, terminators or translation amplifiers.

[0040] The subject of the invention is in addition a gene structure containing at least one of the aforementioned nucleotide sequences and regulatory sequences operatively linked therewith which control expression of the coded sequences in the host cell.

[0041] In addition the present invention relates to a vector containing a nucleotide sequence of the aforementioned kind with its regulator nucleotide sequence operatively linked thereto as well as additional nucleotide sequences for the selection of host cells capable of effecting transformation, for replication within the host cell or for integration in the corresponding host cell genome. In addition, the vector according to the invention can contain a genome structure of the aforementioned type. Suitable vectors are those which replicate in coryneform bacteria like for example pZ1 (Menkel E, Thierbach G, Eggeling L, Sahm H., 1989, *Appl Environ Microbiol* 55(3): 684-688), pEKEx2 (Eikmanns et al., Gene 102: 93-98 (1991), or pXMJ19 (Jacoby M., Burkovski A (1999) Construction and application of new *Corynebacterium glutamicum* vectors, *Biotechnol. Technique*

13:437-441). Other plasmid vectors can be used in the same manner. These identifications are however not limiting for the present invention.

[0042] Utilizing the nucleic acid sequence according to the invention, corresponding probes or primers can be synthesized and used, for example, to amplify and isolate analogous genes from other microorganisms, preferably *coryneform* bacteria, for example with the aid of the PCR technique.

[0043] The subject matter of the present invention is thus also a probe for identifying and/or isolating genes coded for proteins participating in the biosynthesis of L-serine, whereby these probes are produced starting from the nucleic acid sequences according to the invention of the aforementioned type and which contain a suitable marker for detection. In the probe, a partial segment of the sequences according to the invention, for example a conserved region, can be used which for example has a length of 10 to 30 or preferably 12 to 15 nucleotides and under stringent conditions can hybridize with homologous nitride sequences. Numerous suitable markers are known from the literature. The skilled worker in the art can be guided thereto by among others the Handbook of Gait: Oligonucleotide synthesis: a practical approach (IRL Press, Oxford, UK, 1984) and Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Deutschland, 1994) or for example, the Handbook "The DIG System Users Guide for Filter Hybridization" the Firma Roche Diagnostics (Mannheim, Deutschland) and Liebl et al. (International Journal of Systematic Bacteriology (1991) 41: 255:260).

[0044] The subject matter of the present invention includes, further, an L-serine dehydratase which shows reduced L-serine decomposition by comparison with the wild type L-serine dehydratase and which is coded by a nucleic acid sequence according to the invention or its variants of the aforementioned type. The present invention thus includes an L-serine dehydratase or an L-serine dehydratase mutant with an amino acid sequence in accordance with sequence ID No. 2 whose amino acids from position 135 to position 274, for example, as a consequence of a directed mutagenesis in the DNA plane, is altered or is a modified form of this polypeptide sequence or an isoform thereof or a mixture thereof. By "altered" in the framework of the present invention one should understand that complete or partial removal or replacement of the amino acids from position 135 to position 274 is contemplated.

[0045] Under isoforms we understand enzymes with the same or comparable substrate specificity and effectiveness specificity but which differ with respect to the primary structure.

[0046] Under modified forms are to be understood enzymes according to the invention with changes in the sequence, for example, at the N-terminus or C-terminus of the polypeptide or in the regions of the conserved amino acids without however negatively affecting the function of the enzyme. These changes can be in the form of amino acid replacement in accordance with methods known *per se*.

[0047] The polypeptides according to the invention are characterized by the fact that they derive from coryneform bacteria and preferably are of the *corynebacterium* or *brevibacterium* family and especially of the *Corynebacterium*

glutamicum or *Brevibacterium* types and especially preferably derive from *Corynebacterium glutamicum*. Examples of the coryneform bacteria in the strain culture of the wild type are for instance

- [0048] *Corynebacterium acetoacidophilum* ATCC 13870;
- [0049] *Corynebacterium acetoglutamicum* ATCC 15806;
- [0050] *Corynebacterium callunae* ATCC 15991;
- [0051] *Corynebacterium glutamicum* ATCC 13032;
- [0052] *Brevibacterium divaricatum* ATCC 14020;
- [0053] *Brevibacterium lactofermentum* ATCC 13869;
- [0054] *Corynebacterium lilium* ATCC 15990;
- [0055] *Brevibacterium flavum* ATCC 14067;
- [0056] *Corynebacterium melassecola* ATCC 17965;
- [0057] *Brevibacterium saccharolyticum* ATCC 14066;
- [0058] *Brevibacterium immariophilum* ATCC 14068;
- [0059] *Brevibacterium roseum* ATCC 13825;
- [0060] *Brevibacterium thiogenitalis* ATCC 19240; and
- [0061] *Microbacterium ammoniaphilum* ATCC 15354.

[0062] Examples of mutants or production strands suitable for the production of L-serine are organisms from the group of *arthrobacter*, *pseudomonas*, *nocardia*, *methylbacterium*, *hyphomycobium*, *alcaligenes* or *klebsiella*. The present invention has been characterized by listing the aforesaid bacteria strains, but this list should not be considered limiting of the invention.

[0063] The present invention comprises, further, a genetically altered microorganism characterized in that it contains a nucleotide sequence coding for the L-serine dehydratase which is in part or completely deleted or mutated or expressed to a reduced extent by comparison with the naturally occurring nucleotide sequence or which is not expressed at all.

[0064] The invention comprises further a microorganism which is characterized in that the *sdaA* gene is partially or completely deleted or mutated or which is expressed to a reduced extent by comparison with the naturally occurring *sdaA* gene or which is not expressed at all.

[0065] The invention encompasses as well a genetically altered microorganism containing in replicatable form a gene structure or a vector of the aforesaid type.

[0066] The subject of the present invention is moreover also a genetically modified microorganism containing a polypeptide according to the invention of the aforesaid type and which in comparison to the corresponding genetically unmodified microorganism has reduced or no L-serine decomposition.

[0067] A microorganism which, according to the invention has been genetically modified is characterized further in that it is a *coryneform bacterium*, preferably of the family *Corynebacterium* or *Brevibacterium* and especially preferably of the species *Corynebacterium glutamicum* or *Brevibacterium flavum*.

[0068] Basically the genes can, using methods known per se like for example the polymerase chain reaction (PCR), be

amplified by the aid of short synthetic nucleotide sequences (primers) and then isolated. The production of the primers used can be effected generally based upon known gene sequences from existing homologies in conserved regions of the gene and/or taking into consideration the GC content of the DNA of the microorganism investigated.

[0069] A further procedure for isolating coding nucleotide sequences is the complementation of so-called defect mutants of the organism to be investigated which at least phenotypically show a function drop in the activity of the gene investigated or the corresponding protein. Under a complementation is to be understood the preservation of the gene defect of the mutant and the substantial reproduction of the original configuration before mutagenesis which can be achieved by the insertion of functional genes or gene fragments from the microorganism to be investigated. A classical mutagenesis process for producing defect mutants or mutants with a reduced L-serine dehydratase or an L-serine dehydratase which has been shut down is for example the treatment of the bacteria cell with chemicals like for example N-Methyl-N-Nitro-N-Nitrosoguanidine or the use of UV radiation. Such methods of mutation resolution are generally known and can be found among others in Miller (A Short Course in Bacterial Genetics, A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria (Cold Spring Harbor Laboratory Press, 1992)) or the Handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981)).

[0070] The present invention relates moreover to a method for the microbial production of L-serine whereby the nucleic acids in the microorganisms which code for the L-serine dehydratase in part or completely are deleted or mutated or expressed to a lesser extent or practically not at all by comparison with the naturally available nucleic acids, using these genetically altered microorganisms for the microbial production of L-serine, and isolating the correspondingly formed L-serine from the culture medium.

[0071] The genetically altered microorganisms produced in accordance with the invention can be used for the purpose of culturing L-serine in continuous cultures or discontinuously in batch processes (set cultivation) or in a fed batch process or a repeated fed batch process. A collection of known cultivation methods can be found in the textbook of Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

[0072] The culture medium used must be suitable to suitably satisfy the requirements of the respective strain. Descriptions of culture media for various microorganisms can be found in the handbook "Manual of Methods for General Bacteriology" der American Society for Bacteriology" der American Society for Bacteriology (Washington D.C., USA, 1981) as carbon sources, sugars and carbohydrates like for example glucose, saccharose, lactose, fructose, maltose, molasses, starch and cellulose can be used, oils and fats like for example soy oil, soy flour oil, peanut oil, coconut fats can be used, fatty acids like for example palmitic acid, stearic acid and linoleic acids can be used, alcohols like for example glycerine and ethanol can be used and organic acids like for example acetic acid can be used.

These substances can be employed individually or as mixtures. As nitrogen sources, organic nitrogen containing compounds like peptones, yeast extract, meat extract, malt extract, corn spring water, soybean meal and urea, or inorganic compounds like ammonium sulfate, ammonium chloride ammonium phosphate, ammonium carbonate and ammonium nitride are used. The nitrogen sources can be used individually or as mixtures. As phosphorous sources, phosphoric acid, potassium dihydrogen phosphate or dipotassium phosphate or the corresponding sodium-containing salts are used. The culture medium must contain further salts of metal like for example magnesium sulfate or iron sulfate which are required for growth. Finally essential nutrients like amino acids and vitamins are added to the above-mentioned substances. The culture medium can in addition have suitable precursors added to it. The additives can be introduced into the culture in the form of one time addition or can be fed to the culture suitably during cultivation. For pH control of the culture basic compounds like sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia can be used or acid compounds like phosphoric acid or sulfuric acid can be used in a suitable way. For control of foaming, antifoaming agents like for example fatty acid polyglycol esters can be used. To maintain the stability of plasmids suitable selectively effective substances, for example antibiotics can be added to the medium. To maintain the aerobic conditions, oxygen or oxygen-containing mixtures like for example air are introduced into the culture. The temperature of the culture is normally between 20° C. and 45° C. and preferably 25° C. to 40° C. The culture is maintained for a duration until L-serine production is a maximum. This duration is normally from 10 hours to 160 hours.

[0073] The analysis of the L-serine formation can be carried out by anion exchange chromatography with subsequent ninhydrin derivatization as described by Spackman et al. (Analytical Chemistry, 30 (1958), 1190) or the analysis can be effected by reverse phase HPLC as described by Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174.

[0074] The microorganisms which are the subject of the present invention can produce L-serine from glucose, saccharose, lactose, mannose, fructose, maltose, molasses, starch, cellulose or from glycerine and ethanol. The method can use the *coryneforme* bacteria representatives which have already been described in detail. A selection of the results of the fermentation has been given in Table 1. The genetically altered microorganisms of the invention show a substantially improved L-serine production by comparison with the corresponding nontransformed microorganism (wild type) or the microorganisms which contain only the vector without the gene insert. In a special variation of the present invention it has been shown that *C. glutamicum* ATCC 13032ΔpanBCΔsdaA gives rise to at least a 4-fold increase in the L-serine accumulation in the medium by comparison with the control strain (Table 1). Through the common overexpression of other genes, which act positively on the L-serine biosynthesis pathway, a 16-fold increase in L-serine production can be achieved.

[0075] Amino acid production strains, in accordance with the present invention should be understood to be *Corynebacterium glutamicum* strains or homologous microorganisms which are altered by classical and/or molecular genetic methods so that metabolic flow is amplified in the direction

of the biosynthesis of amino acids or their derivatives (metabolic engineering). For example, with these amino acid production strains, one or more genes and/or the corresponding enzyme have their regulation altered or are rendered deregulated at different and correspondingly complex regulated key positions in the metabolic pathway. The present invention includes thereby all such already known amino acid production strains preferably of the *corynebacterium* family or homologous organisms.

[0076] Further, such production strains are encompassed within the invention which the skilled worker in the art will recognize by analogy with other microorganisms, for example, enterobacteria, bacillaceen or yeast types can be produced by current methods.

[0077] The Figures show examples of plasmids which can be used as well as experimental results with respect to nucleic acids or microorganisms according to the invention.

[0078] It shows:

[0079] FIG. 1 The integration plasmid pK19mobsacB-DeltasdaA Markings on the outer edge of the plasmid indicate the respective restriction sites. The portion within the circle indicates the following gene:

kan	kanamycin resistance
sacB	Sucrase
OriT	Transfer origin
sdaA'	5' end of the sdaA gene
sdaA''	3' end of the sdaA gene

[0080] FIG. 2: A graph of the ratio between growth (square symbol □) and L-serine breakdown (circle symbol ○) of *C. glutamicum* 13032ΔpanBCΔsdaA, clone 1 (□, ○) and *C. glutamicum* 13032ΔpanBCΔsdaA, clone 2 (■, ●) compared with *C. glutamicum* 13032ΔpanBC, clone 1 (□, ○) and *C. glutamicum* 13032ΔpanBC, clone 2 (■, ●). The abscissa X represents the fermentation in hours (h). The ordinate y_1 is the growth of the microorganisms measured in terms of optical density at 600 nm. The ordinate y_2 gives the L-serine concentration in mM.

[0081] FIG. 3: The expression plasmid pEC-T18mob2-SerA-fbrCB. The indicia on the outer edge of the plasmid show the resective restriction sites. The indicia within the circle represent the following genes:

SerC	Phosphoserine Transaminase
SerB	Phosphoserine Phosphatase
Rep	Replication origin
Per	Partition cell partition gene
Tet	Tetracycline resistance gene
RP4-mob	Mobilization origin
OriV	Source of DNA replication
SerA-fbr	3-phosphoglycerate dehydrogenase

EXAMPLES

1. The Construction of sdaA-Deletion Mutant of *C. glutamicum* ATCC13032 ΔpanBC.

[0082] The starting point was *Corynebacterium glutamicum* with a nuclotide sequence (Genbank-Acces-

sion-Number BAB99038; SEQ-ID-No. 1) whose derivative polypeptide sequence showed 40% identity with the described L-serine dehydratase of *E.coli* (NCBI-Accession-Number P1095). By gene protected mutagenesis by the method of Link et al (Link A J, Phillips D, Church G M, Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. *J. Bacteriol.* 1997 October; 179(20):6228-37) and Schafer et al. (Gene 145: 69-73 (1994)) the sdaA-gene of *C. glutamicum* was deleted. The following primers were derived from the sdaA coryne-bacterial sequence (NCBI Accession-Number AP005279):

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sdaA-1:
5'-TCGTGCAACTTCAGACTC-3'
(AP005279 nucleotide 73635 - 73653);

sdaA-2:
5'-CCCATCCACTAAACTAAACACGTCTATAATGAACCCACC-3'
(AP005279 complementary to nucleotide 74121 - 74139);

sdaA-3:
5'-TGTAAAGTTAGTGGATGGGCCACTAATGGTGTGCG-3'
(AP005279 complementary to nucleotide 74553 - 74571);

sdaA-4:
5'-CGGGAAAGCCCAAGGTGGT-3'
(AP005279 nucleotide 75044 - 75062)

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[0083] Primers sdaA-1 and sdaA-2 flank respectively the beginning and the end of the sdaA-3 make available respective complementary linker regions (see relevant text) which enable in a two-stage PCR process (cross over PCR) a deletion of the sdaA gene in vitro. In a first PCR reaction with the chromosomal DNA of *C. glutamicum*, the primer combination sdaA-1 and sdaA-2 as well as sdaA-3 and sdaA-4 are used. The PCR reaction is carried out in 30 cycles in the presence of 200 μ M deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP), each with 600 nM of the corresponding oligonucleotide sdaA-1 and sdaA-4 as well as 60 nM of oligonucleotide sdaA-2 and sdaA-3, 100 ng of chromosomal DNA from *Corynebacterium glutamicum* ATCC13032, 1/10 volumes 10-fold of reagent buffer and 2.6 units of heat stabilized Taq-/Owi-DNA-Polymerase-Mischung mixture (Expand High Fidelity PCR System of Firm of Roche Diagnostics, Mannheim, Deutschland) in a Sthermocycler (PTC-100, MJ Research, Inc., Watertown, USA) under the following conditions: 94° C. for 30 seconds, 50° C. for 30 seconds and 72° C. for 40 seconds. The elongation step at 42° C. was extended after 10 cycles by about 5 seconds per cycle. After the PCR reaction, the DNA fragments containing each having a length of 500 bp were isolated with QIAExII Gelextraction kit (Qiagen) in accordance with the requirements of the manufacturer on an 0.8% agarose gel and both fragments were used as templates in the second PCR. As primers the primers sdaA-1 and sdaA-4 were used. This time the reaction was carried out in 35 cycles in the presence of 200 μ M deoxynucleotide triphosphates, 600 nM each of the corresponding oligonucleotides, 2-mg each of the isolated template DNA from the first PCR, 1/10 volume of 10 fold reaction buffer and 2.6 units of Taq-/Pwo-DNA-Polymerase mixture under the following conditions: 94° C. for 30 seconds, 50° C. for 30 seconds and 72° C. for 80 seconds. Again the elongation steps after 10 cycles were extended by 5 seconds each. After PCR reaction

to 1000 bp long DNA fragments which contain the inactive sdaA gene with a 420 bp long central deletion was isolated on a 0.8% agarose gel and cloned, blunt end with the aid of a Sure Clone Kit (Amersham Pharmacia Biotech) in the SmaI-restriction site of the inactivation vector pK19mobsacB (Schafer et al Gene 145: 69-73 (1994) which can replicate only in an *E.coli* but not in *C. glutamicum*. The obtained plasmid pK19mobsacB Δ sdaA (FIG. 1) is tested by restriction mapping for correctness. The cloning was effected in the *Escherichia coli* strain DH5 α cmcr (Grant et al., Proceedings of the National Academy of Sciences of the United States of America USA (1990) 87: 4645-4649).

[0084] Then the plasmid is incorporated by electroporation in *C. glutamicum* 13032 Δ panBC (Radmacher E, Vaitsikova A, Burger U, Krumbach K, Sahm H, Eggeling L. Linking central metabolism with increased pathway flux: L-valine accumulated by *Corynebacterium glutamicum*. *Appl Environ Microbiol.* 2002 68(5):2246-50) and subject to selection with integration of the vector. This strain is pantothenate auxotropic as a result of the deletion of the pantothenate biosynthesis genes panB and panC and is characterized in that it has an amplified accumulation of pyruvate about 50 mM alanin and 8 mM valine because of the pantothenate limitation. In addition the strain can form about 100 μ M L-serine and is suitable as a starting strain for the construction of L-serine producers. It contains Kanamycin resistant clones of *C. Glutamicum* 13032 Δ panBC by which inactivation vector is integrated in the genome. To allow selection of the excision of the vector, kanamycin-resistant clones are plated out on saccharose containing LB medium (Sambrook et al., Molecular cloning. A laboratory manual (1989) Cold Spring Harbour Laboratory (Press) with 15 g/l Agar, 2% glucose/10% saccharose) and colonies are obtained in which the vector has again been lost as a result of a second recombination event. (Jager et al. 1992, *Journal of Bacteriology* 174: 5462-5465). Two of these clones whose nucleotides have sdaA genes deleted from positions 506 to 918 are designated and 13032 Δ panBC Δ sdaA, clone 1 and 13032 Δ panBC Δ sdaA, clone 2 and are used in the further investigations.

2. The Influence of the sdaA Deletion Upon L-serine Decomposition

[0085] In the following, a test was made whether the deleted sdaA gene indeed participates in L-serine decomposition. For this purpose a growth experiment was carried out with each of the two clones of the strains *C. glutamicum* 13032 Δ panBC Δ sdaA in comparison with strain *C. glutamicum* 13032 Δ panBC on minimal medium (Keilhauser et al., *Journal of Bacteriology* 175 (1993) 5595-5603) which additionally contains 2% glucose 1 μ M pantothenate and 100 mM L-serine. The growth and consumption of L-serine were followed. The results are given in FIG. 2.

[0086] The results in FIG. 2 show that the deletion of the sdaA genes results in about 40% reduced decomposition of L-serine.

3. Influence of the Deletion of the sdaA Gene on L-serine Formation

[0087] To test what the influence was of the deletion of the L-serine dehydratase gene upon L-serine formation the strains 13032 Δ panBC Δ sdaA (clone 1, clone 2) and

13032ΔpanBC (clone 1, clone 2) with the plasmid pEC-T18mob2-*serA*^{fbr}*serC**serB* the plasmid is formed (FIG. 3) from the vector pEC-T18mob2 (Tauch, A., Kirchner, O., Loffler, B., Gotker, S., Puhler A., and Kalinowski J. Efficient Electrotransformation of *Corynebacterium diphtheriae* with a MiniReplicon Derived from the *Corynebacterium glutamicum* Plasmid pGA1. Curr. Microbiol. 45(5), 362-367 (2002)), of the *corynebacterial* gene *serA*^{fbr} (Peters-Wendisch P., Netzer R., Eggeling L., Sahm H. 3-Phosphoglycerate dehydrogenase from *Corynebacterium glutamicum*: the C-terminal domain is not essential for activity but is required for inhibition by L-serine. Appl Microbiol Biotechnol. 2-2 December; 60(4): 437-41) as well as *serC* and *serB* (German patent application 100 44 831.3 of 11 Sep. 2000.

[0088] After electroporation, the strains 13032ΔpanBCΔsdaApSerA^{fbr}CB and 13032ΔpanBCpSerA^{fbr}CB were obtained.

[0089] For testing L-serine output the two strains 13032ΔpanBCpSerA^{fbr}CB are cultivated in complex medium (CgIII with 2% glucose and 5 µg/l tetracycline) and the fermentation medium CGXII (J Bacteriol (1993) 175: 5595-5603), each seeded from the preculture to the medium contained in addition 50 µg/l kanamycin and 1 µM pantothenate. As controls, the two starting strains 13032ΔpanBC and 13032ΔpanBCΔsdaA were cultured in the same manner although the medium did not contain tetracycline. For each at least two independent fermentations were carried out. After culturing for 30 hours at 30° C. of a rotating shaker at 120 RPM, the L-serine quantity accumulated in the medium was determined. The determination of the amino acid concentration was carried out by means of high pressure liquid chromatography (J Chromat (1983) 266: 471-482). The results of the fermentation are shown in Table 1 and indicate that the exclusion of L-serine dehydratase led to a 4-fold increase in the L-serine accumulation in the medium independently of whether the L-serine biosynthesis genes *serA*^{fbr}, *serC* and *serB* were overexpressed. The overexpression of the L-serine biosynthesis genes *serA*^{fbr}, *serC* and *serB* however resulted in 16 fold increase in L-serine accumulation in the culture supernatant generally. Thus the use of the constructed and described deletion mutant ΔsdaA resulted in a method which improved the L-serine formation decisively.

TABLE 1

Accumulation of L-serine in the culture supernatant of <i>Corynebacterium glutamicum</i> 13032ΔpanBC and 13032ΔpanBCΔsdaA after expression of the genes <i>serA</i> ^{fbr} , <i>serC</i> and <i>serB</i>		
Strain	OD ₆₀₀	L-Serine [mM]
13032ΔpanBC	40	0.1
13032ΔpanBCΔsdaA	42	0.4
13032ΔpanBCpserA ^{fbr} CB	30	1.6
13032ΔpanBCΔsdaApserA ^{fbr} CB	30	6.6

4. Determination of the L-serine Dehydratase Activity

[0090] For determining the L-serine dehydratase activity the wild type strands WT pXMJ19 (Jacoby M., Burkovski A (1999) Construction and application of new *Corynebacterium glutamicum* vectors. Biotechnol. Technique 13:437-441), overexpression strand WT pXMJ19_sdaA and the deletion strains ΔsdaA pXMJ19 were cultured in CgXII minimal medium as in Keilhauer et al., (1993). The medium contained 30 mg/l protocatechuic acid, 100 mM glucose and 100 mM L-serine. The cells were cultivated in the presence of 1 mM isopropyl-beta-D-thiogalactopyranoside and in the exponential growth phase at an optical density of 6-8, measured by a Pharmacia Biotech ultraspec 3000 spectral photometer were harvested. They were then centrifuged for 10 minutes at 4500 rpm and 4° C., suspended in 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer (pH 8.0) and centrifuged again. Thereafter the cells were taken up in 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer (pH 8.0), 1 mM FeSO₄ and 10 mM dithiothreitol. The cell breakdown was effected by means of ultrasonic treatment (Branson sonifier 250; duty cycle 25%, output control 2.5, 10 minutes) on ice.

[0091] To determine the L-serine dehydratase activity the reaction set contained 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer (pH 8.0), 10 mM dithiothreitol and 10-100 µl new extract. The detection of the pyruvate formation from the serine was effected as described (Ohmori et al., 1991). The reaction was started by adding 50 mM L-serine and after 10 minutes was stopped by the addition of 1,2-diamino-4,5-dimethoxybenzene reagent in a ratio of 1:1. The reagent, as described in Ohmori et al 1991 was comprised of 4 mg 1,2-diamino-4,5-dimethoxybenzol dissolved in 42.4 ml H₂O, 3.5 ml β-mercaptoethanol and 4.1 ml HCl (37% ig) then incubation was carried out for 2 hours at 102° dry heat.

[0092] Detection and quantification of the 2-hydroxy-6,7-dimethoxy-3-methylquinoxaline derivative produced by the pyruvate was carried out by means of high pressure liquid chromatography also as described. (Ohmori et al., 1991). The protein determination in the raw extract followed by means of the Bradford method (Bradford 1976) using the protein assays (The firm Bio-Rad). The specific L-serine dehydratase activity of the two strands are given in Table 2.

TABLE 2

Specific Activity of the L-Serine Dehydratase in the Strains 13032 WT pXMJ19_sdaA (Overexpressed), 13032 WT pXMJ19 (Wild type with empty vectors) and 13032 ΔsdaA pXMJ19.	
<i>C. Glutamicum</i> Strain	spec. Activity [nmol/min * mg]
13032 WT pXMJ19_sdaA	0.221
13032 WT pXMJ19	0.003
13032 ΔsdaA pXMJ19	0

[0093]

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 2

<210> SEQ ID NO 1

<211> LENGTH: 1449

<212> TYPE: DNA

<213> ORGANISM: *Corynebacterium glutamicum*

<400> SEQUENCE: 1

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ctattttagca tcggtatcgg accatcatcc tcacataccg tcggcccat gagagccgccc      180
ctcacgtata tctctgaatt tcccagctcg catgtcgata tcacgttgca cggatccctt      240
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gaaccaacga tagttccat ttagtgcgtca ccctcaccgg gcgcgcggat tcctgcgaaaa      360
gttctgtga acgggcacccaa gggacgggtc tcgtattccc tgacgttgcgcgcgcgcgcgc      420
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<210> SEQ ID NO 2

<211> LENGTH: 449

<212> TYPE: PRT

<213> ORGANISM: *Corynebacterium glutamicum*

<400> SEQUENCE: 2

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1				5				10				15			

Ser Ser His Thr Val Gly Pro Met Arg Ala Ala Leu Thr Tyr Ile Ser

-continued

20	25	30
Glu Phe Pro Ser Ser His Val Asp Ile Thr Leu His Gly Ser Leu Ala		
35	40	45
Ala Thr Gly Lys Gly His Cys Thr Asp Arg Ala Val Leu Leu Gly Leu		
50	55	60
Val Gly Trp Glu Pro Thr Ile Val Pro Ile Asp Ala Ala Pro Ser Pro		
65	70	75
Gly Ala Pro Ile Pro Ala Lys Gly Ser Val Asn Gly Pro Lys Gly Thr		
85	90	95
Val Ser Tyr Ser Leu Thr Phe Asp Pro His Pro Leu Pro Glu His Pro		
100	105	110
Asn Ala Val Thr Phe Lys Gly Ser Thr Thr Arg Thr Tyr Leu Ser Val		
115	120	125
Gly Gly Gly Phe Ile Met Thr Leu Glu Asp Phe Arg Lys Leu Asp Asp		
130	135	140
Ile Gly Ser Gly Val Ser Thr Ile His Pro Glu Ala Glu Val Pro Cys		
145	150	155
Pro Phe Gln Lys Ser Ser Gln Leu Ala Tyr Gly Arg Asp Phe Ala		
165	170	175
Glu Val Met Lys Asp Asn Glu Arg Leu Ile His Gly Asp Leu Gly Thr		
180	185	190
Val Asp Ala His Leu Asp Arg Val Trp Gln Ile Met Gln Glu Cys Val		
195	200	205
Ala Gln Gly Ile Ala Thr Pro Gly Ile Leu Pro Gly Gly Leu Asn Val		
210	215	220
Gln Arg Arg Ala Pro Gln Val His Ala Leu Ile Ser Asn Gly Asp Thr		
225	230	235
240		
Cys Glu Leu Gly Ala Asp Leu Asp Ala Val Glu Trp Val Asn Leu Tyr		
245	250	255
Ala Leu Ala Val Asn Glu Glu Asn Ala Ala Gly Gly Arg Val Val Thr		
260	265	270
Ala Pro Thr Asn Gly Ala Ala Gly Ile Ile Pro Ala Val Met His Tyr		
275	280	285
Ala Arg Asp Phe Leu Thr Gly Phe Gly Ala Glu Gln Ala Arg Thr Phe		
290	295	300
Leu Tyr Thr Ala Gly Ala Val Gly Ile Ile Ile Lys Glu Asn Ala Ser		
305	310	315
320		
Ile Ser Gly Ala Glu Val Gly Cys Gln Gly Glu Val Gly Ser Ala Ser		
325	330	335
Ala Met Ala Ala Ala Gly Leu Cys Ala Val Leu Gly Gly Ser Pro Gln		
340	345	350
Gln Val Glu Asn Ala Ala Glu Ile Ala Leu Glu His Asn Leu Gly Leu		
355	360	365
Thr Cys Asp Pro Val Gly Gly Leu Val Gln Ile Pro Cys Ile Glu Arg		
370	375	380
Asn Ala Ile Ala Ala Met Lys Ser Ile Asn Ala Ala Arg Leu Ala Arg		
385	390	395
400		
Ile Gly Asp Gly Asn Asn Arg Val Ser Leu Asp Asp Val Val Val Thr		
405	410	415
Met Ala Ala Thr Gly Arg Asp Met Leu Thr Lys Tyr Lys Glu Thr Ser		
420	425	430

-continued

Leu Gly Gly Leu Ala Thr Thr Leu Gly Phe Pro Val Ser Met Thr Glu
 435 440 445

cys

1. A nucleic acid which is replicatable in a microorganism of the family *Corynebacterium* and optionally a recombinant nucleic acid, characterized in that it has a nucleotide sequence coding for L-serine dehydratase which is partially or completely mutated or expressed to a lesser degree than the naturally occurring nucleotide sequence or which is not expressed at all.

2. A nucleic acid according to claim 1, characterized in that the sdaA gene sequence is partially or completely deleted or mutated or expressed to a lesser extent by comparison with the naturally occurring sequence or not expressed at all.

3. A nucleic acid according to claim 1, characterized by a nucleotide sequence according to SEQ ID NO 1 whose nucleotides form position 506 to position 918 are completely or partially deleted or mutated, or an allele, homolog or derivative of this nucleotide sequence or a nucleotide sequence hybridizing therewith.

4. A nucleic acid according to claim 1, characterized in that it is isolated from a *coryneform bacterium*.

5. A nucleic acid according to claim 1, characterized in that it is isolated from *Corynebacterium* or *Brevibacterium*.

6. A nucleic acid according to claim 1, characterized in that it is isolated from *Corynebacterium glutamicum* or *Brevibacterium flavum*.

7. A gene structure containing at least one nucleotide sequence according to claim 1 and nucleotide sequences having regulatory sequences operatively linked therewith.

8. A vector containing at least one nucleotide sequence or a gene structure according to claim 7 and additional nucleotide sequences for selection, for replication in the host cell or for integration in the host cell genome.

9. L-serine dehydratase with reduced L-serine dehydratase activity coded with a nucleic acid according to claim 1.

10. L-serine dehydratase according to claim 9 with an amino acid sequence according to sequence ID 2 whose amino acid are altered in positions 135 to 274 or a modified form of this polypeptide sequence or an isoform thereof.

11. L-serine dehydratase according to claim 9, characterized in that it derives from coryneform bacteria.

12. L-serine dehydratase according to claim 9, characterized in that it derives from coryneform bacteria or brevibacteria.

13. L-serine dehydratase according to claim 9, characterized in that it derives from *Corynebacterium glutamicum* or *Brevibacterium flavum*.

14. A microorganism characterized in that it has a nucleotide sequence which codes for an L-serine dehydratase, which is deleted in whole or in part or is mutated or is expressed to a reduced extent by comparison with the naturally occurring nucleotide sequence or is not expressed at all.

15. A microorganism according to claim 14, characterized in that its sdaA gene is wholly or partially deleted or mutated or to a reduced extent by comparison with the naturally occurring sdaA gene or is not expressed at all.

16. A microorganism according to containing in replicatable form a nucleic acid according to claim 1.

17. A microorganism according to claim 14, characterized in that it is a coryneform bacteria.

18. A microorganism according to claim 14, characterized in that it brings to the family a coryneform bacteria or brevibacteria.

19. A microorganism according to claim 14, characterized in that it brings to the family a *Corynebacterium glutamicum* or *Brevibacterium flavum*.

20. A probe for identifying and/or genes for coding which participate in the biosynthesis of L-serine characterized in that they are produced starting with nucleic acids according to claim 1 and contain a suitable marker for detection.

21. A method for the microbial production of L-serine characterized in that

(a) a genetically altered microorganism is produced in which the nucleic acid in the microorganism coding for the L-serine dehydratase is partially or completely deleted or mutated or expressed to a reduced extent by comparison with the naturally occurring nucleic acid or is not expressed at all,

(b) this genetically altered microorganism from step (a) is used for microbial production, and

(c) the L-serine formed is isolated from the culture medium.

22. The method according to claim 21, characterized in that the sdaA gene sequence is partially or completely deleted or mutated or expressed to a reduced extent by comparison with the naturally occurring nucleotide sequence or is not expressed at all.

23. The method according to claim 21, characterized in that the nucleotide according to Sequence ID NO 1 is completely or partially deleted or mutated from position 506 to 918 or expressed to a reduced extent by comparison with the naturally occurring nucleotide sequence or not expressed at all.

24. The method according to claim 21, characterized in that a microorganism from the group of *Corynebacterium*, *Brevibacterium*, *Arthrobacter*, *Pseudomonas*, *Nocardia*, *Methylobacteria*, *Hyphomicrobium*, *Alkaligenes* or *Klebsiella* is used.

25. A method wherein a nucleic acid according to claim 1 is used.

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