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(54) **Microorganisms and method for L-arginine production by fermentation**

(57) The present invention relates to a microorganism having an L-arginine producing ability, said microorganism being a microorganism synthesizing L-arginine through the biosynthetic linear or cyclic pathway, and bearing a recombinant DNA comprising a gene *argJ* coding for an enzyme having an ornithine acetyltransferase activity.

It also relates to a method for producing L-arginine comprising the steps of cultivating the microorganism as defined above.

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Description

Technical Field

[0001] The present invention relates to a new method for L-arginine production by fermentation.

[0002] L-arginine is an industrially useful amino acid as ingredient of liver function promoting agents, transfusion solutions, food additives and the like.

Background of the art

[0003] In microorganisms, biosynthesis of L-arginine proceeds in eight enzymatic steps starting from the precursor L-glutamate and follows two different pathways, the linear pathway or the cyclic acetyl pathway depending on the microorganism concerned (Cunin et *al.,* 1986; Davis, 1986). In both biosynthetic pathways the first step is *N*-transacetylation of glutamate catalyzed by the enzymes displaying *N*-acetylglutamate synthase activity.

[0004] In the linear pathway, the acetylglutamate synthase activity is provided by the enzyme acetylCoA: L-glutamate N-acetyltransferase (EC 2.3.1.1.) encoded by the *argA* gene and in this pathway the intermediate *N*-acetyl L-ornithine is converted into L-ornithine at the fifth enzymatic step through deacetylation by N^2-acetyl-L-ornithine amidohydrolase (EC 3.5.1.16) encoded by the *argE* gene.

[0005] Thus, in microorganisms as *Escherichia coli*, L-arginine is synthesized from L-glutamate *via N*-acetylglutamate, *N*-acetylglutamylphosphate, *N*-acetylglutamate semialdehyde, N-acetylornithine, ornithine, citrulline and argininosuccinate. These intermediates are synthesized through consecutive reactions catalyzed by enzymes commonly known under the names *N*-acetylglutamate synthase, *N*-acetylglutamate kinase, *N*-acetylglutamylphosphate reductase, acetylornithine aminotransferase, *N*-acetylornithinase, ornithine carbamyltransferase, argininosuccinate synthase and argininosuccinase. These enzymes are encoded by *argA, argB, argC*, *argD, argE, argF*, *argG* and *argH* genes, respectively.

[0006] In the cyclic acetyl pathway, the acetyl-group of N-acetylornithine is transferred to L-glutamate by the enzyme ornithine acetyltransferase (N^2-acetyl-L-ornithine: L-glutamate *N*-acetyltransferase; EC 2.3.1.35) encoded by the *argJ* gene. Owning this enzyme, arginine biosynthetic pathway is recycled between the first and the fifth enzymatic steps and such a cyclic acetyl pathway is energetically advantageous over the linear pathway since *N*-acetyl ornithine can be used as the acetyl-group donor once the pathway is initiated from acetyl-CoA as a donor.

[0007] The cyclic acetyl pathway directs the L-arginine flow in procaryotic organisms as *Corynebacterium glutamicum* (Udaka and Kinoshita, 1958), cyanobacteria (Hoare and Hoare, 1966), *Pseudomonas aeruginosa* (Haas *et al.,* 1972), *Neisseria gonorrhoeae* (Shinners and Catlin, 1978), methanogenic archaea (Meile and Leisinger, 1984), *Thermotoga maritima* (Van de Casteele *et al.,* 1990), representatives of *Bacillus* (Sakanyan et *al.,* 1992), *Streptomyces coelicolor* (Hindle *et al.,* 1994), *Thermus thermophilus* (Baetens *et al.*, 1998), an archaeon *Mekhanococcus jannaschii* (Marc *et al.,* 2000) and in some eukaryotic organisms(De Deken, 1962). The nucleotide or amino acid sequences sharing similarity with the *argJ* gene or its product are also available for entirely or partially sequenced genomes and the similarity is indicative of the existence of the cyclic acetyl pathway in these organisms.

[0008] The *argJ*-encoded product, which exhibits the only ornithine acetyltransferase, is considered as a monofunctional enzyme and properties of such enzyme have been described (Haas *et al.,* 1972; Sakanyan et al., 1996; Baetens *et al.*, 1998; Marc *et al.,* 2000). However, some microorganisms harbour the alternative version of the *argJ* gene encoding the enzyme which possesses, in addition to the ornithine acetyltransferase activity, the *N*-acetylglutamate synthase activity as well. Such genes and corresponding bifunctional enzymes have been described for *Neisseria gonorrhoeae* (Picard and Dillon, 1989; Martin and Mulks, 1992), *B. stearothermophilus* (Sakanyan *et al.*, 1990 and, 1993), *Saccharomyces cerevisiae* (Crabeel *et al.,* 1997), *T. neapolitana* (Marc *et al.,* 2000).

[0009] The monofunctional ArgJ enzymes can be distinguished from bifunctional enzymes by two means: (i) by enzymatic assay using two acetyl-group donors, *N*-acetyl L-ornithine and acetyl-CoA; (ii) by complementation test using *argE* and *argA* mutants of *Escherichia coli* for the cloned *argJ* gene. The monofunctional ArgJ enzyme transfers the only acetyl group from *N*-acetyl L-ornithine to L-glutamate and complements the only *argE* mutant, whereas the bifunctional ArgJ enzyme transfers the acetyl-group both from *N*-acetyl L-ornithine and acetyl-CoA and complements both *argE* and *argA* mutant strains.

[0010] Both biosynthetic pathways are subjected to genetic and enzymatic regulation, respectively by a specific transcriptional repressor and by inhibition of enzymatic steps by L-arginine or intermediate products (Maas, 1994; Glansdorff, 1996). Moreover, the early metabolic steps preceding the L-glutamate precursor formation and late degradation steps following the L-arginine degradation are under the control of regulatory mechanisms. Consequently, synthesis of L-arginine and the production yield of this amino acid can be modulated by introduction of mutations at various targets in the genome of a given microorganism or by affecting the cultivation conditions of a given microorganism or by affecting the membrane permeability of a given microorganism.

[0011] Conventional L-arginine production by fermentation has been carried out using microbial strains producing L-arginine, especially representatives of coryneform bacteria; using coryneform bacteria resistant to certain antimetabolic agents including 2-thioazoalanine, α-amino-β-hydroxyvaleric acid, arginine hydroxamate, cysteine analogues, sulfonamide derivatives and the like; using coryneform bacteria exhibiting auxotrophy for some amino acids including for L-proline, L-histidine, L-threonine, L-isoleucine, L-methionine, or L-tryptophan, as well as using coryneform bacteria exhibiting both the mentioned above resistances and auxotrophies for amino acids. In this respect, reference may be made to the following patents : FR 2 084 059, 2 119 755, 2 490 674, 2 341 648, 2 225 519, EP 0 379903 B1, EP 0 378 223 B1, EP 0 336387 B1.

[0012] On the other hand, there have been disclosed methods for producing L-arginine by using a microorganism belonging to the genus *Corynebacterium, Brevibacterium* or *Escherichia* transformed by a recombinant DNA containing a well-defined gene of arginine biosynthesis that allows to enhance the gene-encoded enzyme activity for a given limiting step. The wild-type strain or the mutant for the transcriptional repressor or the mutant which carries a relevant resistance or auxotrophy have been used as recombinant host cell for fermentations.

[0013] Most of the recombinant microorganisms used for producing L-arginine belong to the genus *Corynebacterium* or *Brevibacterium*. In this respect, reference may be made to the following patents: FR 2 143 238; FR 2 484 448; EP 0 259858 B1; EP 0 261627 B1; EP 0 332233 A1; EP 0 999267 A1; EP 1016710 A2 .

[0014] However, the *Escherichia coli* K12 strain, with the entirely sequenced genome (Blattner *et al.*, 1997) and applicability of various genetic approaches and more advantageous vectors to manipulate in this strain or its derivatives, is an attractive host as well for the production of amino acids including L-arginine. The increased production of L-arginine by recombinant *Escherichia coli* strains can be achieved by using the cloned *argA* gene on plasmid vectors and followed by isolation of feed-back resistant mutations by the described method for *E. coli* (Eckhard and Leisinger, 1975; Rajagopal et *al.,* 1998). In this respect, reference may be made to EP 1 016 710 A2.

[0015] Thus, L-arginine production by recombinant microorganisms has been improved by enhancing the number of copies of the gene coding for *N*-acetylglutamate synthase activity, namely by a wild type *argA* gene or its feedback resistant mutants.

[0016] However, the application of the mutant *argA* gene is limited in the context of a possibility of further increasing the productivity of L-arginine by recombinant strains.

[0017] It has now been found that it is possible to produce L-arginine with a microorganism having an L-arginine producing ability, said microorganism being a microorganism synthesizing L-arginine and bearing a recombinant DNA comprising a gene *argJ* coding an enzyme with an ornithine acetyltransferase activity.

Summary of the invention

[0018] The present invention provides a microorganism having L-arginine producing ability, which carries a recombinant DNA comprising an *argJ* gene encoding the ornithine acetyltransferase.

[0019] The present invention also provides the above mentioned microorganism, wherein the *argJ* gene codes for a monofunctional enzyme or preferably for a bifunctional enzyme.

[0020] Preferably, the *argJ* gene codes for a mono - or bi-functional enzyme, devoid of inhibition by L-arginine.

[0021] More preferably, the *argJ* gene is derived from a thermophilic microorganism.

[0022] The present invention also provides a method for producing L-arginine comprising the steps of culturing the above mentioned microorganism in a medium to produce and accumulate L-arginine and collect L-arginine from the medium.

[0023] The term "L-arginine-producing ability" used in the present specification means the ability of the microorganism of the present invention to accumulate L-arginine in a culture medium when it is cultured in said medium.

Brief description of the drawings

[0024]

- Fig 1 is the restriction maps of plasmids pACYC184; pJ-B; pJ-T and pJ-M.

Detailed description of the invention

[0025] The microorganism of the present invention is a microorganism having L-arginine- producing ability, in which said ability is provided by the *argJ* gene encoding ornithine acetyltransferase introduced therein by recombinant DNA techniques.

[0026] Preferably, the *argJ* genes useful in the present invention are the genes encoding enzymes with ornithine acetyltransferase activity or enzymes with an ornithine acetyltransferase and N-acetylglutamate synthetase activities,

said activities of the mono - or bi-functional enzymes being devoid of inhibition by L-arginine.

[0027] The *argJ* gene is advantageously derived from a thermophilic microorganism such as for example *Methanococcus jannasschii* or *Bacillus stearothermophilus* or *Thermotoga neapolitana.*

[0028] Sequences of said genes are disclosed in the following papers incorporated herein as reference: *-argJ* of *Methanococcus jannaschii* : Bult et al.,1996; *-argJ* of *Bacillus stearothermophilus* NCIB8224: Sakanyan et al., 1993; *-argJ* of *Thermotoga neapolitana* : Dimova et al., 2000.

[0029] Examples of appropriate argJ genes are those derived from Bacillus *stearothermophilus NCIB8224, ATCC12980, ATCC7953*, *ATCC10149, Thermotoga neapolitana DSM5068, ATCC49049, Methanococcus jannaschii DSM2661.*

[0030] Preferred *argJ* genes are those derived from *Bacillus stearothermophilus* or *Thermotoga neapolitana.*

[0031] The microorganism producing L-arginine is any microorganism capable of synthesizing L-arginine through either the biosynthetic linear pathway or the cyclic pathway and which harbours the cloned *argJ* gene introduced therein by genetic engeenering.

[0032] Said microorganism may be selected for example from coryneform bacteria, such as those belonging to the genus *Brevibacterium* or the genus *Corynebacterium* or bacteria belonging to the genus *Escherichia.*

[0033] Preferably, said microorganism is a microorganism synthesizing arginine through the linear biosynthetic pathway and more particularly belongs to the genus *Escherichia.*

[0034] Examples of bacteria of the genus *Escherichia* appropriate for the present invention are listed as follows: *Escherichia coli K12* strain and its derivatives, notably *Escherichia coli* P4XB2(Hfr, *metB, relA*, *argR)* (Sakanyan et al., 1996). Said strain *Escherichia coli P4XB2* was deposited at the "Collection Nationale de Culture de Microorganismes" (CNCM) of Pasteur Institute on October 9, 2000, under number I 2571.

[0035] Preferably, the host strain is devoid of the transcriptional repression($argR^-$) involved in the negative control of L-arginine biosynthesis pathway in microorganims.

[0036] The *argJ* gene is amplified by PCR (polymerase chain reaction, see White T.J. *et al.* Trends Genet., 5, 185 (1989) utilising appropriate primers and thereafter ligated with a DNA vector according to the methods well-known to the man skilled in the art. Such methods are disclosed by Sambrook *et al.* in Molecular cloning , Cold Spring Harbor Laboratory Press (1989).

[0037] The vector used for the cloning of *argJ* may be a plasmid autonomously replicable in the microorganisms with a low or moderated or high number of copies; a specific example thereof is the plasmid pACYC184 described in Sambrook et *al.,* in Molecular cloning, Cold Spring Harbor Laboratory Press (1989). A phage vector may also be used. Integration of *argJ* gene onto the chromosomal DNA of the host bacterium can also be performed by homologous recombination without using any vector system. A shuttle vector autonomously replicable in different microorganisms synthesizing L-arginine may also be used for the introduction of *argJ* into the host cells other than *Escherichia*

[0038] In order to prepare recombinant DNA molecules by ligating a gene carrying DNA fragment and a DNA vector, the vector is digested by restriction enzyme(s) corresponding to the termini of the amplified gene. Ligation is generally performed by using a ligase, such as T4DNA polyrucleotide ligase.

[0039] The DNA vector is preferably an expression vector containing preferably a promoter, which may be followed by a ribosome binding site upstream of the gene to be expressed. This vector also contains an origin of replication and a selection marker.

[0040] The promoter may be weak or moderate or strong. The latter is subjected to a controlled action and provide, therefore a controlled gene expression. Appropriate promoters are for example *tet* or *amp* promoters and the like.

[0041] The selection marker is advantageously a gene responsible for resistance to antibiotics such as tetracyclin, ampicillin, chloramphenicol and the like.

[0042] The recombinant DNA comprising the appropriate means for the expression of the *argJ* gene in the microorganism concerned is introduced in that microorganism by conventional methods such as electroporation, $CaCl_2$-mediated transformation and the like.

[0043] According to a variant of embodiment of the invention, the microorganism of the invention may additionally harbour a recombinant DNA comprising the *argA* gene coding for N-acetylglutamate synthase and a DNA vector prepared according to the above methods. The *argA* gene can be taken from *Escherlchia coli, Corynebacterium glutamicum*, *Pseudomonas aeruginosa* and the like.

[0044] Furthermore, the DNA vector may additionally contain a gene for utilization of a source of carbon other than glucose, such as a gene coding for sucrase, levanase, levane sucrase and the like, preferably a gene coding for levanase.

[0045] The method of the present invention for producing L-arginine comprises the steps of cultivating the microorganism of the present invention, in a culture medium, to produce and accumulate the amino acid in the medium, and recovering the amino acid from the medium.

[0046] In the method of the present invention, the cultivation of the microorganism belonging to the genus *Escherichia*, the collection and purification of amino acid from the liquid medium may be performed in a manner similar

to those of the conventional methods for producing an amino acid by fermentation using a coryneform bacterium or *Escherichia coli* or *Bacillus subtilis.* A medium used for cultivation may be either a synthetic medium or a natural medium, so long as the medium includes a carbon and a nitrogen source and minerals, and , if necessary, nutrients which the bacterium used requires for growth in appropriate amounts. The carbon source may include various carbohydrates such as glucose and sucrose, and various organic acids. The carbon source is preferably sucrose. Depending on assimilatory ability of the used bacterium, alcohol including ethanol and glycerol may be used. As the nitrogen source, ammonia, various ammonium salts such as ammonium sulfate, other nitrogen compounds such as amines, a natural nitrogen source such as peptone, soybean hydrolyte and digested fermentative microbe are used. As minerals, mono-potassium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, calcium carbonate are used.

[0047] The cultivation is preferably carried out under aerobic conditions such as a shaking culture, and an aeration and stirring culture. The temperature of culture is usually 20 to 40°C, preferably 28 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 3-day cultivation leads to the accumulation of the given amino acid in the medium.

[0048] Recovering L-arginine can be performed by conventional methods, for example by removing solids such as cells from the medium by centrifugation or membrane filtration after cultivation, and then collecting and purifying L-arginine by ion exchange, concentration and crystalline fraction methods and the like.

[0049] The present invention will be now disclosed in more detail in the following examples given only for illustrative purposes.

Example 1: Construction of plasmids carrying the *argJ* gene

[0050] The following *argJ* genes cloned from the moderate thermophilic bacterium *Bacillus stearothermophilus* (Sakanyan *et al.,* 1993) and the hyperthermophilic bacterium *Thermotoga neapolitana* (Dimova *et al.*, 2000) have respectively the DNA sequences SEQ ID N°1 and SEQ ID N°3 which code for the proteins having the amino-acid sequences SEQ ID N°2 and SEQ ID N°4 respectively. The *argJ* sequence from the hyperthermophilic archaeon *Methanococcus jannaschii* (Bult *et al.,* 1996) has the DNA sequence SEQ ID N°5 which codes for the protein having the amino-acid SEQ ID N°6. The primers corresponding to the 5' and 3' ends of the three *argJ* genes derived from these three microorganisms have been synthesized. The oligonucleotides corresponding to the beginning of the *argJ* gene containing a GGAG Shine/Dalgarno site have the following sequences:

BS 5′-GAAGGAGAGTATACCATGACGATCACAAAACAAACGG-3′ (SEQ ID N°7)

TN 5′-GAAGGAGAGTATACCATGTTCGTTCCGAGGGGATTCAG-3′ (SEQ ID N°8)

MJ 5′-GAAGGAGAGTATACCATGAGAGTTATTGATGGTGGAG-3′ (SEQ ID N°9)

The oligonucleotides corresponding to the end of the *argJ* gene containing a GGATCC *BamH*I site have the following sequences:

BS 5′-AAAGGATCCTTACGTCCGATAGCTGGCG-3′(SEQ ID N°10)

TN 5′-AAAGGATCCTCATGTCCTGTACCTCCCG-3′(SEQ ID N°11)

MJ 5′-AAAGGATCCTTAAGTTGTATATTCAGCG-3′(SEQ ID N°12)

Amplification of the *argJ* gene from different DNA templates was carried out by PCR with DNA polymerase Pfu (Stratagene). The conditions used were as follows:

Initial denaturation	95°C, 5 min	
Denaturation	94°C, 1 min	
Annealing	47°C, 1 min	30 cycles
Extension	72°C, 2 min	
Final extension	72°C, 7 min	4°C

The PCR products were subsequently phosphorylated, digested with *Bam*HI and then mixed with the plasmid vector pACYC184 preliminary digested with the enzyme *Eco*RV, dephosphorylated and then digested with the second enzyme *Bam*HI. After ligation by T4 DNA ligase, the recombinant DNAs were transferred to the *Escherichia coli* K12 XS1D2R strain [F^- Δ(*ppc-argE*) *nalA rpoB* λ^- *hsdR recA]* by electroporation (2500 V, 21 μF, 400 Ω, 10 msec). The recombinant clones were selected on minimal medium M9 (Miller, 1992) without arginine solidified with agar (1.5%), supplemented with 0.2% of succinate and containing the antibiotic chloramphenicol (25 μg/ml). The Cm^r $ArgE^+$ colonies were selected after three days of incubation at 37°C and the recombinant plasmids carrying the *argJ* gene of the corresponding thermophilic microorganism were isolated from such clones. The plasmid DNAs obtained were sequenced to verify the cloned DNA sequences and the plasmids in which the *argJ* gene transcription is orientated under the control of the *tet* gene promoter were selected. Their restriction maps are shown in Figure 1. The plasmids obtained, containing the *argJ* gene of *Bacillus stearothermophilus, Thermotoga neapolitana* or *Methanococcus jannaschli,* were called pJ-B, pJ-T and pJ-M respectively.

Example 2: Genetic analysis of the recombinant plasmids

[0051] By means of genetic and enzymatic analyses, it is possible to recognize the two types of the ArgJ enzyme. The monofunctional enzyme which possesses the only ornithine acetyltransferase activity is able to complement the *argE* mutant of *Escherichia coli* K12, whereas the bifunctional enzyme which exhibits both ornithine acetyltransferase activity and acetylglutamate synthase activity is able to complement the *argE* and *argA* mutants of *Escherichia coli* K12.

[0052] The three plasmids obtained were transferred by electroporation to the *Escherichia coli* K12 XA4 strain, which bears the single *argA* mutation and to the double mutant *Escherichia coli* K12 *XA4::argE* strain, which bears the *argA* and *argE* mutations, using the conditions described in Example 1. The recombinant colonies were selected on LB rich medium solidified with agar (1.2%) and containing the antibiotic chloramphenicol (25 μg/ml). 50 colonies from each dish were resuspended in NaCl solution (0.9%) and then replicated on dishes with a minimal medium M9 solidified with agar (1.2%) and with or without L-arginine (150 μg/ml) but always containing chloramphenicol (25 μg/ml). After two days of incubation at 37°C, all 50 clones of the *Escherichia coli* K12 strains XA4(pJ-B), XA4::*argE*(pJ-B), XA4 (pJ-T) and XA4::*argE*(pJ-T) developed on the selective media described. By contrast, no colonies of the *Escherichia coli* K12 strains XA4(pJ-M) and XA4::*argE*(pJ-M) grew on arginine-free medium, whereas they were clearly visible after two days on medium supplemented with L-arginine These results indicate that the *argJ* gene of *Bacillus stearothermophilus* and *Thermotoga neapolitana* codes for a bifunctional enzyme, whereas the *argJ* gene of *Methanococcus jannaschii* codes for a monofunctional enzyme.

Example 3: Enzymatic analysis

[0053] The *Escherichia coli* K12 strains XS1D2(pJ-B), XS1D2(pJ-T) and XS1D2(pJ-M) were cultivated in a minimal medium M9 devoid of arginine, but supplemented with succinate (0.2%) and containing chloramphenicol (25 μg/ml), at 37°C for 24 hours. The cells were then pelleted, washed twice in Tris-HCl buffer (0.1 M, pH 8) and then lyzed by sonication (15 min per pulse of 10 s at 19 kHz). The enzymatic activities were measured in the following buffer: 0.1 M MES, 0.1 M PIPES, 0.1 M Tris, 0.1 M glycine and 0.1 M K_2HPO_4, using as an acetyl-group donor, acetyl CoA or *N*-acetyl ornithine at 37°C or at 70°C, and the reaction product, i.e. *N*-acetylglutamate, was quantified by HPLC. The samples were analyzed on a Luna C18 column (Phenomenex) on an HPLC system (Kontron) using a mixture of 0.1 M phosphoric acid and methanol (90:10 v:v) with a flow rate of 1 ml/min as the mobile phase. The reaction product was detected at 215 nm. The results given in Table 1 show that the three enzymes possess the ornithine acetyltransferase activity at 37°C and 70°C.

Table 1: Ornithine acetyltransferase and acetylglutamate synthase activities ($\mu moles \cdot min^{-1} \cdot mg^{-1}$ protein) measured at 37°C and 70°C for recombinant thermostable ArgJ enzymes

Strain/ plasmid	37°C			70°C	
	Ornithine acetyltransferase activity	Ornithine acetyltransferase activity in the presence of 10mM of L-arginine	Acetylglutamate synthase activity	Ornithine acetyltransferase activity	Acetylglutamate synthase activity
XS1D2(pJ-B)	4	4	0.5	25	2
XS1D2(pJ-T)	5	5	0.4	190	7
XS1D2(pJ-M)	4.5	4.5	0	165.5	0

The acetylglutamate synthase activity was detected only for the enzymes of *Bacillus stearothermophilus* and *Thermo-*

toga neapolitana at both temperatures.
These results confirm that the ArgJ enzymes from *Bacillus stearothermophilus* and *Thermotoga neapolitana* are indeed bifunctional enzymes, whereas that from *Methanococcus jannaschii* is a monofunctional enzyme. No decreasing of enzymatic activities was detected by addition of 10mM L-arginine.

Example 4: L-arginine Production by recombinant *Escherichia coli* K12 P4XB2 strains

[0054] Plasmids pJ-B, pJ-T and pJ-M were transferred to the *Escherichia coli* K12 P4XB2 strain by electroporation under the conditions described above in Example 1. The corresponding clones were selected on LB rich medium solidified with agar (1.2%) and containing chloramphenicol (25 μg/ml). Three independent colonies of each recombinant strain were chosen for evaluating the amount of L-arginine produced during the fermentations. For this purpose the colonies, taken from the dishes were resuspended in a LB medium containing chloramphenicol and cultivated at 30°C until the optical density reached 0.8 at 600 nm. 1 ml of this preculture was added to 14 ml of fermentation medium having the following composition: 2.8% of $(NH_4)_2SO_4$, 0.2% of K_2HPO_4, 0.5% of yeast extract, 0.05% of $MgSO_4$, 0.001% of $FeSO_4$, 0.001% of $MnSO_4$, 10 μg/ml of thiamine, 100 μg/ml of methionine, 5% of glucose, 2.5% of $CaCO_3$, 25 μg/ml of chloramphenicol; pH 7.2. The fermentation was performed in 750 ml conical flasks on a circular shaker at a speed 320 rpm at 30°C for 40 h. After fermentation, the samples were recovered and the amount of L-arginine was evaluated against a L-arginine calibration-scale, either by paper chromatography or by thin layer chromatography and developing with 0.5% of ninhydrin dissolved in acetone or by spectrophotometry or by an amino acid analyzer.
The results of these fermentations are presented in Table 2.

Table 2:

Production of L-arginine by the *Escherichia coli* K12 P4XB2 strain and its recombinant derivatives harbouring the cloned *argJ* gene	
Strain/plasmid	L-arginine (g/l)
P4XB2	< 0.2
P4XB2 (pJ-M)	0.5
P4XB2 (pJ-B)	9.0
P4XB2 (pJ-T)	9.0

These results revealed that all the *argJ*-carrying plasmids possess the capacity to increase the yield of L-arginine in *Escherlchia coli* K12 host cells. Obviously, this level of production of L-arginine in *Escherichia coli* is much greater in those strains which contain plasmids pJ-B or pJ-T as compared with the *Escherichia coli* K12 strains which contain the pJ-M plasmid. This demonstrates that expression of the gene coding for the bifunctional ArgJ enzyme ensures a greater production yield of L-arginine, compared with the gene coding for a monofunctional ArgJ enzyme.

Example 5: Synthesis of L-arginine in the *Escherichia coli K* 12 strain carrying two plasmids

[0055] The plasmid pARG2S makes it possible to produce L-arginine in *Escherichia coli* K12. This plasmid carries the *argA* gene from *Escherichia coli* K12 and the levanase gene *(sacC)* from *Bacillus subtilis* Marburg 168 on the pBR327-kan vector. The wild-type *argA* gene from *Escherichia coli K12* was cloned by complementation of the *argA* mutant of *Escherichia coli K12* (Nersisyan *et al.,* 1986). The *sacC* gene from *Bacillus subtilis* Marburg 168 was selected in the pQB79,1 cosmid bank (Fouet *et al.,* 1982) by using a minimal medium M9 containing sucrose as a sole carbon source. The *sacC* gene identified within a 6.7 kb *Eco*RI-*Hind*III DNA fragment was inserted in the plasmid pBR327-kan digested by *Eco*RI *and Hind*III. Then, a 1.5 kb *Bam*HI-*Sal*I DNA fragment carrying *arg*A was inserted in the obtained plasmid digested by *Bam*HI *and Sal*I by selection of recombinant clones bearing the pARGS2 plasmid. The pARGS2 plasmid ensures the growth of *Escherichia coli K12 argA* mutant cells on a selective medium M9 with sucrose as a sole carbon source, without or with L-arginine.
[0056] Plasmids pJ-B, pJ-T and pJ-M were transferred to the *Escherichia coli* K12 P4XB2(pARGS2) strain and the recombinant clones were selected on LB medium containing the two antibiotics, chloramphenicol (25 μg/ml) and kanamycin (40 μg/ml). Three colonies of each transformed strain and of the original strain were tested for the production of L-arginine under the conditions used in Example 4, except that the medium contained the only kanamycin (40 μg/ml) for *Escherichia coli* K12 P4XB2(pARGS2) or kanamycin in addition to the composition described for transformed clones.
[0057] The results of the fermentations are given in Table 3.

Table 3:

Production of L-arginine by the *Escherichia coli* K12 P4XB2 strain carrying the plasmid pARGS2 alone or in combination with pJ-M, pJ-B or pJ-T.	
Strain/plasmid	L-arginine (g/l)
P4XB2	< 0.2
P4XB2(pARGS2)	6.5
P4XB2(pARGS2/pJ-M)	7.0
P4XB2(pARGS2/pJ-B)	13
P4XB2(pARGS2/pJ-T)	13

[0058] These results demonstrate that the concomitant presence of any of the. three plasmids carrying the *argJ* gene along with the pARGS2 plasmid in the same *Escherichia coli* host strain provides higher production of L-arginine. However, the L-arginine yield is greater in the *Escherichia coli* K12 P4XB2 strain harbouring pARGS2 and pJ-B or pJ-T plasmids than in the *Escherichia coli* K12 P4XB2 strain harbouring pARGS2 and pJ-M plasmids. These results reveal that the co-existence of the *argA* gene (the pARGS2 plasmid)with the *argJ* gene coding for the bifunctional enzyme ornithine acetyltransferase (the pJ-B or pJ-T plasmids) in the same strain, assures a greater yield of L-arginine than with the *argJ* gene coding for a monofunctional enzyme (the pJ-M plasmid).

Example 6: Production of L-arginine in a fermentation medium containing sucrose

[0059] Plasmid pARGS2 enables the *Escherichia coli* K12 cells to consume sucrose as a carbon source. The wild-type *Escherichia coli* K12 strain and its derivatives are naturally unable of developing in a minimal medium in which glucose is replaced with sucrose.

[0060] The strains described in Example 5 were used to perform fermentations for the production of L-arginine under the conditions described above, except that the glucose is replaced with sucrose (6%) and the cultivation was prolonged for 44 h. The results are given in Table 4.

Table 4:

Production of L-arginine by recombinant *Escherichia coli* K12 P4XB2 strains on sucrose-containing fermentation medium	
Strain/plasmid	L-arginine (g/l)
P4XB2	< 0.2
P4XB2(pARGS2)	8.5
P4XB2(pARGS2/pJ-M)	8.5
P4XB2(pARGS2/pJ-B)	14.0
P4XB2(pARGS2/pJ-T)	14.0

[0061] These results again reveal that the bifunctional ArgJ enzyme as compared with the monofunctional enzyme provides higher yields of L-arginine in *Escherichia coli* K12 strains carrying the second plasmid pARGS2 with the *argA* and the sucrose-consumpting gene *sacC* during fermentation in a medium in which glucose is replaced by sucrose.

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Claims

1. A microorganism having an L-arginine producing ability, said microorganism being a microorganism synthesizing L-arginine through the biosynthetic linear or cyclic pathway, and bearing a recombinant DNA comprising a gene *argJ* coding for an enzyme having an ornithine acetyltransferase activity.

2. A microorganism having an L-arginine producing ability, said microorganism being a microorganism synthesizing L-arginine through the biosynthetic linear pathway, and bearing a recombinant DNA comprising a gene *argJ* coding for an enzyme having an ornithine acetyltransferase activity.

3. The microorganism according to claims 1 or 2, wherein the *argJ* gene codes for a bifunctional enzyme having both ornithine acetyltransferase activity and acetylglutamate synthetase activity.

4. The microorganism according to claims 1 to 3, wherein the enzyme is devoid of inhibition by L-arginine.

5. A microorganism having a L-arginine producing ability, said microorganism synthesizing L-arginine through biosynthetic linear or cyclic pathway and bearing a recombinant DNA comprising a gene *argJ* encoding for a bifunctional enzyme with an ornithine acetyltransferase activity and acetylglutamate synthetase activity.

6. The microorganism according to claim 1,which belongs to the genus *Escherlchia coli.*

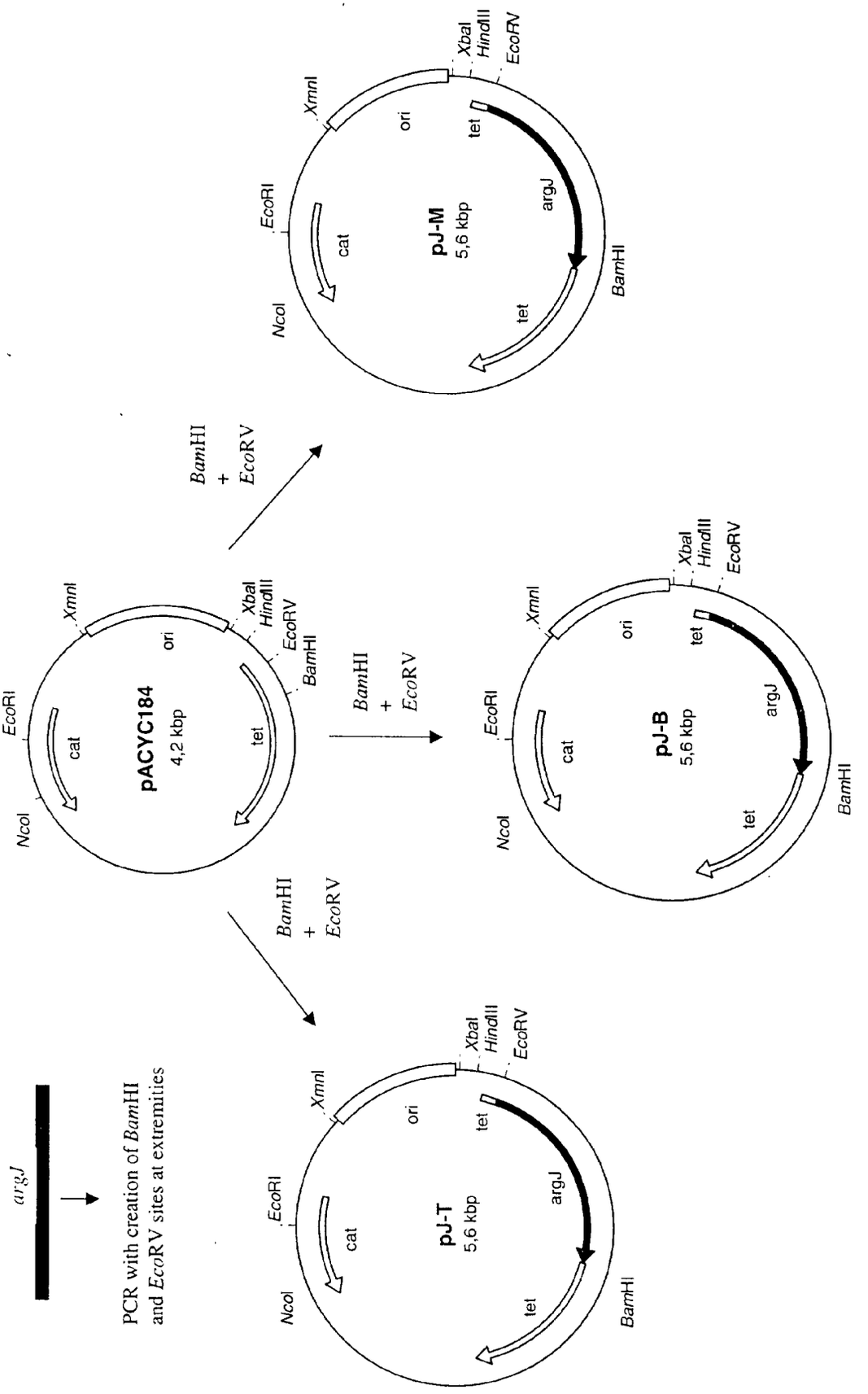
7. The microorganism according to claim 1, wherein the *argJ* gene is derived from a thermophilic microorganism.

8. The microorganism according to anyone of claims 1 to 7, wherein the *argJ* gene is derived from the thermophilic microorganism belonging to the species *Bacillus stearothermophilus* and *Thermokoga neapolitana.*

9. The microorganism according to any one of claims 1 to 5, which harbours a further recombinant DNA comprising gene a *argA* coding for the *N*-acetylglutamate synthetase.

10. The microorganism according to any one of claims 1 to 9, wherein the recombinant DNA is a plasmid DNA present at a low or moderate copy number.

11. A method for producing L-arginine comprising the steps of cultivating the microorganism as defined in any one of claims 1 to 10, in a culture medium to produce and accumulate L-arginine in the medium and recovering L-arginine from the medium.


argJ
PCR with creation of BamHI and EcoRV sites at extremities
BamHI + EcoRV
pACYC184
4,2 kbp
pJ-M
5,6 kbp
pJ-B
5,6 kbp
pJ-T
5,6 kbp
EcoRI
NcoI
XmnI
ori
cat
tet
XbaI
HindIII
EcoRV
BamHI
argJ

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 00 40 3003

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
D,X	RAJAGOPAL B S ET AL: "Use of inducible feedback-resistant N-acetylglutamate synthetase (argA) genes for enhanced arginine biosynthesis by genetically engineered Escherichia coli K-12 strains." APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 64, no. 5, May 1998 (1998-05), pages 1805-1811, XP002169946 ISSN: 0099-2240 * the whole document *	1-8,10, 11	C12N15/54 C12P13/10
X,D	MARC F ET AL: "Characterization and kinetic mechanism of mono- and bifunctional ornithine acetyltransferases from thermophilic microorganisms." EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 267, no. 16, August 2000 (2000-08), pages 5217-5226, XP002169947 ISSN: 0014-2956 * page 5221 - page 5225 *	1-8,10	
X	SAVCHENKO A ET AL: "The Bacillus stearothermophilus argCJBD operon harbours a strong promoter as evaluated in Escherichia coli cells" GENE: AN INTERNATIONAL JOURNAL ON GENES AND GENOMES,GB,ELSEVIER SCIENCE PUBLISHERS, BARKING, vol. 212, no. 2, 8 June 1998 (1998-06-08), pages 167-177, XP004122918 ISSN: 0378-1119 * page 172 *	1-8,10	TECHNICAL FIELDS SEARCHED (Int.Cl.7) C12N C12P

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	18 June 2001	Cupido, M

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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Application Number
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X,D	BAETENS MARGOT ET AL: "Genes and enzymes of the acetyl cycle of arginine biosynthesis in the extreme thermophilic bacterium Thermus thermophilus HB27." MICROBIOLOGY (READING), vol. 144, no. 2, February 1998 (1998-02), pages 479-492, XP002169948 ISSN: 1350-0872 * page 482, right-hand column *	1-8,10	

TECHNICAL FIELDS SEARCHED (Int.Cl.7)

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