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(54) Titre : GENES DE CORYNEBACTERIUM GLUTAMICUM POUR LA BIOSYNTHESE DE L'ACIDE FOLIQUE ET LEUR UTILISATION POUR LA PRODUCTION MICROBIENNE D'ACIDE FOLIQUE

(54) Title: GENES FROM CORYNEBACTERIUM GLUTAMICUM FOR THE BIOSYNTHESIS OF FOLIC ACID AND THEIR USE FOR THE MICROBIAL PRODUCTION OF FOLIC ACID

(57) Abrégé/Abstract:
The invention relates to nucleotide sequences of four genes (folE, folP, folB and folK) from Corynebacterium glutamicum for the biosynthesis of folic acid and their use for the microbial production of folic acid.

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(54) **Title:** GENES FROM CORYNEBACTERIUM GLUTAMICUM FOR THE BIOSYNTHESIS OF FOLIC ACID AND THEIR USE FOR THE MICROBIAL PRODUCTION OF FOLIC ACID

(54) **Bezeichnung:** GENE AUS *CORYNEBACTERIUM GLUTAMICUM* FÜR DIE FOLSÄUREBIOSYNTHESE UND IHR EINSATZ ZUR MIKROBIELLEN HERSTELLUNG VON FOLSÄURE

(57) **Abstract:** The invention relates to nucleotide sequences of four genes (*folE, folP, folB* and *folK*) from *Corynebacterium glutamicum* for the biosynthesis of folic acid and their use for the microbial production of folic acid.

(57) **Zusammenfassung:** Die vorliegende Erfindung besteht in Nucleotidsequenzen von vier Genen (*folE, folP, folB und folK*) aus *Corynebacterium glutamicum* für die Folsäurebiosynthese und ihr Einsatz zur mikrobiellen Herstellung von Folsäure.

GENES FROM CORYNEBACTERIUM GLUTAMICUM FOR THE BIOSYNTHESIS OF FOLIC ACID AND THEIR USE FOR THE MICROBIAL PRODUCTION OF FOLIC ACID

The present invention is concerned with the process for producing folic acid by fermentation using a genetically manipulated organism. This invention consists of the nucleotide sequences of four genes (*folE*, *folP*, *folB* and *folK*) from *Corynebacterium glutamicum* for folic acid biosynthesis and the use thereof for the microbial production of folic acid. These four genes form an operon and are transcribed in the following sequence: *folE*, *folP*, *folB*, *folK*.

Folic acid is essential for animal organisms. Its derivative tetrahydrofolate is a very versatile carrier of activated one-carbon units in cells of the animal organism. Folic acid consists of three groups: a substituted pteridine ring, *p*-aminobenzoate and glutamate. Mammals are unable to synthesize a pteridine ring. They absorb folic acid from the diet and from microorganisms in their intestinal tract. Folic acid deficiency leads mainly to lesions in the mucous membranes.

The commercial importance of folic acid is in the animal feed and human food markets. Folic acid is employed mainly as a dietary supplement.

Microorganisms can be employed for the fermentative production of folic acid. They can be optimized in their efficiency of folic acid biosynthesis by genetic manipulation of the folic acid biosynthetic pathway. Genetic manipulation means in this connection that the number of copies and/or the rate of transcription of the genes of the folic acid biosynthetic pathway is

increased. As a consequence thereof, the proportion of gene product increases, and thus the intracellular enzymic activity does too. Increased enzymic activity leads to an increased rate of dietary (e.g. glucose)

conversion into folic acid and thus also to an increased product concentration. For genetic manipulation, the nucleotide sequences of the genes of the folic acid biosynthetic pathway must be identified. This invention is concerned with four novel gene sequences for folic acid biosynthesis from *Corynebacterium glutamicum* and with their use for the microbial production of folic acid.

One part of the invention comprises the *folE* gene product. SEQ ID NO. 2 describes a polypeptide sequence. The *folE* gene product encodes a polypeptide of 202 amino acids with a molecular weight of 22 029 Da. The present invention is also concerned with functional derivatives of this polypeptide obtainable by replacing one or more amino acids, preferably up to 25% of the amino acids, most suitably up to 15% of the amino acids, in SEQ ID NO. 2 by deletion, insertion or substitution or by a combination of deletion, insertion and substitution. The term functional derivative means that the enzymatic activity of the derivative is still of the same order of magnitude as that of the polypeptide having the sequence SEQ ID NO. 2.

Another part of the invention comprises the *folP* gene product. SEQ ID NO. 4 describes a polypeptide sequence. The *folP* gene product encodes a polypeptide of 285 amino acids with a molecular weight of 29 520 Da. The present invention is also concerned with functional derivatives of this polypeptide obtainable by replacing one or more amino acids, preferably up to 40% of the amino acids, most suitably up to 25% of the amino acids, in SEQ ID NO. 4 by deletion, insertion or substitution or by a combination of deletion, insertion and substitution. The term functional derivative means that the enzymatic activity of the derivative is still of the same order of magnitude as that of the polypeptide having the sequence SEQ ID NO. 4.

Another part of the invention comprises the *folB* gene product. SEQ ID NO. 6 describes a polypeptide sequence. The *folB* gene product encodes a polypeptide of 131 amino acids with a molecular weight of 14 020 Da. The present invention is also concerned with functional derivatives of this polypeptide obtainable by replacing one or more amino acids, preferably up to 30% of the amino acids, most suitably up to 20% of the amino acids, in SEQ ID NO. 6 by deletion, insertion or substitution or by a combination of deletion, insertion and substitution. The term functional derivative means that the enzymatic activity of the derivative is still of the same order of magnitude as that of the polypeptide having the sequence SEQ ID NO. 6.

Another part of the invention comprises the *folK* gene product. SEQ ID NO. 8 describes a polypeptide sequence. The *folK* gene product encodes a polypeptide of 160 amino acids with a molecular weight of 18 043 Da. The present invention is also concerned with functional derivatives of this polypeptide obtainable by replacing one or more amino acids, preferably up to 40% of the amino acids, most suitably up to 30% of the amino acids, in SEQ ID NO. 8 by deletion, insertion or substitution or by a combination of deletion, insertion and substitution. The term functional derivative means that the enzymatic activity of the derivative is still of the same order of magnitude as that of the polypeptide having the sequence SEQ ID NO. 8.

Another part of the invention comprises the polynucleotide sequences which encode the polypeptides described above. The polynucleotide sequences can be generated starting from sequences isolated from *Corynebacterium glutamicum* (i.e. SEQ ID NOS. 1, 3, 5 and 7) in which these sequences are modified by site-directed mutagenesis or a total chemical synthesis is carried out after back-translation of the corresponding

polypeptide using the genetic code.

These polynucleotide sequences can preferably be employed for the transformation of host organisms, and in this connection preferably of microorganisms, specifically in the form of gene constructs which comprise at least one copy of one of these polynucleotides together with at least one regulatory sequence. Regulatory sequences comprise promoters, terminators, enhancers and ribosome binding sites.

Preferred host organisms for transformation with these gene constructs are *Corynebacterium* and *Bacillus* species. It is also possible to employ any eukaryotic microorganism, preferably yeast strains of the genus *Ashbya*, *Candida*, *Pichia*, *Saccharomyces* and *Hansenula*.

Another part of the invention comprises the process for producing folic acid by cultivating a host organism which is transformed in the manner described above, and subsequently isolating the folic acid.

The processes and the procedures for cultivating microorganisms and for isolating folic acid from a microbial production are familiar to trained staff.

The invention is described in more detail in the following examples, as is its use for the genetic manipulation of microorganisms, to increase the efficiency of folic acid synthesis.

Example 1

Construction of a genome library from *Corynebacterium glutamicum* ATCC 13032

DNA from the genome of *Corynebacterium glutamicum* ATCC 13032 can be obtained by standard methods which

have already been described, for example by J. Altenbuchner and J. Cullum (1984, Mol. Gen. Genet. 195: 134-138). The genome library can be produced in accordance with standard protocols (e.g. Sambrook, J. et al. (1989) Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory Press) with any cloning vector, e.g. pBluescript II KS- (Stratagene) or ZAP Express™ (Stratagene). It is moreover possible to use any fragment size, preferably *Sau3AI* fragments with a length of 2-9 kb, which can be incorporated into cloning vectors with digested *BamHI*.

Example 2

Analysis of the nucleic acid sequence of the genome library

Individual *E. coli* clones can be selected from the genome library constructed in example 1. *E. coli* cells are cultivated by standard methods in suitable media (e.g. LB supplemented with 100 mg/l ampicillin), and the plasmid DNA can then be isolated. Cloning of genome fragments from the DNA of *Corynebacterium glutamicum* into pBluescript II KS- (see example 1) allows the DNA to be sequenced with the aid of the oligonucleotides 5'-AATTAACCCTCACTAAAGGG-3' and 5'-GTAATACGACTCACTATAGGGC-3'.

Example 3

Computer analysis of the sequences of the isolated nucleic acids

The nucleotide sequences can be connected together for example with the aid of the BLASTX algorithm (Altschul et al. (1990) J. Mol. Biol. 215: 403-410). It is possible in this way to discover novel sequences and elucidate the function of these novel genes.

Example 4

Identification of an *E. coli* clone which comprises a nucleotide sequence of the gene for GTP cyclohydrolase I (EC 3.5.4.16)

Analysis of the *E. coli* clones as described in example 2, which was followed by analysis, as described in example 3, of the sequences obtained thereby revealed a sequence which is described by SEQ ID NO. 1. On use of the BLASTX algorithm (see example 3), this sequence revealed similarity with GTP cyclohydrolases I (FolE; EC 3.5.4.16) from various organisms. The greatest similarity was with the GTP cyclohydrolase I (FolE) from *Mycobacterium tuberculosis* (NRDB 006273; 72% agreement at the amino acid level).

Example 5

Identification of an *E. coli* clone which comprises a nucleotide sequence of the gene for dihydropteroate synthase (EC 2.5.1.15)

Analysis of the *E. coli* clones as described in example 2, which was followed by analysis, as described in example 3, of the sequences obtained thereby revealed a sequence which is described by SEQ ID NO. 3. On use of the BLASTX algorithm (see example 3), this sequence revealed similarity with dihydropteroate synthases (FolP; EC 2.5.1.15) from various organisms. The greatest similarity was with the dihydropteroate synthase (FolP) from *Mycobacterium tuberculosis* (NRDB 006274; 53% agreement at the amino acid level).

Example 6

Identification of an *E. coli* clone which comprises a nucleotide sequence of the gene for dihydroneopterin aldolase (EC 4.1.2.25)

Analysis of the *E. coli* clones as described in example 2, which was followed by analysis, as described in example 3, of the sequences obtained thereby revealed a sequence which is described by SEQ ID NO. 5. On use of the BLASTX algorithm (see example 3), this sequence revealed similarity with dihydroneopterin aldolases (FolB; EC 4.1.2.25) from various organisms. The greatest similarity was with the dihydroneopterin aldolase (FolB) from *Mycobacterium tuberculosis* (NRDB O06275; 61% agreement at the amino acid level).

Example 7

Identification of an *E. coli* clone which comprises a nucleotide sequence of the gene for 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase (EC 2.7.6.3)

Analysis of the *E. coli* clones as described in example 2, which was followed by analysis, as described in example 3, of the sequences obtained thereby revealed a sequence which is described by SEQ ID NO. 7. On use of the BLASTX algorithm (see example 3), this sequence revealed similarity with 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinases (FolK; EC 2.7.6.3) from various organisms. The greatest similarity was with the 2-amino-4-hydroxy-6-hydroxy-methyldihydropteridine pyrophosphokinase (FolK) from *Mycobacterium leprae* (EMBL AL023093; 43% agreement at the amino acid level).

Example 8

Use of the genes for GTP cyclohydrolase I, for dihydropteroate synthase, for dihydroneopterin aldolase and for 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase from *Corynebacterium glutamicum* for producing folic acid

The genes for GTP cyclohydrolase I, for dihydropteroate synthase, for dihydroneopterin aldolase and for 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase from *Corynebacterium glutamicum* can be introduced with the aid of suitable cloning and expression systems into *Corynebacterium glutamicum* or into any other microorganism. Genetically manipulated microorganisms which differ from the wild-type organism in relation to the activity or the number of gene copies can be produced. These novel genetically manipulated strains can be employed for producing folic acid.

Sequence list

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(2) Title: Genes from *Corynebacterium glutamicum* for folic acid biosynthesis and their use for the microbial production of folic acid

(3) Number of sequences: 8

SEQ ID NO. 1: DNA (*folE*)

ATGAAGGAGACAACCGTGGATAACCACGCTGCAGTTCGCGAGTTCGATGAGGAGCGCGCAACAGC
TGCGATTCGTGAGTTGCTCATCGCTGTGGGTGAGGATCCAGATCGCGAAGGCCTGTTGGAAACCC
CAGCTCGAGTGGCTAGGGCGTACAAGGAAACTTTCGCGGGTCTGCATGAGGATCCCACCACTGTG
CTGGAGAAGACGTTCTCTGAGGGCCATGAAGAGTTGGTTCTGGTTCGTGAGATCCCGATTTACTC
CATGTGTGAGCACCACTTGGTGCCGTTCTTTGGCGTGGCGCACATTGGTTACATTCCGGGTAAGT
CCGGCAAGGTGACTGGCCTGTCCAAGCTGGCGCGTTTAGCGGATATGTTTGCTAAGCGACCTCAG
GTTCAGGAGCGCTTGACCTCCCAAATTGCGGATGCTCTCGTCGAAAAGCTTGATGCCCAGGCCGT
GGCCGTGGTGATTGAAGCTGAGCACCTGTGCATGGCCATGCGCGGAATCCGTAAGCCTGGTGCTG
TGACCACGACGTCTGCGGTGCGCGGCGGTTTTAAGAACAACGCTGCCTCCCGCGCTGAGGTGTTC
TCCCTGATTCGGGGGCACTAA

SEQ ID NO. 2: amino acid (FolE)

MKETTVDNHAAVREFDEERATAAIRELLIAVGEDPDREGLLETPARVARAYKETFAGLHEDPTTV
LEKTFSEGHEELVLVREIPIYSMCEHHLVPFFGVAHIGYIPGKSGKVTGLSKLARLADMFAKRPQ
VQERLTSQIADALVEKLDAQAVAVVIEAEHLCMAMRGIRKPGAVTTTSAVRGGFKNNAASRAEVF
SLIRGH

SEQ ID NO. 3: DNA (*folP*)

ATGAACGTATCCTCTTTGACCATCCCGGGACGCTGTTTGGTCATGGGAATTGTCAATGTCACTGA
GGATTCCTTTTCGGACGGTGGCAAGTACATTGACGTTGATCAGGCGATCGCGCATGCCAAGGAAT
TGGTGGCTGCTGGCGCCGACATGATTGATGTCGGCGGCGAGTCCACCCGGCCTGGGGCAGTGCGC
GTCGACGCGTCCGTGGAACGGGACCGGGTTGTGCCGGTCATTAAGGCGCTTCACGACGCCGGCAT
CCACACTTCCGTAGACACCATGCGGGCCTCCGTGGCGCAGGCTGCCGCGGGCGCTGGCGTCTCCA
TGATCAACGACGTCTCTGGCGGTTTGGCTGATCCTGAGATGTTTTCTGTCATGGCGGAAGCGCAA
ATTCCCGTGTGTTTGATGCACTGGCGCACCCTCCAATTCGGTGATGCCGCAGGTCAGGCAGATCA
CGGTGGAGACGTTGTAGCCGATGTGCACGCAGTGCTTGATGATCTTGTCGCCCGCGCCACCGCTG
CTGGTGTGGCCGAAAACCAGATCGTGCTTGATCCAGGTTTGGGTTTTGCCAAATCACGTGAAGAC
AACTGGCGTTtGCTGCAAGCACTGCCCGAGTTTATTTCTGGACCTTTCCCCATCCTGGTGGGAGC
ATCCCGGAAGCGATTCCTGGCTGGCGTGCGCAAAGACCGTGGCCTAGATGTCACCCCCATTGATG
CCGACCCAGCAACCGCAGCGGTGACCGCAGTGTCTGCACATATGGGAGCATGGGGTGTGCGCGTG
CACGATGTCCCAGTATCAAGGGACGCTGTTGATGTTGCCGCATTGTGGCGAAGTGGAGGAACTCA
CCATGGCTGA

SEQ ID NO. 4: amino acid (FolP)

MNVSSLTIPGRCLVMGIVNVTEDSFSDGGKYIDVDQALAHAKELVAAGADMIDVGGESTRPGAVR
VDASVERDRVVPVIKALHDAGIHTSVDTMRASVAQAAAGAGVSMINDVSGGLADPEMFSVMAEAQ
IPVCLMHWRTLQFGDAAGQADHGGDVVADVHAVLDDLVAPATAAGVAENQIVLDPGLGFAKSRED
NWRLLQALPEFISGPFPILVGASRKRFLAGVRKDRGLDVTPIDADPATAAVTAVSAHMGAWGVRV
HDVPVSRDAVDVAALWRSGGTHHG

SEQ ID NO. 5: DNA (*FolB*)

ATGGCTGATCGTATTGAACTTAAAGGCCTTGAATGCTTCGGACACCACGGTGTGTTCGACTTTGA
AAAAGAGCAAGGCCAGCCCTTCATTGTGGATGTCACCTGCTGGATGGATTTCGATGCCGCAGGTG
CCAGCGATGACCTTTCCGACACCGTAGATTACGGCGCGTTGGCATTGTTGGTTGCTGAAATCGTG
GAAGGCCCATCCAGGGATTTGATCGAGACGGTGGCCACGGAATCTGCGGATGCTGTGATGGCTAA
ATTTGATGCGCTTCATGCGGTGGAAGTAACCATCCATAAGCCCAAAGCACCGATCCCACGTACTT
TTGCTGACGTCGCGGTGGTTGCCCGACGTTCCAGGAAATCCATGGCTGCTGGAAGGAGCAACGCC
TAA

SEQ ID NO. 6: amino acid (FolB)

MADRIELKGLECFGHHGVFDFEKEQGQPFIVDVTCWMDFDAAGASDDLSDTVDYGALALLVAEIV
EGPSRDLIETVATESADAVMAKFDALHAVEVTIHKPKAPIPRTFADVAVVARRSRKSMAAGRSNA

SEQ ID NO. 7: DNA (*folK*)

ATGCATGCAGTTTTGTCCATCGGTTCCAACATGGATGATCGCTACGCGCTGCTCAACACAGTGAT
CGAGGAATTCAAAGATGAGATCGTGGCGCAGTCTGCGATCTACTCAACCCCACCGTGGGGCATTG
AGGATCAGGATGAATTCCTCAACGCAGTGCTCGTTGTTGAGGTTGAAGAAACCCCCATCGAGTTG
CTGCGCCGTgGCCAAAAACTCGAAGAAGCCGCCGAGCGGGTCCGCGTCCGCAAATGGGGGCCACG
CACCCTCGATGTGGATATCGTGCAGATCATTAAAGATGGGGAAGAGATCCTTTCTGAGGATCCCG
AACTGACCTTGCCACACCCTTGGGCTTGGCAGCGTGCCTTCGTGTTGATCCCTTGGTTGGAAGCA
GAACCTGATGCCGTCCTGCACGGCACGACCATTGCAGAACATGTGGATAATCTTGATCCCACAGA
CATTGAAGGTGTCACCAAGATTTAA

SEQ ID NO. 8: amino acid (folK)

MHAVLSIGSNMDDRYALLNTVIEEFKDEIVAQSAIYSTPPWGIEDQDEFLNAVLVVEVEETPIEL
LRRGQKLEEAAERVRVRKWGPRTLDVDIVQIIKDGEEILSEDPELTLPHPWAWQRAFVLIPWLEA
EPDAVLHGTTIAEHVDNLDPTDIEGVTKI

We claim:

1. A polypeptide having GTP cyclohydrolase I activity and selected from the following group:

(a) a polypeptide having the amino acid sequence which is described in SEQ ID NO. 2

(b) a polypeptide which is modified by comparison with that in (a) by deletion, insertion or substitution of one or more amino acids.

2. A polypeptide having dihydropteroate synthase activity and selected from the following group:

(a) a polypeptide having the amino acid sequence which is described in SEQ ID NO. 4;

(b) a polypeptide which is modified by comparison with that in (a) by deletion, insertion or substitution of one or more amino acids.

3. A polypeptide having dihydroneopterin aldolase activity and selected from the following group:

(a) a polypeptide having the amino acid sequence which is described in SEQ ID NO. 6

(b) a polypeptide which is modified by comparison with that in (a) by deletion, insertion or substitution of one or more amino acids.

4. A polypeptide having 2-amino-4-hydroxy-6-hydroxy-methyldihydropteridine pyrophosphokinase activity and selected from the following group:

(a) a polypeptide having the amino acid sequence which is described in SEQ ID NO. 8

(b) a polypeptide which is modified by comparison with that in (a) by deletion, insertion or substitution of one or more amino acids.

5. A polynucleotide which encodes a polypeptide corresponding to claim 1, 2, 3 or 4.

6. A gene construct having at least one copy of a polynucleotide corresponding to claim 5, together with at least one regulatory sequence.

7. A host organism which is transformed with a gene construct corresponding to claim 6.

8. A process for producing folic acid by cultivating a host organism corresponding to claim 7 with subsequent isolation of the folic acid.