PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/52, C12P 13/08, C12N 15/70, 15/90, 1/21 // (C12N 1/21, C12R 1:19)

A1

(11) International Publication Number: **WO 98/04715**

(43) International Publication Date: 5 February 1998 (05.02.98)

(21) International Application Number: PCT/US97/13359

(22) International Filing Date: 30 July 1997 (30.07.97)

(30) Priority Data:
60/022,407 30 July 1996 (30.07.96) US

(71) Applicant: ARCHER-DANIELS-MIDLAND COMPANY [US/US]; 4666 Faries Parkway, Decatur, IL 62526 (US).

(72) Inventors: WANG, Ming-Der; 12037 Mil Pitrero Road, San Diego, CA 92128 (US). BRADSHAW, Jill, S.; 899 Harold Circle, Decatur, IL 62526 (US). SWISHER, Stacia, L.; 1643 E. Hinsdale Avenue, Decatur, IL 62526 (US). LIAW, Hungming, James; 1013 Galen Drive, Champaign, IL 61821 (US). HANKE, Paul, D.; 309 North Abbey, Urbana, IL 61801 (US). BINDER, Thomas, P.; 2323 West Packard, Decatur, IL 62522 (US).

(74) Agents: GOLDSTEIN, Jorge, A. et al.; Sterne, Kessler, Goldstein & Fox P.L.L.C., Suite 600, 1100 New York Avenue, N.W., Washington, DC 20005-3934 (US).

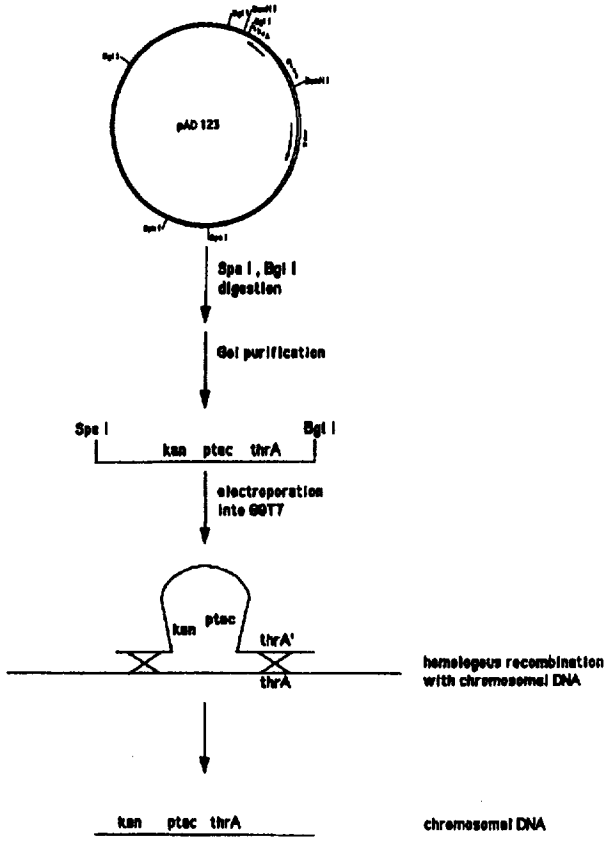
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: NOVEL STRAINS OF *ESCHERICHIA COLI*, METHODS OF PREPARING THE SAME AND USE THEREOF IN FERMENTATION PROCESSES FOR L-THREONINE PRODUCTION

(57) Abstract

The present invention relates to novel strains of *Escherichia coli* and fermentation processes involving these microorganisms. More specifically, the present invention relates to genetically-modified *Escherichia coli* strains and the use thereof for the production of the amino acids, particularly members of the aspartate family of amino acids such as threonine. The present invention also relates to methods of preparing *E. coli* strains for use in the fermentative production of amino acids.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

NOVEL STRAINS OF *ESCHERICHIA COLI*, METHODS OF PREPARING THE SAME AND USE THEREOF IN FERMENTATION PROCESSES FOR L-THREONINE PRODUCTION

Field of the Invention

The present invention relates to novel strains of *Escherichia coli* and fermentation processes involving these microorganisms. More specifically, the present invention relates to genetically-modified *Escherichia coli* strains and the use thereof for the production of the amino acids, particularly members of the aspartate family of amino acids such as threonine. The present invention also relates to methods of preparing *E. coli* strains for use in the fermentative production of amino acids.

Background of the Invention

In *Escherichia coli*, the amino acids L-threonine, L-isoleucine, L-lysine and L-methionine derive all or part of their carbon atoms from aspartate (aspartic acid) via the following common biosynthetic pathway (G.N. Cohen, "The common pathway to lysine, methionine and threonine," pp. 147-171 in *Amino Acids: Biosynthesis and Genetic Regulation*, K.M. Herrmann and R.L. Somerville, eds., Addison-Welesley Publishing Co., Inc., Reading, Mass. (1983)):

aspartate → aspartyl phosphate → aspartate semialdehyde → homoserine →→MET/THR/ILE
↓
LYS

The first reaction of this common pathway is catalyzed by one of three distinct aspartate kinases (AK I, II, or III), each of which is encoded by a separate gene and differs from the others in the way its activity and synthesis are regulated. Aspartate kinase I, for example, is encoded by *thrA*, its activity is inhibited by threonine, and its synthesis is repressed by threonine and isoleucine in combination. AK II, however, is encoded by *metL* and its synthesis repressed by methionine (although its activity is not inhibited by methionine or by paired combinations of methionine, lysine, threonine and isoleucine (F. Falcoz-Kelly *et al.*, *Eur. J. Biochem.* *8*:146-152 (1969); J.C.

Patte *et al.*, *Biochim. Biophys. Acta 136*:245-257 (1967)). AK III is encoded by *lysC* and its activity and synthesis are inhibited and repressed, respectively, by lysine.

Two of the AKs, I and II, are not distinct proteins, but rather a domain of a complex enzyme that includes homoserine dehydrogenase I or II, respectively, each of which catalyzes the reduction of aspartate semialdehyde to homoserine (P. Truffa-Bachi *et al.*, *Eur. J. Biochem. 5*:73-80 (12968)). Homoserine dehydrogenase I is therefore also encoded by *thrA*, its synthesis is repressed by threonine plus isoleucine and its activity is inhibited by threonine. HD II is similarly encoded by *metL* and its synthesis is repressed by methionine.

Threonine biosynthesis includes the following additional reactions: homoserine → homoserine phosphate → threonine. The phosphorylation of homoserine is catalyzed by homoserine kinase, a protein which consists of two identical 29 kDa subunits encoded for by *thrB* and whose activity is inhibited by threonine (B. Burr *et al.*, *J. Biochem. 62*:519-526 (1976)). The final step, the complex conversion of homoserine phosphate to L-threonine is catalyzed by threonine synthase, a 47 kDa protein encoded for by *thrC* (C. Parsot *et al.*, *Nucleic Acids Res. 11*:7331-7345 (1983)).

The *thrA*, *thrB* and *thrC* genes all belong to the *thr* operon, a single operon located at 0 minutes on the genetic map of *E. coli* (J. Thèze and I. Saint-Girons, *J. Bacteriol. 118*:990-998 (1974); J. Thèze *et al.*, *J. Bacteriol. 117*:133-143 (1974)). These genes encode, respectively, for aspartate kinase I-homoserine dehydrogenase I, homoserine kinase and threonine synthase. Biosynthesis of these enzymes is subject to multivalent repression by threonine and isoleucine (M. Freundlich, *Biochem. Biophys. Res. Commun. 10*:277-282 (1963)).

A regulatory region is found upstream of the first structural gene in the *thr* operon and its sequence has been determined (J.F. Gardner, *Proc. Natl. Acad. Sci. USA 76*:1706-1710 (1979)). The *thr* attenuator, downstream of the transcription initiation site, contains a sequence encoding a leader peptide; this

sequence includes eight threonine codons and four isoleucine codons. The *thr* attenuator also contains the classical mutually exclusive secondary structures which permit or prevent RNA polymerase transcription of the structural genes in the *thr* operon, depending on the levels of the charged threonyl- and isoleucyl-tRNAs.

Because of the problems associated with obtaining high levels of amino acid production via natural biosynthesis (*e.g.* repression of the *thr* operon by the desired product), bacterial strains have been produced having plasmids containing a *thr* operon with a *thrA* gene that encodes a feedback-resistant enzyme. With such plasmids, L-threonine has been produced on an industrial scale by fermentation processes employing a wide variety of microorganisms, such as *Brevibacterium flavum*, *Serratia marcescens*, and *Escherichia coli*.

For example, the *E. coli* strain BKIIM B-3996 (Debabov *et al.*, U.S. Patent No. 5,175,107), which contains the plasmid pVIC40, makes about 85 g/L in 36 hr. The host is a threonine-requiring strain because of a defective threonine synthase. In BKIIM B-3996, it is the recombinant plasmid, pVIC40, that provides the crucial enzymatic activities, *i.e*, a feedback-resistant AK I-HD I, homoserine kinase and threonine synthase, needed for threonine biosynthesis. This plasmid also complements the host's threonine auxotrophy.

E. coli strain 29-4 (E. Shimizu *et al.*, *Biosci. Biotech. Biochem. 59*:1095-1098 (1995)) is another example of a recombinant *E. coli* threonine producer. Strain 29-4 was constructed by cloning the *thr* operon of a threonine-overproducing mutant strain, *E. coli* K-12 (βIM-4) (derived from *E. coli* strain ATCC 21277), into plasmid pBR322, which was then introduced into the parent strain (K. Wiwa *et al.*, *Agric. Biol. Chem. 47*:2329-2334 (1983)). Strain 29-4 produces about 65 g/L of L-threonine in 72 hr.

Similarly constructed recombinant strains have been made using other organisms. For example, the *S. marcescens* strain T2000 contains a plasmid having a *thr* operon which encodes a feedback-resistant *thrA* gene product and produces about 100 g/L of threonine in 96 hrs (M. Masuda *et al.*, *Applied Biochemistry and Biotechnology 37*:255-262 (1992). All of these strains contain

plasmids having multiple copies of the genes encoding the threonine biosynthetic enzymes, which allows overexpression of these enzymes. This overexpression of the plasmid-borne genes encoding threonine biosynthetic enzymes, particularly a *thrA* gene encoding a feedback-resistant AK I-HD I, enables these strains to produce large amounts of threonine. Other examples of plasmid-containing microorganisms are described, for example, in U.S. Patent Nos. 4,321,325; 4,347,318; 4,371,615; 4,601,983; 4,757,009; 4,945,058; 4,946,781; 4,980,285; 5,153,123; and 5,236,831.

Plasmid-containing strains such as these, however, have problems that limit their usefulness for commercial fermentative production of amino acids. For example, a significant problem with these strains is ensuring that the integrity of the plasmid-containing strain is maintained throughout the fermentation process because of potential loss of the plasmid during cell growth and division. To avoid this problem, it is necessary to selectively eliminate plasmid-free cells during culturing, such as by employing antibiotic resistance genes on the plasmid. This solution, however, necessitates the addition of one or more antibiotics to the fermentation medium, which is not commercially practical for large scale fermentations.

Another significant problem with plasmid-containing strains is plasmid stability. High expression of enzymes whose genes are coded on the plasmid, which is necessary for commercially practical fermentative processes, often brings about plasmid instability (E. Shimizu *et al.*, *Biosci. Biotech. Biochem.* *59*:1095-1098 (1995)). Plasmid stability is also dependent upon factors such as cultivation temperature and the level of dissolved oxygen in the culture medium. For example, plasmid-containing strain 29-4 was more stable at lower cultivation temperatures (30°C vs. 37°C) and higher levels of dissolved oxygen (E. Shimizu *et al.*, *Biosci. Biotech. Biochem.* *59*:1095-1098 (1995)).

Non-plasmid containing microorganisms, while less efficacious than those described above, have also been used as threonine producers. Strains of *E. coli* such as H-8460, which is obtained by a series of conventional mutagenesis and selection for resistance to several metabolic analogs makes

about 75 g/L of L-threonine in 70 hours (Kino *et al.*, U.S. Patent No. 5,474,918). Strain H-8460 does not carry a recombinant plasmid and has one copy of the threonine biosynthetic genes on the chromosome. The lower productivity of this strain compared to the plasmid-bearing strains, such as BKIIM B-3996, is believed to be due to lower enzymatic activities (particularly those encoded by the *thr* operon) as these non-plasmid containing strains carry only a single copy of threonine biosynthetic genes. Other examples of suitable non-plasmid containing microorganisms are described, for example, in U.S. Patent Nos. 5,376,538; 5,342,766; 5,264,353; 5,217,883; 5,188,949; 5,164,307; 5,098,835; 5,087,566; 5,077,207; 5,019,503; 5,017,483; 4,996,147; 4,463,094; 4,452,890; 3,732,144; 3,711,375; 3,684,654; 3,684,653; 3,647,628; 3,622,453; 3,582,471; 3,580,810; 3,984,830; and 3,375,173.

In both the non-plasmid and plasmid containing strains of *E. coli*, the *thr* operon is controlled by the particular strain's respective native threonine promoter. As described above, the expression of the native promoter is regulated by an attenuation mechanism controlled by a region of DNA which encodes a leader peptide and contains a number of threonine and isoleucine codons. This region is translated by a ribosome which senses the levels of threoninyl-tRNA and isoleucinyl-tRNA. When these levels are sufficient for the leader peptide to be translated, transcription is prematurely terminated, but when the levels are insufficient for the leader peptide to be translated, transcription is not terminated and the entire operon is transcribed, which, following translation, results in increased production of the threonine biosynthetic enzymes. Thus, when threonyl-tRNA and/or isoleucinyl-tRNA levels are low, the *thr* operon is maximally transcribed and the threonine biosynthetic enzymes are maximally made.

In the *E. coli* threonine-producing strain BKIIM B-3996, the threonine operon in the plasmid is controlled by its native promoter. As a result, the *thr* operon is only maximally expressed when the strain is starved for threonine and/or isoleucine. Since starvation for threonine is not possible in a threonine-

producing strain, these strains have been rendered auxotrophic for isoleucine in order to obtain a higher level of enzymatic activity.

Another way of overcoming attenuation control is to lower the level(s) of threonyl-tRNA and/or isoleucinyl-tRNA in the cell. A *thrS* mutant, for example, having a threonyl-tRNA synthase which exhibits a 200-fold decreased apparent affinity for threonine, results in overexpression the *thr* operon, presumably due to the low level of threonyl-tRNA (E.J. Johnson *et al.*, *J. Bacteriol. 129*:66-70 (1977)).

In fermentation processes using these strains, however, the cells must be supplemented with isoleucine in the growth stage because of their deficient isoleucine biosynthesis. Subsequently, in the production stage, the cells are deprived of isoleucine to induce expression of the threonine biosynthetic enzymes. A major drawback, therefore, of using native threonine promoters to control expression of the threonine biosynthetic enzymes is that the cells must be supplemented with isoleucine.

The antibiotic borrelidin is also known to reduce the enzymatic activity of threonyl tRNA-synthetase, and thereby inhibit the growth of *E. coli* (G. Nass *et al.*, *Biochem. Biophys. Res. Commun. 34*:84 (1969)). In view of this reduced activity, certain borrelidin-sensitive strains of *E. coli* have been employed to produce high levels of threonine (Japanese Published Patent Application No. 6752/76; U.S. Patent No. 5,264,353). Addition of borrelidin to the culture was found to increase the yield of L-threonine. Borrelidin-sensitive strains of *Brevibacterium* and *Corynebacterium* have also been used to produce high levels of threonine (Japanese Patent No. 53-101591).

Borrelidin-resistant mutants of *E. coli* similarly exhibit changes in threonyl tRNA-synthestase activity. More specifically, borrelidin-resistant *E. coli* have been shown to exhibit one of the following features: (i) constitutively increased levels of wild-type threonyl tRNA-synthetase; (ii) structurally altered threonyl tRNA-synthetase; or (iii) some unknown cellular alteration, probably due to a membrane change (G. Nass and J. Thomale, *FEBS Lett. 39*:182-186

(1974)). None of these mutant strains, however, has been used for the fermentative production of L-threonine.

In view of the discussion above, there remains a need in the art for microorganism strains which efficiently produce amino acids such as threonine, but without the problems associated with the state of the art.

Summary Of The Invention

It is therefore an object of the present invention to provide microorganisms which efficiently produce L-threonine in high yields, but which do not require any recombinant plasmids containing genes that encode threonine biosynthetic enzymes and preferably have no amino acid nutritional requirements. Other objects, features and advantages of the present invention will be set forth in the detailed description of preferred embodiments that follows, and in part will be apparent from the description or may be learned by practice of the invention. These objects and advantages of the invention will be realized and attained by the methods particularly pointed out in the written description and claims hereof.

These and other objects are accomplished by the methods of the present invention, which, in a first embodiment, is directed to a process for producing amino acids such as L-threonine, which comprises the steps of culturing a strain of *E. coli* in a medium and recovering the amino acid from the medium. The strain of *E. coli* used in this process has the following characteristics: (i) it contains a genetic determinant of amino acid biosynthesis, such as the threonine operon (which encodes the threonine biosynthetic enzymes), on the chromosome under control of a non-native promoter; and (ii) it does not require any recombinant plasmids containing genes that encode threonine biosynthetic enzymes to produce threonine.

Another embodiment of the present invention is directed to a biologically pure culture of a strain of *E. coli* having the above characteristics.

An additional embodiment of the present invention is directed to a process for producing amino acids such as L-threonine, which comprises the steps of

culturing a strain of *E. coli* in a medium and recovering the amino acid from the medium, wherein the strain of *E. coli* is resistant to borrelidin.

A further embodiment of the present invention is directed to a method of producing a strain of *E. coli* useful for the fermentative production of amino acids such as threonine which comprises the steps of (a) introducing genetic material from an amino acid-producing microorganism into the chromosome of an *E. coli* auxotroph so as to render that *E. coli* prototrophic; (b) inserting a non-native promoter into the chromosome before the chromosomal location of the amino acid biosynthetic genes to control the expression thereof; and, optionally, (c) removing amino acid nutritional requirements for and/or regulatory hindrances to amino acid biosynthesis from the chromosome.

It is to be understood that both the foregoing general description and the following detained description are exemplary and explanatory only and are intended to provide further explanation of the invention as claimed.

Brief Description of Drawings

Figure 1 depicts the construction of plasmid pAD103 from Kohara's lambda 676 and plasmid pUC19.

Figure 2 depicts the construction of plasmid pAD106 from plasmid pAD103 and plasmid pUC4k.

Figure 3 depicts the construction of plasmid pAD115 from plasmid pAD103 and plasmid pkk223-3.

Figure 4 depicts the construction of plasmid pAD123 from plasmid pAD115 and plasmid pAD106.

Figure 5 depicts the intergration of the promoter region from plasmid pAD123 into the chromosome of *E. coli*.

Detailed Description of Preferred Embodiments of the Invention

In a first embodiment, the present invention is directed to novel bacterial strains which may be used in fermentation processes for the production of

amino acids. The novel bacterial strains of the present invention have the following characteristics:

(i) the cells contain at least one *thr* operon, *i.e.*, at least one set of the genes encoding the threonine biosynthetic enzymes, on the chromosome under the control of a non-native promoter; and

(ii) the cells do not require any recombinant plasmids encoding the threonine biosynthetic enzymes in order to produce threonine.

Preferably, the inventive strains are capable of producing at least about 50 g/L of L-threonine in about 30 hours, more preferably at least about 70 g/L in about 30 hours,even more preferably at least about 80 g/L in about 30 hours, and most preferably about at least about 90 g/L in about 30 hours. Preferably, the inventive strains are capable of producing at least about 90 g/L in about 48 hours, more preferably at least about 100 g/L in about 48 hours, and most preferably at least about 110 g/L of threonine in about 48 hours.

Preferably, the inventive strains are capable of producing L-threonine at a rate of at least about 2 g/L/hr, more preferably at least about 2.5 g/L/hr, even more preferably at least about 3 g/L/hr, and most preferably at least about 3.6 g/L/hr.

In a particularly preferred embodiment, the novel bacterial strains also have no amino acid nutritional requirements for fermentative production of threonine, *i.e.*, the cells do not require amino acids supplements for growth and threonine production.

According to the present invention, the inventive bacterial strain does not require any recombinant plasmids containing one or more genes that encode threonine biosynthetic enzymes for threonine production, *i.e.*, the strain is capable of producing threonine without the need for one or more of the threonine biosynthetic enzymes to be encoded by genes contained in a recombinant plasmid. The inventive strains may, of course, optionally contain one or more recombinant plasmids as desired. For example, while such plasmids are not required for threonine production, the inventive strains may nevertheless contain recombinant plasmids that encode for threonine

biosynthetic enzymes in order to increase threonine production. The inventive strains may likewise contain recombinant plasmids encoding other enzymes involved in threonine biosynthesis, such as aspartate semialdehyde dehydrogenase (*asd*).

Preferably, the inventive bacterial strains are strains of *Escherichia coli*. More preferably, the inventive bacterial strains are strains of *E. coli* that exhibit resistance to the macrolide anitbiotic borrelidin. A particularly preferred example of the inventive bacterial strains is *E. coli* strain kat-13, which was deposited at the Agricultural Research Service Culture Collection (NRRL), 1815 North University Street, Peoria, Illinois 61604, USA, on June 28, 1996 and assigned accession number NRRL B-21593.

The threonine (*thr*) operon on the chromosome of the cells of the inventive bacterial strains encodes the enzymes necessary for threonine biosynthesis. Preferably, the threonine operon consists of an AK-HD gene (*thrA* or *metL*), a homoserine kinase gene (*thrB*), and a threonine synthase gene (*thrC*). More preferably, the *thr* operon consists of *thrA* (the AK I-HD I gene), *thrB* and *thrC*. Suitable *thr* operons may be obtained, for example, from *E. coli* strain ATCC 21277 and strain ATCC 21530. The *thr* operon from strain ATCC 21277 is particularly preferred. Multiple copies of the *thr* operon may be present on the chromosome.

Preferably, the *thr* operon contains at least one non-attenuated gene, *i.e.*, expression of the gene is not suppressed by the levels (extra- and/or intracellular) of one or more of the threonine biosynthetic enzymes and/or the products thereof (*e.g.* threonine and isoleucine). The inventive strain may also contain a *thr* operon that contains a defective *thr* attenuator (the regulatory region downstream of the transcription intitation site and upstream of the first structural gene) or a *thr* operon that lacks the *thr* attenuator altogether.

In a particularly preferred embodiment of the present invention, the *thr* operon encodes one or more feedback-resistant threonine biosynthetic enzymes, *i.e.*, the activity of the enzyme is not inhibited by the extra- and/or intracellular levels of the intermediates and products of threonine biosynthesis. Most

preferably, the *thr* operon contains a gene that encodes a feedback-resistant AK-HD, such as a feedback-resistant AK I-HD I. Use of a feedback-resistant AK-HD provides a higher level of enzymatic activity for threonine biosynthesis, even in the presence of the L-threonine being produced.

Expression of the threonine operon(s) in the inventive strains is controlled by a non-native promoter, *i.e.*, a promoter that does not control the expression of the *thr* operon in *E. coli* bacterial strains normally found in nature. Replacing the native promoter of the threonine biosynthetic enzymes with a strong non-native promoter to control the expression of the *thr* operon results in higher threonine production even with only a single, genomic copy of the *thr* operon. In addition, since a non-native promoter is used to control the expression of threonine operon, it is not necessary to render the bacterial strains auxotrophic for isoleucine to achieve this higher threonine production. Illustrative examples of suitable promoters include, but are not limited to: the *lac* promoter; the *trp* promoter; the P_L promoter of λ bacteriophage; the P_R promoter; the *lpp* promoter; and the *tac* promoter. Particularly preferred for use in the inventive bacterial strains is the *tac* promoter.

In addition to the threonine operon, the chromosome in the cells of the inventive bacterial strains preferably also contains at least one gene encoding aspartate semialdehyde dehydrogenase (*asd*). Most preferably, the chromosome in the cells of the present invention contains at least one *asd* gene, at least one *thrA* gene, at least one *thrB* gene and at least one *thrC* gene. The chromosome may, of course, contain multiple copies of one or more of these genes.

Threonine dehydrogenase (*tdh*) catalyzes the oxidation of L-threonine to α-amino-β-ketobutyrate. Accordingly, in an especially preferred embodiment, the chromosome of the inventive cells further contains at least one defective threonine dehydrogenase (*tdh*⁻) gene. The defective *tdh* gene may be a gene having a reduced level of expression of threonine dehydrogenase or a gene that encodes a threonine dehydrogenase mutant having reduced enzymatic activity relative to that of native threonine dehydrogenase. Preferably, the defective *tdh* gene employed in the inventive strain does not express threonine

dehydrogenase. Illustrative examples of suitable *tdh* genes that do not express threonine dehydrogenase include a *tdh* gene having a chloramphenicol acetyltransferase (*cat*) gene inserted into it or a *tdh* gene having transposon Tn5 inserted into it, as described in U.S. Patent No. 5,175,107.

The bacterial strains of the present invention may be prepared by any of the methods and techniques known and available to those skilled in the art. Illustrative examples of suitable methods for constructing the inventive bacterial strains include mutagenesis using suitable agents such as NTG; gene integration techniques, mediated by transforming linear DNA fragments and homologous recombination; and transduction mediated by the bacteriophage P1. These methods are well known in the art and are described, for example, in J.H. Miller, *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1972); J.H. Miller, *A Short Course in Bacterial Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1992); M. Singer and P. Berg, *Genes & Genomes*, University Science Books, Mill Valley, California (1991); J. Sambrook, E.F. Fritsch and T. Maniatis, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989); P.B. Kaufman *et al.*, *Handbook of Molecular and Cellular Methods in Biology and Medicine*, CRC Press, Boca Raton, Florida (1995); *Methods in Plant Molecular Biology and Biotechnology*, B.R. Glick and J.E. Thompson, eds., CRC Press, Boca Raton, Florida (1993); and P.F. Smith-Keary, *Molecular Genetics of Escherichia coli*, The Guilford Press, New York, NY (1989).

In a particularly preferred embodiment of the invention, *E. coli* strain 472T23, which requires threonine for growth, may be converted to a threonine producer using P1-mediated transduction to introduce the threonine operon of *E. coli* strain ATCC 21277, which may be obtained from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, USA. This *thr* operon consists of a feedback resistant aspartate kinase-homoserine dehydrogenase gene (*thrA*), a homoserine kinase gene (*thrB*), and a threonine synthase gene (*thrC*).

To improve threonine production in the inventive strains, the defective threonine dehydrogenase gene from *E. coli* strain CGSC6945 (relevant genotype: *tdh-1::cat1212*; obtained from the *E. coli* Genetic Stock Center, 355 Osborne Memorial Laboratory, Department of Biology, Yale University, New Haven, Connecticut 06520-8104, USA) may be introduced by P1 transduction. The resulting threonine producer may be further improved by mutagenizing with NTG and/or selecting for borrelidin resistance.

Plasmids carrying an antibiotic resistance marker gene, such as *kan* (which encodes for kanomycin resistance), and a strong promoter, such as P_L or *tac*, preferably flanked by DNA upstream of *thrA* and a few hundred base pairs of the wild-type *thrA* gene (*i.e.*, not the whole *thrA* gene), may be constructed and used as a vehicle to deliver the desired DNA fragments into the chromosome. The fragment on the plasmid may be isolated by digestion with a suitable restriction enzyme and purified, and then introduced, through transformation or electroporation, into a strain to remove the control region of threonine operon and replace it by homologous recombination with the desired fragment, *i.e.*, an antibiotic resistance marker gene and a strong promoter at the beginning the *thrA* gene. This fragment may then be transferred into the borrelidin resistant strain by P1 transduction.

The isoleucine requirement of the strain of the preferred host, 472T23, may be eliminated, for example, by introducing a wild type allele of the marker through P1 transduction. Unwanted nutritional requirements of other hosts may be removed in a similar manner or according to other methods known and available to those skilled in the art.

A second embodiment of the present invention is directed to the use of the above-described bacterial strains in fermentation processes for the production of amino acids of the aspartate family. L-threonine, for example, is obtained by culturing the inventive bacterial strains in a synthetic or natural medium containing at least one carbon source, at least one nitrogen source and, as appropriate, inorganic salts, growth factors and the like.

Illustrative examples of suitable carbon sources include, but are not limited to: carbohydrates, such as glucose, fructose, sucrose, starch hydrolysate, cellulose hydrolysate and molasses; organic acids, such as acetic acid, propionic acid, formic acid, malic acid, citric acid, and fumaric acid; and alcohols, such as glycerol.

Illustrative examples of suitable nitrogen sources include, but are not limited to: ammonia, including ammonia gas and aqueous ammonia; ammonium salts of inorganic or organic acids, such as ammonium chloride, ammonium phosphate, ammonium sulfate and ammonium acetate; and other nitrogen-containing, including meat extract, peptone, corn steep liquor, casein hydrolysate, soybean cake hydrolysate and yeast extract.

After cultivation, the L-threonine that has accumulated in the culture broth can be separated according to any of the known methods, *e.g.*, by use of ion-exchange resins as described in U.S. Patent No. 5,342,766. This method involves first removing the microorganisms from the culture broth by centrifugation and then adjusting the pH of the broth to about 2 using hydrochloric acid. The acidified solution is subsequently passed through a strongly acidic cation exchange resin and the adsorbent eluted using dilute aqueous ammonia. The ammonia is removed by evaporation under vacuum, and the resulting solution is condensed. Addition of alcohol and subsequent cooling provides crystals of L-threonine.

Other amino acids of the aspartate family can be produced by methods similar to that described in detail above. Isoleucine, for example, can be prepared from the inventive bacterial strains containing, on the chromosome or on a plasmid, an amplified *ilvA* gene or *tdc* gene, both of which encode threonine deaminase, the first enzyme involved in the bioconversion of threonine to isoleucine. Amplification of this gene, for example, by use of a *ilvA* gene encoding a feedback-resistant enzyme, leads to increased biosynthesis of isoleucine.

Similarly, methionine can be prepared by microorganisms such as *E. coli* that contain at least one *met* operon on the chromosome, *i.e.* the *metL* gene

(which encodes AK II-HD II), the *metA* gene (homoserine succinyltransferase), the *metB* gene (cystathionine γ-synthase), the *metC* gene (cystathionine β-lyase) and the *metE* and *metH* genes (homocysteine methylase). These genes, including feedback-resistant variants thereof, and, optionally, a non-native promoter can be introduced into the chromosome of the host microorganism according to one or more of the general methods discussed above and/or known to those skilled in the art. Lysine can likewise be prepared by microorganisms that contain a gene encoding the lysine biosynthetic enzymes (preferably a feedback-resistant lysine biosynthetic enzyme encoded by *lysC* and/or *dapA*) and, optionally, a non-native promoter.

A third embodiment of the present invention is directed to the use of borrelidin-resistant bacterial strains in fermentation processes for the production of L-threonine. Preferably, the borrelidin-resistant strains are mutants of an *E. coli* strain. A particularly preferred embodiment of such a mutant is *E. coli* strain kat-13, which was deposited at the Agricultural Research Service Culture Collection (NRRL), 1815 North University Street, Peoria, Illinois 61604, USA, on June 28, 1996 and assigned accession number NRRL B-21593.

Borrelidin resistance may be determined by any of the accepted methods known to those skilled in the art. For example, borrelidin-resistant strains can be isolated by plating the candidate strains on minimal medium containing about 139 μM borrelidin, as described in G. Nass and J. Thomale, *FEBS Lett.* *39*:182-186 (1974). In addition, borrelidin resistance in certain strains is manifested as a change in one or more phenotypic characteristics of the cells. For example, borrelidin-resistant mutants of *E. coli* strain 6-8 and its derivatives appear round, rather than as rods. In such cases, evidence of a change in a phenotypic characteristic may be sufficient to adequately identify borrelidin-resistant strains.

The borrelidin-resistant mutants useful in this embodiment of the present invention are capable of producing threonine. The genes that encode the threonine biosynthetic enzymes may be present on the chromosome or contained in plasmids or mixtures thereof. Multiple copies of these genes may also be

present. Preferably, the genes that encode the threonine biosynthetic enzymes are resistant to attenuation control and/or encode feedback-resistant enzymes.

As noted above, the inventive borrelidin-resistant strains may contain one or more recombinant plasmids as desired. For example, the inventive microorganisms may contain recombinant plasmids that encode for threonine biosynthetic enzymes. The inventive bacterial strains may likewise contain recombinant plasmids encoding other enzymes involved in threonine biosynthesis, such as aspartate semialdehyde dehydrogenase (*asd*), or enzymes to augment growth.

Additionally, the borrelidin-resistant strains may be modified as desired, for example, in order to increase threonine production, remove nutritional requirements, and the like, using any of the methods and techniques known and avaiable to those skilled in the art. Illustrative examples of suitable methods for modifying borrelidin-resistant *E. coli* mutants and variants include, but are not limited to: mutagenesis by irradiatiton with ultraviolet light or X-rays, or by treatment with a chemical mutagen such as nitrosoguanidine (N-methyl-N'-nitro-N-nitrosoguanidine), methylmethanesulfonate, nitrogen mustard and the like; gene integration techniques, such as those mediated by transforming linear DNA fragments and homologous recombination; and transduction mediated by bacteriophages such as P1. These methods are well known in the art and are described, for example, in J.H. Miller, *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1972); J.H. Miller, *A Short Course in Bacterial Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1992); M. Singer and P. Berg, *Genes & Genomes*, University Science Books, Mill Valley, California (1991); J. Sambrook, E.F. Fritsch and T. Maniatis, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989); P.B. Kaufman *et al.*, *Handbook of Molecular and Cellular Methods in Biology and Medicine*, CRC Press, Boca Raton, Florida (1995); *Methods in Plant Molecular Biology and Biotechnology*, B.R. Glick and J.E. Thompson, eds., CRC Press, Boca Raton, Florida (1993); and P.F. Smith-

Keary, *Molecular Genetics of Escherichia coli*, The Guilford Press, New York, NY (1989).

Preferably, the borrelidin-resistant mutants of the present invention are modified so as to include a non-native promoter upstream of and in operable link with one or more of the genes that encode the threonine biosynthetic enzymes, regardless of whether these genes are on the chromosome and/or contained in plasmids.

According to a particularly preferred mode of this embodiment of the present invention, L-threonine is obtained by culturing at least one borrelidin-resistant bacterial strain in a synthetic or natural medium containing at least one carbon source, at least one nitrogen source and, as appropriate, inorganic salts, growth factors and the like, as described above. Accumulated threonine can be recovered by any of the methods known to those skilled in the art.

The following examples are illustrative only and are not intended to limit the scope of the invention as defined by the appended claims. It will be apparent to those skilled in the art that various modifications and variations can be made in the methods of the present invention without departing from the spirit and scope of the invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided they come within the scope of the appended claims and their equivalents.

All patents and publications referred to herein are expressly incorporated by reference.

Example 1: Preparation of E. coli strain kat-13

A. Transfer of the threonine operon of E. coli strain ATCC 21277 into the chromosome of E. coli strain 472T23.

E. coli strain ATCC 21277 (U.S. Patent No. 3,580,810), available from the American Type Culture Collection,12301 Parklawn Drive, Rockville, Maryland 20852, USA, is amino-β-hydroxyvaleric acid (AHV) resistant but requires proline, thiamine, isoleucine, methionine to grow in a minimal medium. ATCC 21277 is reported to accumulate 6.20 g/L of threonine in a

fermentation process. The threonine operon of ATCC 21277 consists of an aspartate kinase I-homoserine dehydrogenase I gene (*thrA*) that encodes a feedback-resistant enzyme, a homoserine kinase gene (*thrB*), and a threonine synthase gene (*thrC*).

E. coli strain 472T23, which is deposited in the USSR Collection of Commercial Microorganisms at USSR Antibiotics Research Institute under Reg. No. BKIIM B-2307, is reported to require threonine and isoleucine to grow in a minimal medium which contains glucose, ammonia, vitamin B1, and mineral salts. This strain cannot produce threonine because it carries a defective *thrC* gene, an essential gene for threonine biosynthesis. The strain 472T23 also carries a defective threonine deaminase gene, *ilvA*, which codes for the first enzyme in isoleucine biosynthesis.

Bacteriophage P1 lysate was prepared by growing phage on ATCC 21277. Strain 472T23 was then infected with this P1 lysate, in which a small number of the phage particles carried the threonine operon of ATCC 21277. Following infection, bacteria synthesizing threonine were selected by spreading on minimal medium E [glucose 0.05 g/L; $MgSO_4 \cdot 7H_2O$ 0.2 g/L; citric acid$\cdot H_2O$ 2.0 g/L; K_2HPO_4 10.0 g/L; $NaHNH_4PO_4 \cdot 4H_2O$ 3.5 g/L; agar 15.0 g/L] agar plates supplemented with 0.25 g/L isoleucine. Several threonine prototrophic transductants, which carried the threonine operon of ATCC 21277, were now able to grow in a minimal plates supplemented only with isoleucine.

These transductants were screened by shake-flask fermentation for threonine production as described below in Example 2. One of them, G9, producing threonine, was selected for further strain development.

B. Transfer of a defective threonine dehydrogenase (tdh⁻) gene inserted with a chloramphenicol acetyltransferase (cat) gene into the chromosome of E. coli strain G9.

Strain CGSC6945, carrying a defective threonine dehydrogenase gene (*tdh*⁻), was obtained from the *E. coli* Genetic Stock Center, 355 Osborne Memorial Laboratory, Department of Biology, Yale University, New Haven, Connecticut 06520-8104, USA. The threonine dehydrogenase gene is defective

because inserted into it is the chloramphenicol acetyltransferase (*cat*) gene. To transfer this defective gene to G9, P1 phage were grown on CSCG6945, and the lysate was used to infect G9. Several chloramphenicol-resistant transductants of G9 were selected and screened for threonine production with shake-flask fermentation as described below in Example 2. One of them, G909, with a higher threonine titer than G9, was selected for further development.

C. Insertion of a non-native promoter into the chromosome of E. coli strain G909.

In order to deliver the *tac* promoter into the chromosome of G909, homologous recombination between a linear DNA fragment and the chromosome of an exonucleaseV minus strain (*recD*) was employed.

The linear DNA fragment contained 1.5 kb of the sequence upstream (5' end) of the threonine operon, a kanamycin resistant marker, the *tac* promoter sequence, and about 480 bp of the *thrA* gene. This fragment, which provided 5' end homology, a selection marker (kanamycin resistance), a strong and controllable promoter to the threonine operon (*tac*), and 3'end homology, respectively, was generated as follows.

The threonine operon of the wild type *E. coli* W3110 was cloned into the restriction enzyme SphI site of plasmid pUC19 by using the DNA of the lambda clone 676 from Dr. Yuji Kohara, Department of Molecular Biology, School of Science, Nagoya University, Chikusa-ku, Nagoya, Japan. The DNAs of lambda clone 676 and pUC19 were then digested with SphI. The pUC19 fragment was subsequently dephosphorylated with shrimp alkaline phosphatase (SAP) and agarose-gel purified. The 6.9 kb fragment of threonine operon from lambda clone was also purified. These two fragments were subsequently ligated by T4 DNA ligase to generate plasmid pAD103.

An upstream flanking region for homologous recombination and kanamycin resistance marker was then constructed. pAD103 was digested with restriction enzyme BstEII, XbaI and blunt-ended with klenow fragment treatment. The 1.5 kb fragment containing only the 5'end (upstream) of the threonine operon (but not the *thr* operon itself or its control region) was isolated

and ligated to the fragment of kanamycin resistance gene from pUC4K (Pharmacia), which was digested with restriction enzyme SalI and klenow fragment treated to fill-in the 3' overhangs to generate intermediate plasmid pAD106.

pAD103 was also digested with restriction enzyme TaqI and blunt-ended with klenow fragment treatment. The fragment containing the native ribosome binding site and about 480 bp of the coding sequence of the *thrA* gene was isolated and then ligated to a fragment of pKK233-3 (Pharmacia), which had been digested with restriction enzyme SmaI and dephosphorylated with SAP, to obtain plasmid pAD115, which contained the DNA sequence of the *tac* promoter, the ribosome binding sites and a few hundred bases of the *thrA* gene.

pAD115 was subsequently digested with restriction enzyme BamHI and 0.75 kb of the DNA fragment which contained the desired DNA sequences was isolated. pAD106 was also digested with BamHI and then dephosphorylated with SAP. The two fragments were then ligated to provide plasmid pAD123, which contained the DNA sequence upstream of the threonine operon, a kanamycin resistance marker gene, the *tac* promoter, and about 480 bp of the beginning of the *thrA* gene.

pAD123 was then digested with SpeI, BglI and the fragment containing the desired DNA sequences was isolated.

The exonuclease V minus strain (*recD*) was prepared by growing P1 phage on *E. coli* strain KW251 (relevant genotype: *argA81::Tn10*, *recD1014*; obtained from Pharmacia), which contains a *recD* gene with a cotransducible transposon Tn10 insertion in *argA*. The lysate which was prepared from the phage was then used to infect strain G9 and the tetracycline-resistant transductant G9T7 was isolated.

The DNA fragment from plasmid pAD123 was delivered to *E. coli* strain G9T7 by electroporation. A kanamycin-resistant strain of G9T7 was isolated and a P1 phage lysate was made by growing phage on this strain. The P1 phage lysate was then used to transduce G909. One of the kanamycin-

resistant transductants of G909, tac3, which showed a higher threonine titer in the presence of IPTG in shake-flask study, was isolated.

P1 phage lysate was subsequently prepared with strain tac3 and then used to infect strain 6-8 (described below). The kanamycin-resistant transductants were selected and one of them, strain 6-8tac3, which produced a even higher titer than tac3 in a shake-flask study, was isolated.

D. NTG mutagenesis and the isolation of borrelidin-resistant mutants from E. coli strains G909 and 6-8.

The cells of strain G909 were mutagenized by N-methyl-N'-nitro-N-nitrosoguanidine (NTG) treatment (50 mg/L, 30 min. at 36°C) using conventional methods. The resulting cells were then spread on minimal medium E agar plate containing 0.25 g/L of L-isoleucine and 0.1% v/v of borrelidin. After incubation for 3-5 days at 36°C, the large colonies that formed on the plate, which included strain 6-8, were selected for testing for borrelidin resistance and L-threonine production.

To test for borrelidin resistance, each strain was cultivated in 20 ml of the seed medium SM [32.5 g/L glucose; 1 g/L $MgSO_4 \cdot 7H_2O$; 24.36 g/L K_2HPO_4; 9.52 g/L KH_2PO_4; 5 g/L $(NH_4)_2SO_4$; 15 g/L yeast extract; pH 7.2] at 36°C for 17 hr with shaking. The cells were harvested and washed with minimal medium E. The cell suspension was then inoculated into a sterilized tube containing 3 ml of minimal medium E and 0, 0.1, 0.5, or 1 mM borrelidin. After 24 hr cultivation at 36°C with shaking, growth was determined by measuring the optical density at 660 nm. The results are shown below relative to growth in the absence of borrelidin.

borrelidin (mM)	G909	6-8
0	100.0	100.0
0.1	24.2	134.5
0.5	2.9	141.0
1	0.9	184.5

E. Removal of isoleucine requirement and lactose repressor gene (lacI).

By introducing the non-native *tac* promoter and a feedback-resistant *thrA* gene, expression of the *thr* operon (*thrA*, *thrB*, *thrC*) is no longer controlled by the attenuation mechanism. As a result, starvation for isoleucine and/or the presence of an *ilvA*⁻ auxotrophic marker is no longer required for threonine production.

Accordingly, the wild type *ilvA* marker was introduced by transduction into 6-8tac3 to fix the isoleucine requirement of the strain, *i.e.*, to eliminate the need for isoleucine-supplemented medium for cell growth. P1 phage lysate made from CGSC7334 (relevant genotype: *lacI42::Tn10*, *lacZU118*; obtained from the *E. coli* Genetic Stock Center, 355 Osborne Memorial Laboratory, Department of Biology, Yale University, New Haven, Connecticut 06520-8104, USA) was used to infect 6-8tac3 and transductants positive for isoleucine biosynthesis were selected. These transductants produced approximately the same amount of L-threonine as strain 6-8tac3 in a shake-flask study. One of these transductants, 6-8tac3ile+ was selected for further development.

Since the threonine operon of 6-8tac3ile is under the control of the *tac* promoter, isopropyl-β-D-thiogalactoside (IPTG) was necessary to induce the cells to fully express the *thr* operon. The use of IPTG to induce expression of the *thr* operon, however, is less preferred according to the methods of the present invention.

Accordingly, to eliminate this unnecessary regulatory hindrance, a defective *lac* repressor (*lacI*) gene is introduced by infecting 6-8tac3ile+ with P1 phage made from CGSC7334. The resultant transductants (6-8tac3lacI-) were tested for resistance to tetracycline and tetracycline-resistant colonies were selected.

Example 2: Shake-flask fermentation study of threonine production.

A comparison of threonine production among the various *E. coli* strains was determined by their performance in shake-flask fermentation. The strains

being tested were grown on LB agar medium [10 g/L of trypton, 5 g/L of extract, 15 g/L agar]. After 1 to 2 days of growth, the cells were suspended in 5 ml of seed medium [dextrose 32.5 g/L; K_2HPO_4 24.35 g/L; KH_2PO_4 9.5 g/L; yeast extract 15 g/L; $(NH_4)_2SO_4$ 5 g/L; $MgSO_4 \cdot 7H_2O$ 1 g/L] at pH 7.2. The seed was grown for 24 hours with a stirring speed of 250 rpm at 37°C. 15 ml of fermentation medium [dextrose 40 g/L; yeast extract 2 g/L; citric acid 2 g/L; $(NH_4)_2SO_4$ 25 g/L; $MgSO_4 \cdot 7H_2O$ 2.8 g/L; $CaCO_3$ 20 g/L; trace metal solution 2 mL] at pH 7.2 was then added to the seed and the fermentation process performed at 37°C with a stirring speed of 250 rpm. After cultivation, the amount of L-threonine that had accumulated in the culture broth was analyzed by HPLC (ISCO Model 2353 pump, Rainin Model RI-1 refractive index detector, and aminex Hp87-CA column).

The amount of L-threonine produced by each of the tested strains is presented below.

Strain	L-threonine produced (g/L)
G909	4.95
6-8	11.45
tac3	12.9 (induced by IPTG)
	10.6 (non-induced)
6-8 tac3 ile+	12.7 (induced by IPTG)
6-8 tac3 lacI-	13.9
kat 13	14.0

Example 3: Fermentation study

The *E. coli* strains of the present invention and their precursor strains were tested for L-threonine production by fermentation.

G909 was tested under the following conditions. 0.5L of aqueous culture medium containing 30g/L of tryptic soy broth and 5g/L of yeast extract in a 2L baffled shake flask was inoculated with 1.5 mL of G909 and incubated on shaker at 35°C and 200 rpm for 8.5 hours. 0.9 mL (0.03%) of the mature

inoculum was added to a glass fermentor containing 3.0L of the seed fermentor medium [10 g/L d.s. of corn steep liquor, 0.4 g/L of L-isoleucine, 2.5 g/L of KH_2PO_4, 2.0 g/L of $MgSO_4$·7H2O, 0.5 g/L of $(NH_4)_2SO_4$, 0.192 g/L of anhydrous citric acid, 0.03 g/L of $FeSO_4$·$7H_2O$, 0.021 g/L of $MnSO_4$·H_2O and 80g/L of dextrose]. Incubation was conducted under the following conditions: a temperature of 39°C for the first 18 hours, and then 37°C for the duration; pH of 6.9 (maintained by addition of NH_4OH); air flow of 3.5LPM; agitation of 500 rpm initially, which was then increased to maintain the DO at 20%; and back pressure of 1-2 psi. Completion of the seed fermentor stage was determined by depletion of dextrose. 315 mL (15%) of the mature inoculum from the seed fermentor was added to a glass fermentor containing the same medium (main fermentor medium) listed above with the following exceptions: volume was 2.1L and 0.34 g/L of L-isoleucine was added. Incubation was conducted for 48 hours under the following conditions: temperature of 37°C; pH of 6.9 (maintained with NH_4OH); air flow of 3.5LPM until 20 hours then increased to 4.0LPM; agitation of 500 rpm initially, which was then increased to maintain the DO at 20%; back pressure of 1-2 psi; and dextrose level of 10 g/L (maintained by feeding with a 50% w/w dextrose solution). The fermentation was terminated after 48 hours. G909 produced the following results: a final titer of 62.3 g/L of threonine with a total productivity of 274 g and a yield of 23.2%.

tac3 was tested under the same conditions as G909 described above with the following exception: 1 mg/L of IPTG was added at the start of the main fermentor stage. With addition of IPTG, tac3 produced a final titer of 85.7g/L of threonine with a total productivity of 355g and a yield of 28.8%.

6-8 was tested under the same conditions as G909 described above. 6-8 produced the following results: a final titer of 74.1 g/L threonine with a total productivity of 290g and a yield of 28.3%.

6-8tac3 was tested under the same conditions as tac3 described above, including the addition of IPTG. 6-8tac3 produced the following results: a final

titer of 99.3 g/L threonine with a total productivity of 421g and a yield of 35.1%.

6-8tac3ile+ was tested under the same conditions as 6-8tac3 as described above, with the following exception: no L-isoleucine was required in either the seed fermentor stage or the main fermentor stage. Due to an agitation failure at 22.5 hours, only the titer at 22 hours was recorded (62 g/L threonine).

kat-13 was tested under the same conditions as 6-8tac3 as described above with the following exception: no IPTG was added. Under these conditions, kat-13 produced a final titer of 102 g/L threonine with a total productivity of 445 g and a yield of 33.1%.

The relevant genotypes of the constructed strains, supplements required for fermentative production of threonine, and the titers recorded are presented in the Table below:

Strain	Relevant Genotype	Supplements for production	titer at 30 hours	titer at 48 hours	Yield
G9	ilvA⁻,	Ile	ND	ND	ND
G909	ilvA⁻, tdh::Cm	Ile	53	62.3	23.2
tac3	ilvA⁻, tdh::Cm, *ptacthrABC*	Ile, IPTG	86	85.7	28.8
6-8	ilvA⁻, tdh::Cm, Bor-R	Ile	70	74.1	28.3
6-8tac3	ilvA⁻, tdh::Cm, *ptacthrABC*, Bor-R	Ile, IPTG	75	99.3	35.1
6-8tac3ile+	tdh::Cm, Bor-R, *ptacthrABC*	IPTG	62 (at 22 hours)	NA	NA
kat13	tdh::Cm, Bor-R, *ptacthrABC* lacI⁻	None	92.1	102	33.1

Bor-R: borrelidin Resistance

ND: Not done

NA: Not available

ptacthrABC: the *thrA*, *thrB* and *thrC* genes under control of the *tac* promoter

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 10, lines 8 - 11

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

Argricultural Research Culture Collection (NRRL)

Address of depositary institution *(including postal code and country)*

1815 North University Street
Peoria, Illinois 61604
United States of America

Date of deposit	Accession Number
June 28, 1996	NRRL B-21593

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

Escherichia coli strain kat-13

In respect of those designations in which a European Patent is sought a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☐ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

Form PCT/RO/134 (July 1992)

(Escherichia coli strain kat - 13)

SINGAPORE

The applicant hereby request that the furnishing of a sample of a microorganism shall only be made available to an expert. The request to this effect must be filed by the applicant with the International Bureau before the completion of the technical preparations for international publication of the application.

NORWAY

The applicant hereby request that, until the application has been laid open to public inspection (by the Norwegian Patent Office), or has been finally decided upon by the Norwegian Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the Norwegian Patent Office not later than at the time when the application is made available to the public under Sections 22 and 33(3) of the Norwegian Patents Act. If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the Norwegian Patent office or any person approved by the applicant in the individual case.

AUSTRALIA

The applicant hereby gives notice that the furnishing of a sample of a microorganism shall only be effected prior to the grant of a patent, or prior to the lapsing, refusal or withdrawal of the application, to a person who is a skilled addressee without an interest in the invention (Regulation 3.25(3) of the Australian Patents Regulations).

FINLAND

The applicant hereby requests that, until the application has been laid open to public inspection (by the National Board of Patents and Registration), or has been finally decided upon by the National Board of Patents and Registration without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art.

ICELAND

The applicant hereby request that, until the application has been laid open to public inspection (by the Icelandic Patent Office), or has been finally decided upon by the Icelandic Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art.

(Escherichia coli strain kat - 13)

DENMARK

The applicant hereby request that, until the application has been laid open to public inspection (by the Danish Patent Office), or has been finally decided upon by the Danish Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the Danish Patent Office not later than at the time when the application is made available to the public under Sections 22 and 33(3) of the Danish Patents Act. If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the Danish Patent office or any person approved by the applicant in the individual case.

SWEDEN

The applicant hereby request that, until the application has been laid open to public inspection (by the Swedish Patent Office), or has been finally decided upon by the Swedish Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the International Bureau before the expiration of 16 months from the priority date (preferably on the Form PCT/RO/134 reproduced in annex Z of Volume I of the PCT Applicant's Guide). If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the Swedish Patent office or any person approved by the applicant in the individual case.

UNITED KINGDOM

The applicant hereby request that the furnishing of a sample of a microorganism shall only be made available to an expert. The request to this effect must be filed by the applicant with the International Bureau before the completion of the technical preparations for international publication of the application.

NETHERLANDS

The applicant hereby request that until the date of a grant of a Netherlands patent or until the date on which the application is refused or withdrawn or lapsed, the microorganism shall be made available as provided in Rule 31F(1) of the Patent Rules only by the issue of a sample to an expert. The request to this effect must be furnished by the applicant with the Netherlands Industrial Property Office before the date on which the application is made available to the public under Section 22C or Section 25 of the Patents Act of the Kingdom of the Netherlands, whichever of the two dates occurs earlier.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 10, lines 8 - 11

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution
Argricultural Research Culture Collection (NRRL)

Address of depositary institution *(including postal code and country)*
1815 North University Street
Peoria, Illinois 61604
United States of America

Date of deposit	Accession Number
June 28, 1996	NRRL B-21593

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

Escherichia coli strain kat-13

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☐ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

Form PCT/RO/134 (July 1992)

What is claimed is:

1. A process for producing L-threonine, which comprises the steps of:

(a) culturing a strain of *E. coli* in a medium; and

(b) recovering L-threonine that is produced by said *E. coli*;

wherein said strain of *E. coli*:

(i) contains on the chromosome at least one threonine (*thr*) operon operably linked with at least one non-native promoter; and

(ii) does not require any recombinant plasmids containing one or more genes that encode one or more of the threonine biosynthetic enzymes in order to produce threonine.

2. The process of claim 1, wherein said *E. coli* is capable of producing at least about 50 g/L of L-threonine in about 30 hours.

3. The process of claim 1, wherein said non-native promoter is selected from the group consisting of the *tac* promoter, the *lac* promoter, the *trp* promoter, the *lpp* promoter, the P_L promoter and the P_R promoter.

4. The process of claim 1, wherein said threonine operon contains a gene that encodes a feedback-resistant aspartate kinase-homoserine dehydrogenase.

5. The process according to claim 1, wherein said *E. coli* is strain kat-13.

6. The process according to claim 3, wherein said non-native promoter is the *tac* promoter.

7. The process according to claim 1, wherein said *E. coli* contains a defective threonine dehydrogenase gene on the chromosome.

8. The process of claim 1, wherein said threonine operon is obtained from ATCC 21277.

9. The process of claim 1, wherein said *E. coli* is borrelidin resistant.

10. A method of producing an amino acid-producing strain of *E. coli* carrying no recombinant plasmids that encode one or more biosynthetic enzymes for said amino acid which comprises the steps of:

(a) introducing genetic material from an amino acid-producing microorganism into the chromosome of an *E. coli* strain;

(b) inserting a non-native promoter into said chromosome before the chromosomal location of and in operable link with the amino acid biosynthetic genes to control the expression thereof; and

(c) optionally, removing regulatory hindrances to and/or nutritional requirements for amino acid biosynthesis from said chromosome.

11. A strain of the microorgansim *E. coli* having the following characteristics:

(i) its chromosome contains at least one threonine (*thr*) operon operably linked with at least one non-native promoter; and

(ii) it does not require any recombinant plasmids containing one or more genes that encode one or more of the threonine biosynthetic enzymes in order to produce threonine.

12. The strain of claim 11, wherein said threonine operon consists of a feedback-resistant aspartate kinase I-homoserine dehydrogenase I gene (*thrA*), a homoserine kinase (*thrB*) gene, a threonine synthase gene (*thrC*).

13. The strain of claim 11, wherein said *E. coli* is strain kat-13.

14. The strain according to claim 11, wherein said non-native promoter is the *tac* promoter.

15. The strain of claim 11, wherein said *E. coli* contains a defective threonine dehydrogenase gene on the chromosome.

16. The strain of claim 11, wherein said threonine operon is obtained from ATCC 21277.

17. The strain of claim 11, wherein said *E. coli* is borrelidin resistant.

18. The strain of claim 11, wherein said *E. coli* has the characteristics of strain NRRL B-21593.

19. A process for producing L-threonine, which comprises the steps of:

(a) culturing a strain of borrelidin-resistant *E. coli* in a medium; and

(b) recovering L-threonine that is produced by said *E. coli*.

20. The process of claim 19, wherein said *E. coli* is capable of producing at least about 50 g/L of L-threonine in about 30 hours.

21. The process of claim 19, wherein said *E. coli* contains at least one threonine (*thr*) operon operably linked with at least one non-native promoter.

22. The process of claim 21, wherein said threonine operon contains a gene that encodes a feedback-resistant aspartate kinase-homoserine dehydrogenase.

23. The process according to claim 19, wherein said *E. coli* is strain kat-13.

24. The process according to claim 21, wherein said non-native promoter is the *tac* promoter.

FIGURE 1

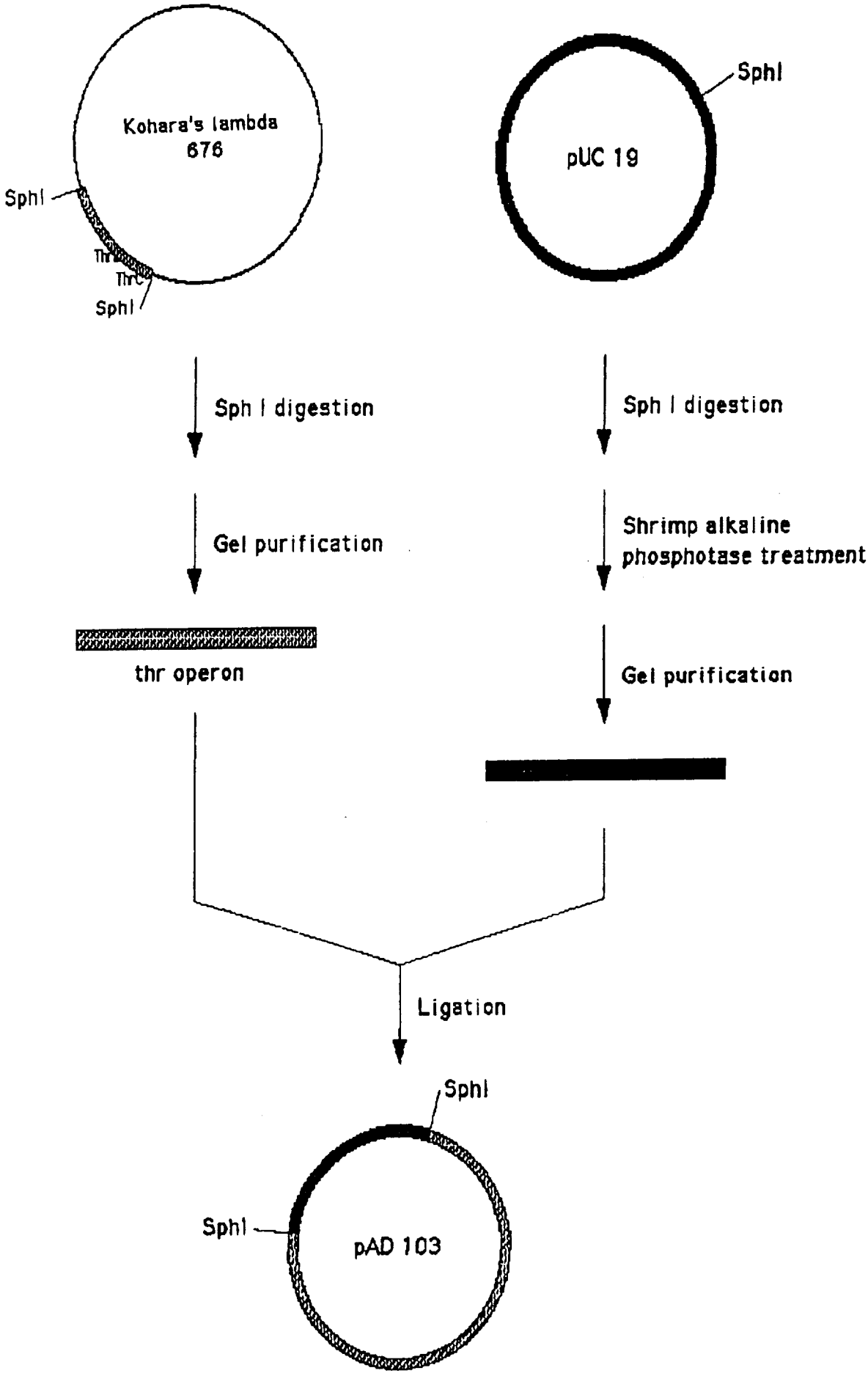

Kohara's lambda
676
SphI
Thr
Thr
SphI
pUC 19
SphI
Sph I digestion
Gel purification
thr operon
Sph I digestion
Shrimp alkaline
phosphotase treatment
Gel purification
Ligation
SphI
SphI
pAD 103

FIGURE 2

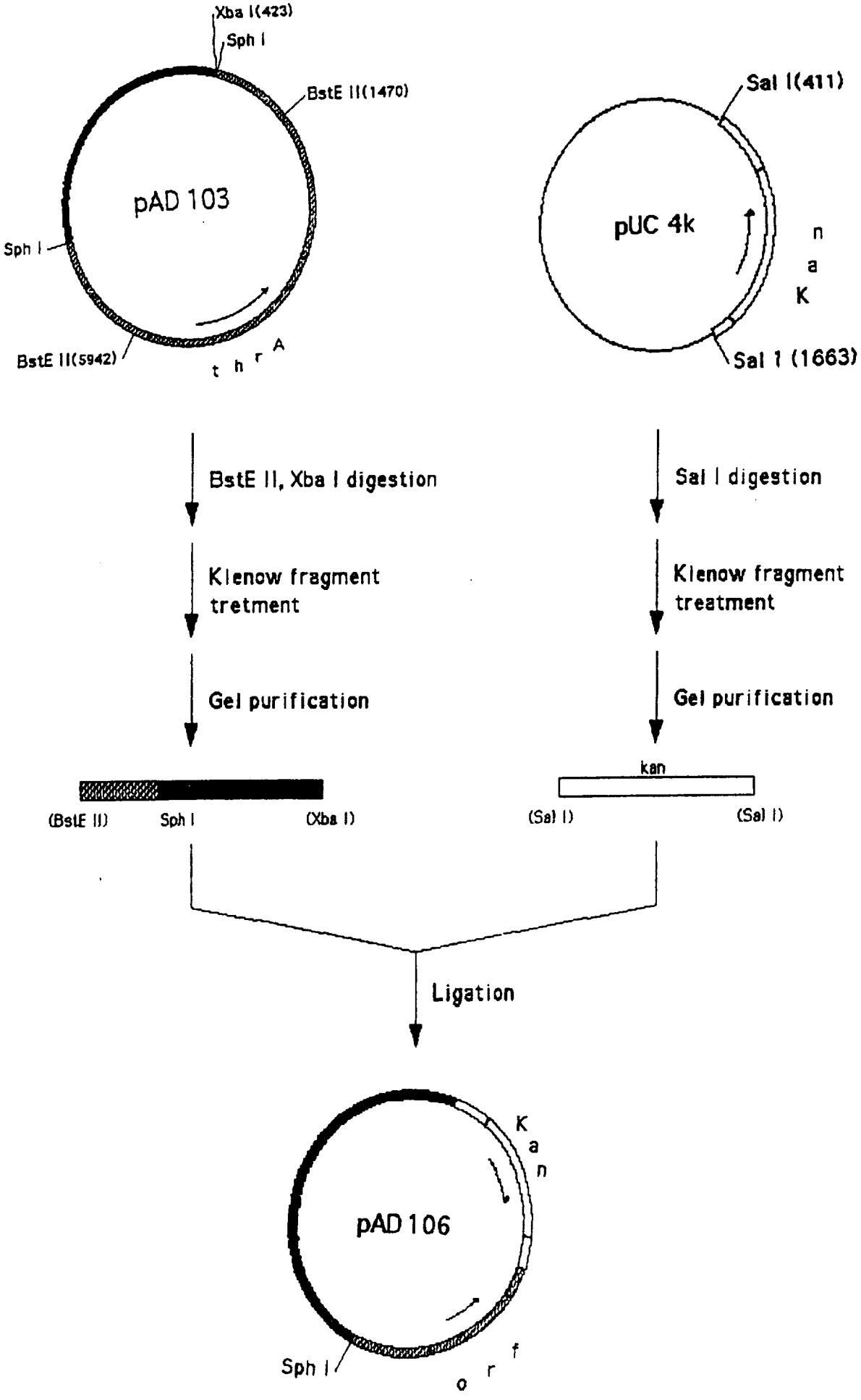

Xba I(423)
Sph I
BstE II(1470)
pAD 103
Sph I
BstE II(5942)
t h r A
Sal I(411)
pUC 4k
n
a
K
Sal I (1663)
BstE II, Xba I digestion
Sal I digestion
Klenow fragment tretment
Klenow fragment treatment
Gel purification
Gel purification
kan
(BstE II)
Sph I
(Xba I)
(Sal I)
(Sal I)
Ligation
K
a
n
pAD 106
Sph I
o r f

FIGURE 3

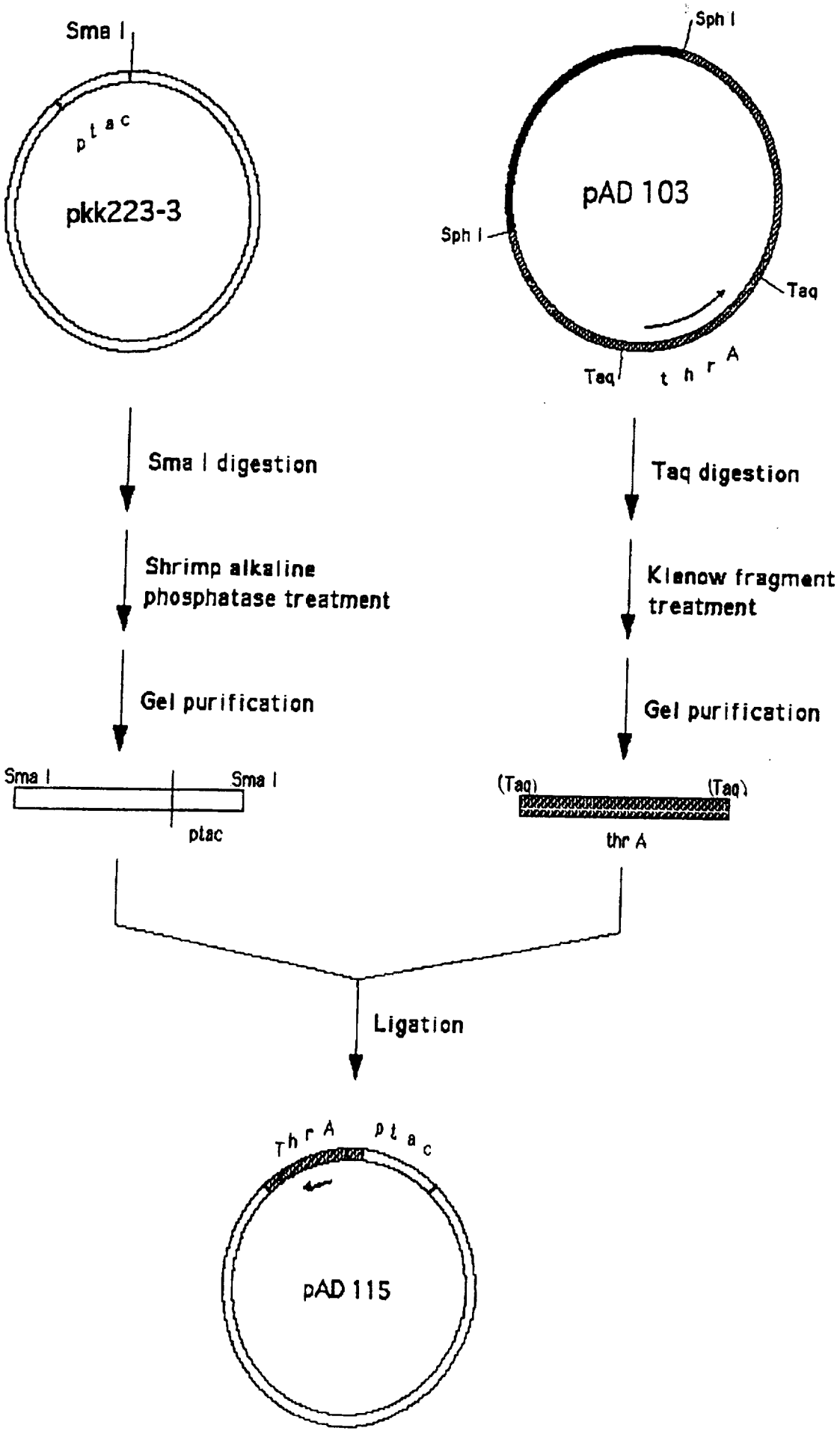

Sma I
ptac
pkk223-3
Sph I
pAD 103
Sph I
Taq
Taq
thr A
Sma I digestion
Shrimp alkaline phosphatase treatment
Gel purification
Taq digestion
Klenow fragment treatment
Gel purification
Sma I
Sma I
ptac
(Taq)
(Taq)
thr A
Ligation
Thr A
ptac
pAD 115

FIGURE 4

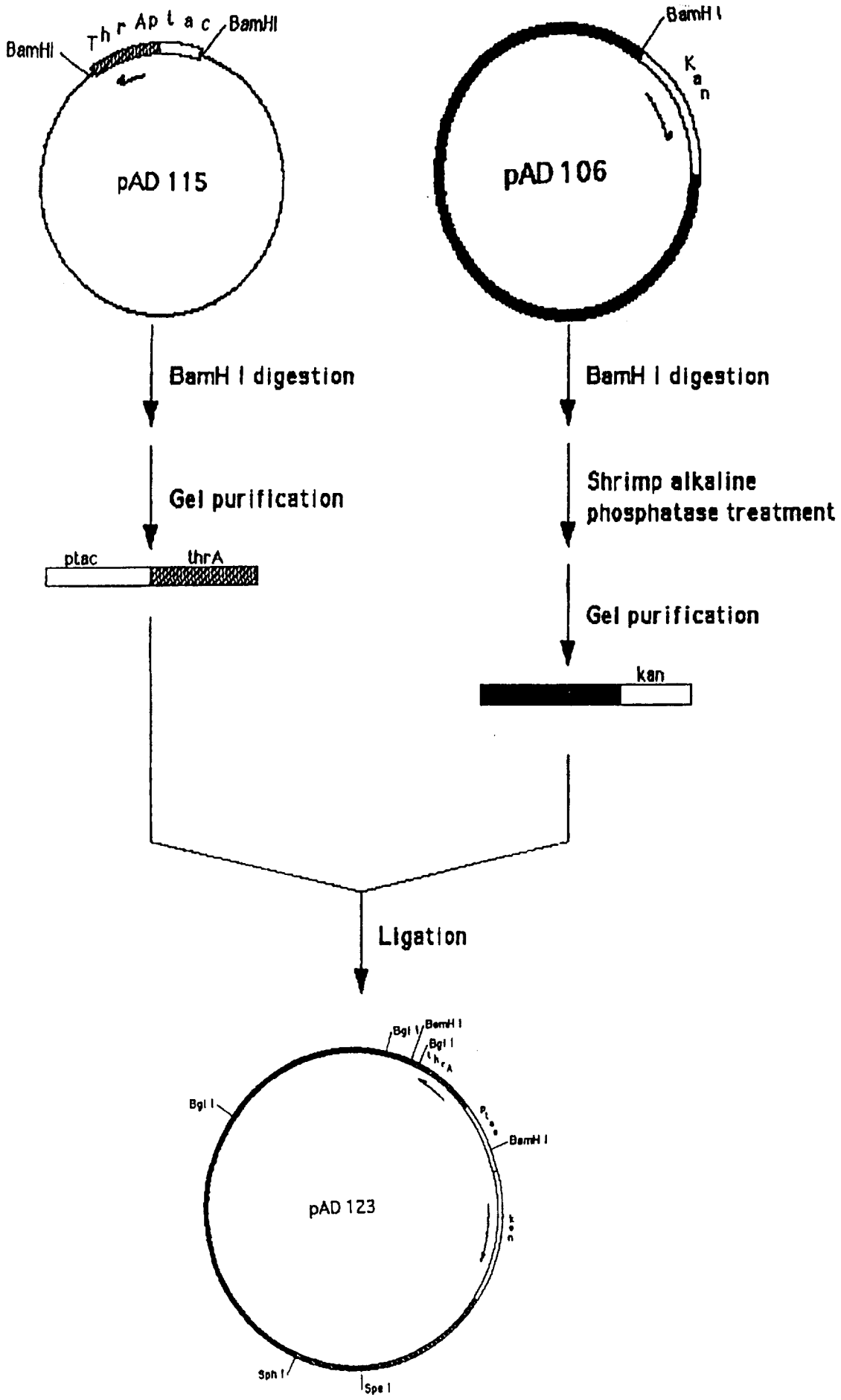
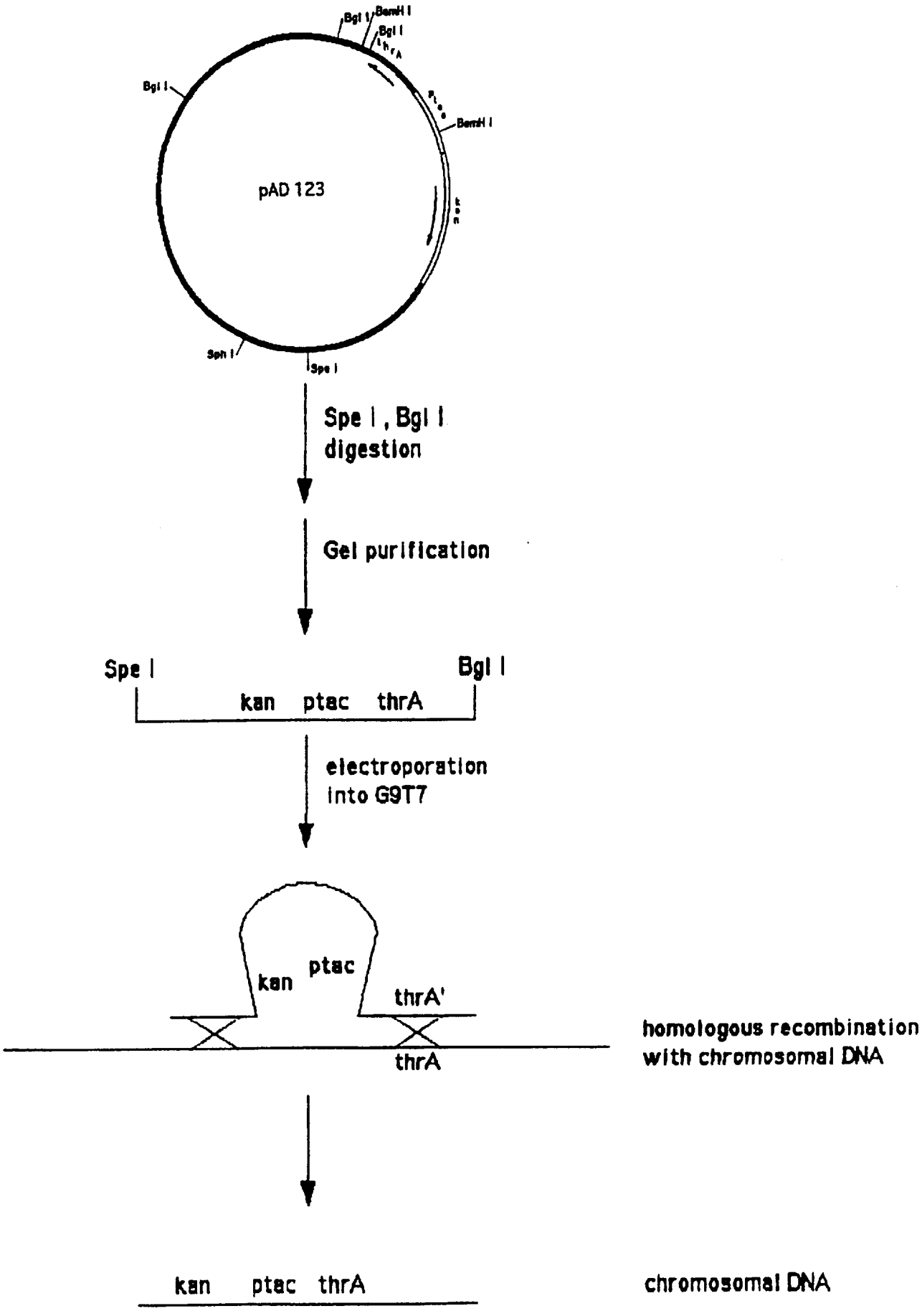

BamHI
Thr
Ap
lac
BamHI
pAD 115
BamH I
Kan
pAD 106
BamH I digestion
BamH I digestion
Gel purification
Shrimp alkaline phosphatase treatment
ptac
thrA
Gel purification
kan
Ligation
Bgl I
BamH I
Bgl I
thrA
Bgl I
Plac
BamH I
pAD 123
kan
Sph I
Spe I

FIGURE 5


Bgl I
BamH I
Bgl I
thrA
Bgl I
Ptac
BamH I
pAD 123
kan
Spe I
Spe I
Spe I , Bgl I
digestion
Gel purification
Spe I
Bgl I
kan ptac thrA
electroporation
into G9T7
kan
ptac
thrA'
thrA
homologous recombination
with chromosomal DNA
kan ptac thrA
chromosomal DNA

INTERNATIONAL SEARCH REPORT

Interna' al Application No
PCT/US 97/13359

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/52 C12P13/08 C12N15/70 C12N15/90 C12N1/21
//(C12N1/21,C12R1:19)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12P

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 593 792 A (AJINOMOTO CO., INC., JAPAN) 27 April 1994 see page 3, line 47 - page 4, line 2; figure 3; table 1 ---	1-4,7, 11,12, 15,20,21
X	EP 0 685 555 A (AJINOMOTO KK) 6 December 1995	10
Y	see abstract see page 7, line 45 - line 51 see claim 7 ---	1-4,7, 11,12,15
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

6

Date of the actual completion of the international search	Date of mailing of the international search report
18 December 1997	08.01.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Oderwald, H

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Interna' al Application No
PCT/US 97/13359

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MIZUKAMI T ET AL: "IMPROVEMENT OF THE STABILITY OF RECOMBINANT PLASMIDS CARRYING THE THREONINE OPERON IN AN L THREONINE-HYPERPRODUCING STRAIN OF ESCHERICHIA-COLI W." AGRIC BIOL CHEM 50 (4). 1986. 1019-1028. CODEN: ABCHA6 ISSN: 0002-1369, XP002047573	19
Y	see page 1019; figure 2 ---	20,21
X	DATABASE WPI Section Ch, Week 9550 Derwent Publications Ltd., London, GB; Class B05, AN 95-392096 XP002047688 & TW 258 753 A (DEV CENT BIOTECHNOLOGY) , 1 October 1995 see abstract ---	10
A	LITTLE S ET AL: "Translational coupling in the threonine operon of Escherichia coli K-12." JOURNAL OF BACTERIOLOGY, (1989 JUN) 171 (6) 3518-22. JOURNAL CODE: HH3. ISSN: 0021-9193., June 1989, XP002047574 see abstract; figure 2 ---	6,14,24
A	US 3 580 810 A (SHIIO ISAMU ET AL) 25 May 1971 see column 1, line 56 - line 60 see column 4, line 56 - line 57 -----	8,16

6

INTERNATIONAL SEARCH REPORT

Int ational application No.
PCT/US 97/ 13359

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

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

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

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

See annex

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

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

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

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

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-18

A method for producing an amino acid, L-threonine, by introducing one or more biosynthetic enzymes for that amino acid into the chromosome of E.coli and the strain obtained thereby.

2. Claims: 19-24

A process for producing L-threonine with a borrelidin-resistant E.coli.

INTERNATIONAL SEARCH REPORT

...rmation on patent family members

Interna' al Application No
PCT/US 97/13359

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0593792 A	27-04-94	DE 69219775 D	19-06-97
EP 0685555 A	06-12-95	CN 1117524 A	28-02-96
		JP 8047397 A	20-02-96
US 3580810 A	25-05-71	FR 1580549 A	05-09-69
		GB 1223470 A	24-02-71