60759

AUSTRALIA
PATENTS ACT 1990
NOTICE OF ENTITLEMENT

We, **Shionogi & Co., Ltd.** AND **Nippon Zeon Co., Ltd.**, the applicant/Nominated Person in respect of Application No. 40903/93 state the following:-

The Nominated Person is entitled to the grant of the patent because the Nominated Person derives title to the invention from the inventors by assignment.

The Nominated Person is entitled to claim priority from the application listed in the declaration under Article 8 of the PCT because the Nominated Person made the application listed in the declaration under Article 8 of the PCT, and because that application was the first application made in a Convention country in respect of the invention.

DATED this FOURTH day of MARCH 1994

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CAVE for and on behalf
of the applicant(s)

(DCC ref: 1641959)

AU9340903

(12) PATENT ABRIDGMENT (11) Document No. AU-B-40903/93
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 657859

(54) Title
FOWL MYCOPLASMA ANTIGEN, GENE THEREOF, RECOMBINANT VECTOR CONTAINING SAID GENE, AND VACCINE PREPARED BY UTILIZING THE SAME

(51)[5] International Patent Classification(s)
C12N 015/31 A61K 039/04 C07K 013/00

(21) Application No 40903/93 (22) Application Date 28.05.93

(87) PCT Publication Number WO93/24646

(30) Priority Data

(31) Number	(32) Date	(33) Country
4-138819	29.05.92	JP JAPAN

(43) Publication Date 30.12.93

(44) Publication Date of Accepted Application 23.03.95

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(57) Claim

1. A substantially pure protein capable of reacting with Mycoplasma gallisepticum immunized serum or Mycoplasma gallisepticum infected serum, having a molecular weight of about 40 kilodaltons encoded by DNA sequence derived from Mycoplasma gallisepticum and having restriction enzyme map shown in Fig. 1, or a protein functionally equivalent thereto.

2. A protein according to claim 1 which has an amino acid sequence shown by Sequence No. 1, or a protein functionally equivalent thereto.

6. A vaccine for Mycoplasma gallisepticum infection comprising as an effective ingredient a protein according to claim 1 or 2.

OPI DATE 30/12/93 APPLN. ID 40903/93
AOJP DATE 10/03/94 PCT NUMBER PCT/JP93/00715
AU9340903

(51) 国際特許分類5
C12P 21/02
A1

(11) 国際公開番号 WO 93/24646

(43) 国際公開日 1993年12月9日 (09.12.1993)

(21) 国際出願番号 PCT/JP93/00715
(22) 国際出願日 1993年5月28日 (28. 05. 93)

(30) 優先権データ
特願平4/138819 1992年5月29日 (29. 05. 92) JP

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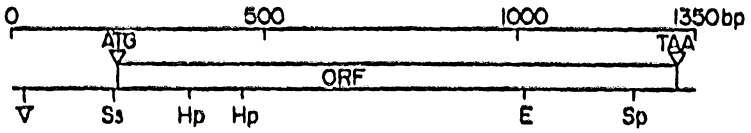
AT (欧州特許), AU, BE (欧州特許), CA, CH (欧州特許),
DE (欧州特許), DK (欧州特許), ES (欧州特許), FR (欧州特許),
GB (欧州特許), GR (欧州特許), IE (欧州特許), IT (欧州特許),
JP, KR, LU (欧州特許), MC (欧州特許), NL (欧州特許),
PT (欧州特許), SE (欧州特許), US.

添付公開書類 国際調査報告書

657859

(54) Title : FOWL MYCOPLASMA ANTIGEN, GENE THEREOF, RECOMBINANT VECTOR CONTAINING SAID GENE, AND VACCINE PREPARED BY UTILIZING THE SAME

(54) 発明の名称 家禽マイコプラズマ抗原、その遺伝子、その遺伝子を含む組み換えベクター、およびそれを利用したワクチン



Hp : HpaI
Ss : SspI
E : EcoRI
Sp : SpeI
V : EcoRV
ATG : 翻訳開始コドン a
TAA : 翻訳終止コドン b

a: ... initiator codon
b: ... terminator codon

(57) Abstract

A highly efficacious vaccine for infectious diseases caused by fowl *Mycoplasma gallisepticum*, prepared by utilizing a substantially pure protein which has a molecular weight of about 40 kDa, is coded for by a DNA sequence originating in *Mycoplasma gallisepticum* and having a specified restriction enzyme cleavage map, and can act on mycoplasma-immune or mycoplasma-infected serum, or other protein having equivalent functions.

(57) 要約

マイコプラズマ免疫血清またはマイコプラズマ感染血清に反応しうる実質的に純粋なタンパク質であって、特定の制限酵素切断点地図を有するマイコプラズマ・ガリセプティカム由来のＤＮＡ配列がコードする分子量約４０キロダルトンのタンパク質、またはそれと機能的に同等のタンパク質を利用した有効性の高い家禽マイコプラズマ・ガリセプティカム感染症用ワクチン。

DESCRIPTION

POULTRY MYCOPLASMA ANTIGENS, GENE THEREOF AND RECOMBINANT VECTORS CONTAINING THE GENE AS WELL AS VACCINES UTILIZING THE SAME

TECHNICAL FIELD

The present invention relates to antigen proteins of Mycoplasma gallisepticum infected to poultry;, recombinant vectors integrated with genes encoding to antigen proteins, hosts transformed by the vectors, as well as poultry vaccines for Mycoplasma gallisepticum infections utilizing the antigen proteins.

BACKGROUND

Mycoplasma gallisepticum infectious disease, that is one of the most serious infections on poultry such as chickens, etc., is characterized by chronic respiratory impairment accompanied by inflammation of the air sac in chicken. When chickens were infected with Mycoplasma gallisepticum, an egg-laying rate and a hatching rate of eggs produced by infected chickens are markedly reduced. As the result, shipping of eggs and egg-laying chickens decrease so that a considerable economic loss is caused. In addition, Mycoplasma gallisepticum infection induces the reduction in immunity so that chickens are liable to suffer from

other infectious diseases to cause complication of severe infectious diseases. Furthermore, Mycoplasma gallisepticum is known to be a pathogen of sinusitis in turkeys.

The present inventors already found proteins react with antisera against Mycoplasma gallisepticum (Japanese Patent Application Laid-Open No. 2-111795). It is expected that these proteins would be useful as vaccines for preventing Mycoplasma gallisepticum infections, but in order to prepare more potent vaccines, it is desired to provide proteins having a higher activity.

DISCLOSURE OF THE INVENTION

As a result of extensive investigations to obtain more effective vaccines for preventing Mycoplasma gallisepticum infections, the present inventors have selected TMG-1 from the proteins disclosed in Japanese Patent Application Laid-Open No. 2-111795 supra. It has then be found that addition of protein of about 11 kilodaltons to TMG-1 markedly increased the antigenicity of Mycoplasma gallisepticum, antisera induced using the addition product as antigen prevent the growth of Mycoplasma gallisepticum, and the protein described above can be expected to be useful as poultry vaccine for preventing Mycoplasma gallisepticum infections and also useful as diagnosis of Mycoplasma gallisepticum

infections for poultry use. The present invention has thus come to be accomplished.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a restriction enzyme cleavage map of DNA fragment which can be used for recombination in the present invention.

Fig. 2 illustratively shows the procedure for cloning TTM-1 DNA to M13 phage.

Fig. 3 illustratively shows the procedure for producing a site-specific mutant prepared using artificially synthesized oligonucleotide primer.

Fig. 4 illustratively shows the procedure for producing plasmid pMTTMIE which expresses protein TTMG-1 encoded by TTM-1'.

BEST MODE FOR PRACTICING THE INVENTION

According to a first aspect of the present invention, there is provided a protein which causes an antigen-antibody reaction with Mycoplasma gallisepticum poultry antisera and has a molecular weight of about 40 kilodaltons (hereinafter abbreviated as kd) encoded by DNA sequence having a restriction enzyme cleavage map shown in Fig. 1. According to a second aspect of the present invention, there is provided a DNA sequence which encodes the amino acid sequence. According to a third aspect of the present invention, there is provided a recombinant vector containing the DNA and a host

transformed or transfected by the recombinant vector. According to a fourth aspect of the present invention, there is provided a poultry vaccine for preventing Mycoplasma gallisepticum infections, comprising the said protein as an effective component.

That is, in the first aspect of the present invention, the protein is the one that causes an antigen-antibody reaction with sera immunized or infected with Mycoplasma gallisepticum and has a molecular weight of about 40 Kd encoded by DNA sequence having a restriction enzyme cleavage map shown in Fig. 1. Specific examples include a protein having an amino acid sequence shown in Sequence No. 1, a fused protein having a C-terminus the amino acid sequence and containing bacteria-derived enzyme proteins such as β-galactosidase, β-lactamase, etc. at the N-terminus thereof.

The protein can be obtained by using the host transformed by or transfected by the recombinant vector that is concerned with the third aspect of the invention. The recombinant vector described above can be obtained by incorporating the DNA fragment as the third aspect of the invention into an expression vector in a conventional manner.

Sources for collecting the DNA fragment may be any of the sources so long as they belong to Mycoplasma gallisepticum. Specific examples include S6 strain (ATCC 15302), PG31 (ATCC 19610) and the like. Specific

example of the DNA fragment used for recombination is a DNA fragment having a restriction enzyme cleavage map shown in Fig. 1 (for example, DNA fragment shown in Fig. 2.

The nucleotide sequence of 202 to 988 in the fragment having DNA sequence shown by Sequence No. 1 is the same as that of protein TMG-1 described in Japanese Patent Application Laid-Open No. 2-111795. The nucleotides of 986 to 988 which correspond to a termination codon of the gene encoding this TMG-1 are modified so as not to be translated as termination codon in the host, and DNA sequence of 999 to 1387 is further added thereto. TGA of 1048 to 1050 is also modified so as not to be translated as termination codon.

NNN in DNA sequence is not particularly restricted unless it is not a termination codon upon expression. However, it is expected in natural Mycoplasma gallisepticum that TGA would be translated into tryptophan (J. Bacteriology, 172(1), 504-506 (1990)). It is thus preferred to modify NNN into a base translated as tryptophan also in host cells, for example, into TGG.

The vector which is used to construct the recombinant vector is not particularly limited but specific examples include plasmids such as pUC8, pUC9, pUC10, pUC11, pUC18, pUC19, pBR322, pBR325, pBR327, pDR540, pDR720, and the like; phages such as λgt11, λgt10, λEMBL3, λEMBL4, Charon 4A and the like.

The method for inserting the DNA fragment described above into these vectors to produce recombinant vectors may be performed in a manner well known to one skilled in the art. For example, the vector is cleaved with a restriction enzyme and ligated directly with the DNA fragments described above, under control of a suitable expression regulatory sequence. As the expression regulatory sequence used, those may be mentioned, for example lac promoter-operator, trp promoter, tac promoter, lpp promoter, PL promoter, amyE promoter, Gal7 promoter, PGK promoter, ADH promoter, etc.

In producing the recombinant vector for the purpose of expressing these proteins derived from Mycoplasma, techniques for producing a recombinant vector by once incorporating the aforesaid DNA fragment into a suitable vector followed by subcloning is well known to one skilled in the art. These subcloned DNA fragment are excised with an appropriate restriction enzyme and ligated with the expression regulatory sequence described above to produce, the recombinant vector capable of producing the protein.

The vector which is used for the subcloning is not critical but specific examples include plasmids such as pUC8, pUC9, pUC10, pUC11, pUC18, pUC19, pBR322, pBR325, pBR327, PDR540, pDR720, pUB110, pIJ702, YEp13, YEp24, YCp19, pAc373, pAcYM1, and the like.

Then, a variety of appropriate hosts are transformed using the obtained recombinant vector to obtain microorganisms that can produce the protein having antigenicity derived from Mycoplasma gallisepticum, or a fused protein containing the same amino acid sequence.

The appropriate host used herein can be chosen taking into account adaptability to expression vector, stability of the products, etc. Specific examples are genus Escherichia (for example, Escherichia coli), genus Bacillus (for example Bacillus subtilis, Bacillus sphaericus, etc.), Actinomyces, Saccharomyces, insect cell, silkworms, etc. The host transformed by an appropriate expression vector can be cultured and proliferated under suitable conditions well known to one skilled in the art.

Upon production of the protein, conditions for inducing the action of expression regulatory sequence can be chosen. More specifically, in the case of lac promoter-operator, such conditions can be effected by adding a suitable quantity of isopropylthio-β-D-galactopyranoside to a culture broth.

The poultry vaccine for Mycoplasma gallisepticum infections from the thus obtained host which is concerned with the fourth aspect of the invention can be prepared by a modification of conventional technique. The host can be cultured under conditions generally used for culturing microorganisms

of this type. In the case of E. coli, the bacteria can be cultured in LB medium at 37°C under aerobic conditions.

After culturing, the protein of the present invention as its first aspect can be purified by means of chromatography, precipitation by salting out, density gradient centrifugation and the like that are well known to one skilled in the art and may optionally be chosen. The thus obtained protein can be used as a vaccine. Alternatively, the transformed host can be inactivated and the inactivated host can be used as vaccine. In this case, the inactivation is carried out in a conventional manner after culture of the host is completed. The inactivation may be attained by heating but it is simpler to add an inactivator to the culture broth. As the inactivator, there may be used Merzonin, ß-propiolactone, tyrosine, salicylic acid, Crystal Violet, benzoic acid, benzetonium chloride, polymyxin, gramicidin, formalin, phenol, etc. The inactivated culture broth is added, if necessary and desired, with a suitable quantity of adjuvant. The inactivated product is then separated with a siphon or by means of centrifugation, etc. As the adjuvant, aluminum hydroxide gel, aluminum phosphate gel, calcium phosphate gel, alum, etc. are employed. The inactivated product thus separated is adjusted with phosphate buffered saline, etc. to a suitable concentration. If necessary and desire, an antiseptic is added to the product. Examples

of the antiseptic which can be used include Merzonin, β-propiolactone, tyrosine, salicylic acid, Crystal Violet, benzoic acid, benzetonium chloride, polymyxin, gramicidin, formalin, phenol, etc.

In order to further enhance the immune activity, adjuvant may also be added to the vaccine obtained. The adjuvant is generally used in a volume of 1 to 99 based on 100 volume of the vaccine.

When the vaccine is used, it may be mixed with diluents, thickeners, etc. in a conventional manner. The vaccine exhibits the effect in a dose of at least 1 µg antigenic protein mass per kg wt. The upper limit is not critical unless the dose shows acute toxicity. The dose can be determined opportunely, for example, under such conditions that the neutralizing antibody titer (log_{10}) is 1.0 to 2.0. No acute toxicity was appreciable in a dose of 5 mg antigenic protein mass per kg wt. to chicken.

The poultry vaccine for Mycoplasma gallinopticum infection obtained in the present invention is inoculated to poultry intramuscularly, subcutaneously or intracutaneously, etc. The vaccine may also be sprayed onto respiratory tract for immunization.

According to the present invention, the proteins having higher antigenicity than those obtained in the prior art can be provided efficiently. The

excellent peptides are effective as vaccines and poultry diagnostics for Mycoplasma gallisepticum infection.

[EXAMPLES]

[Example 1]

Harvest of polypeptide gene TTM-1 in which Mycoplasma gallisepticum is expressed:

(1) Production of genomic DNA of Mycoplasma gallisepticum

Mycoplasma gallisepticum S6 strain was cultured at 37°C for 3 to 5 days in liquid medium prepared by supplementing 20% horse serum, 5% yeast extract, 1% glucose and a trace amount of phenol red as a pH indicator in 100 ml of PPLO broth basal medium. As Mycoplasma gallisepticum proliferated, pH of the culture broth decreased. At the point of time when the color of the pH indicator contained in the culture broth changed from red to yellow, incubation was terminated. The culture medium was centrifuged at 8000G for 20 minutes to collect the cells. The cells were then suspended in 1/10 volume of PBS based on the volume of culture medium. The suspension was again centrifuged at 10,000 rpm for 20 minutes to collect the cells. The collected cells were resuspended in 2.7 ml of PBS and SDS was added thereto in a concentration of 1%. Furthermore 10 µg of RNase was added to the mixture. The mixture was incubated at 37°C for 30 minutes to cause lysis.

The lysate was extracted 3 times with an equal volume of phenol and then 3 times with ethyl ether. The extract was precipitated with ethanol to give 200 µg of genomic DNA of Mycoplasma gallisepticum.

(2) Genomic Southern hybridization of Mycoplasma gallisepticum using TM-1 gene as a probe

After 1 µg of Mycoplasma gallisepticum DNA obtained in (1) was digested with XbaI, the digestion product was subjected to 0.6% low melting point agarose gel electrophoresis. After the electrophoresis, the gel was immersed in an alkali denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 10 minutes to denature DNA and further immersed in a neutralizing solution (3 M sodium acetate, pH 5.5) for 10 minutes to neutralize. Following the neutralization, the DNA was transferred onto a nylon membrane in 6-fold SSC solution (0.7 M NaCl, 0.07 M sodium citrate, pH 7.5). After air drying, the membrane was heated at 80°C for 2 hours. 4-fold SET (0.6 M NaCl, 0.08 M Tris-HCl, 4 mM EDTA, pH 7.8), 10-fold Denhardt, 0.1% SDS, 0.1% $Na_4P_2O_7$, 50 µg/ml of denatured salmon sperm DNA and pUM-1 insert DNA (TM-1 gene: see Japanese Patent Application Laid-Open No. 2-111795) which had been labelled in a conventional manner were added to cause hybridization at 68°C for 14 hours. The nylon membrane was overlaid on an X ray film. Autoradiography revealed that hybridizat on occurred on a fragment of about 3.4 kbp.

(3) Cloning of XbaI-digested fragment of about 3.4 kbp into pUC-19 and colony hybridization

After 4 µg of Mycoplasma gallisepticum DNA obtained in Example 1 (1) was digested with restriction enzyme XbaI, the digestion product was subject to 0.6% low melting point agarose gel electrophoresis. After the electrophoresis, a fragment of about 3.4 kbp was recovered. The fragment was ligated by ligase with pUC-19 cleaved through digestion with XbaI and competent E. coli TG1 strain was transformed. The transformants were cultured at 37°C for 15 hours in LB agar medium containing 0.003% of 5-bromo-4-Chloro-3-indolyl-β-D-galactopyranoside, 0.03 mM of isopropylthio-β-D-galactopyranoside and 40 µg/ml of ampicillin. White colonies grown on the agar medium were transferred onto a nylon membrane followed by hybridization in a manner similar to (2, above. Autoradiography revealed that cloning was effected and, the plasmid was named pUTTM1.

(4) Determination of the entire nucleotide sequence of TTM-1

Sequence of insert DNA fragment was determined by the Dideoxy method of Sanger et at. {Proc. Natl. Acad. Sci., USA, 74, 5463 (1977)} using pUTTM-1 prepared in (3) above. The nucleotide sequence is shown by Sequence No. 1 (provided that NNN in the sequence is both TGA). It is reported that TGA codon is read as tryptophan in the genus Mycoplasma, not as translation termination codon. In view of the sequence, the

molecular weight of the protein encoded by TTM-1 was assumed to be about 40 kilodaltons.

[Example 2]

(1) Production of TTM-1' modified (TGA → TGG) not to read TTM-1-encoding protein TTMG-1 by TGA as translation termination codon

◎ 2-1 Cloning of TTM-1 DNA to M13 phage (Fig. 2)

pUTTM-1 of 1-(3) was digested with restriction enzymes SacI and EcoRI and the digestion product was then subjected to 0.8% low melting point agarose gel electrophoresis. A 1.1 kbp fragment containing the 5'-end of TTM-1 was recovered by treating with phenol-chloroform and precipitating with ethanol, followed by ligation with the fragment obtained by digestion of M13mp11 phage with SacI and EcoRI. The reaction solution was mixed at m.o.i. of 0.1 with a solution obtained by culturing E. coli TG1 at 37°C for 24 hours, adding IPTG thereto in a final concentration of 100 mM and further supplementing IPTG in X-gal concentration of 2%. The resulting mixture was inoculated on soft agar to solidify. Incubation was then performed at 37°C for 24 hours. Among the phage plaques formed, recombinant phage TTM-1N containing 1.1 kbp DNA of TTM-1 was collected from the phage, which color did not change to blue.

Likewise, pUTTM-1 was digested with EcoRI and EcoRV. After 0.8% low melting point agarose gel

electrophoresis, a 0.4 kbp fragment containing the 3'-end of TTM-1 was recovered from the gel. By treating with phenol-chloroform and precipitating with ethanol, the DNA fragment was recovered. M13mp10 phage was ligated with the fragment obtained by digestion with EcoRI and EcoRV using ligase. The reaction solution was treated as in the cloning of 1 kbp DNA. Recombinant phage TTM-1C containing 0.4 kbp DNA of TTM-1 was thus obtained.

(2) Preparation of single stranded DNA from each recombinant phage

The two recombinant phage obtained in (1) above were added at m.o.i. of 0.1, respectively, to E. coli TG1 proliferated at 37°C in 100 ml of 2 x YT medium. After shake culture at 37°C for 5 hours, centrifugation was performed at 5000G for 30 minutes to obtain the cell-free supernatant. 0.2-fold volume of polyethylene glycol/sodium chloride mixture (20% polyethylene glycol #6000, 2.5 M NaCl) was added to the supernatant. After settlement at 4°C for an hour, the mixture was centrifuged at 5000G for 20 minutes to recover the precipitates. The precipitates were dissolved in 500 -l of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). After extraction with phenol-chloroform, single stranded DNA of each recombinant phage was recovered by ethanol precipitation.

(3) Production of site-specific mutant using artificially synthesized oligonucleotide as a primer (Fig. 3)

When the thus obtained DNA is incorporated and expressed in E. coli as it is, the site corresponding to NNN of the nucleotides shown by Sequence No. 1 is recognized as termination codon since this portion is TGA. Thus, the sequences following the portion is not translated. Therefore, in order to modify nucleotide adenine corresponding to the third nucleotide of codon NNN to guanine, the following two oligonucleotides were synthesized to translate the TGA portion as methionine.

Sequence No. 2:

3'-TACGTTCTTCCTGGCAAACCTTACCACTACTT-5'

and,

Sequence No. 3:

3'-CTACAAAGAACCTAAATATCA-5'

The oligonucleotide shown by Sequence No. 2 is annealed to single stranded DNA of TTM-1N and the oligonucleotide shown by Sequence No.3 to single stranded DNA of TTM-1C to cause the desired mutation by the method of Frits Eckstein et al. (Nucleic Acid Research, 8749-8764, 1985). The thus obtained recombinant phages were named TTM-1N' and TTM-1C', respectively. DNAs of TTM-1N' and TTM-1C' phages

obtained were digested with restriction enzymes SacI-EcoRI and EcoRI-BglII, respectively. By 0.8% low melting point agarose gel electrophoresis, the fragments of 1.1 kbp and 0.4 kbp were extracted from the agarose gel and recovered by ethanol precipitation. On the other hand, plasmid pUTTM-1 was also digested with SacI-BglII. The 4.8 kbp fragment bearing vector was extracted by 0.8% low melting point agarose gel electrophoresis and recovered by ethanol precipitation. The thus obtained three fragments were ligated by ligase and competent E. coli TG1 strain was transformed to obtain plasmid pUTTM-1' bearing TTM-1' with mutation at the desired site thereof. Sequencing analysis was performed as in 1-(4). It was thus confirmed that the desired site underwent mutation.

The restriction enzyme map of the thus obtained gene derived from Mycoplasma gallisepticum is shown in Fig. 1.

[Example 3]

Production of expression plasmid pUTM1E of protein TTMG-1 encoded by TTM-1' (Fig. 4)

Digestion of plasmid pBMG6T (Japanese Patent Application Laid-Open No. 2-111795) with restriction enzyme BamHI was followed by a treatment with DNA polymerase I and then digestion with restriction enzyme AvaIII. After 0.8% low melting point agarose gel electrophoresis, DNA of about 5000 bp was recovered from

the gel. By treating with phenol-chloroform and precipitation with ethanol, a fragment containing tac promoter was recovered. On the other hand, plasmid pUTTM1 obtained in (3) was digested with restriction enzymes AvaIII and EcoRV. The digestion product was subjected to 0.8% low melting point agarose gel electrophoresis. DNA of about 600 bp was recovered from the gel and treated with phenol-chloroform. By ethanol precipitation, a fragment containing a part of TTM-1 DNA was recovered.

The two fragments were ligated using ligase and competent E. coli TG1 strain was transformed. The transformants were cultured at 37°C for 15 hours in LB agar medium containing ampicillin. The plasmid was extracted by the method of Birnboim & Doly [Nucleic Acid Research, 7, 1513 (1979)] to produce plasmid pTTM1E bearing tac promoter and TTM-1 DNA.

On the other hand, pBMG6T was digested with restriction enzyme BamHI. After 0.8% low melting point agarose gel electrophoresis, a fragment of about 700 bp containing transcription termination sequence was recovered by ethanol precipitation.

Lastly, pTTM1E was digested with restriction enzyme BglII followed by a treatment with phenol and chloroform. The fragment recovered by ethanol precipitation was ligated by ligase with the aforesaid fragment of about 700 bp containing the transcription termination

sequence. The desired plasmid was selected in a manner similar to pTTM1E and named pMTTM1E.

[Example 4]

Expression of polypeptide encoded by TTM1E

After *E. coli* TG1 strain transformed by pMTTM1E was cultured at 37°C for 12 hours in LB medium supplemented with 50 µl/ml of ampicillin, 1 ml of the culture broth was taken and added to 100 ml of LB medium containing 50 µg/ml of ampicillin followed by incubation at 37°C. Two hours later, 1 mM of isopropylthio-β-D-galactopyranoside was added in a concentration of 1 mM and incubation was continued at 37°C for further 12 hours. After the incubation, *E. coli* was centrifuged at 6000G for 10 minutes. After the cells were collected, the cells were subjected to 10% SDS-PAGE and electrophoresed at 50 mA for 2 hours. After the electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 thereby to detect a new band of about 40 kilodaltons, amounting to about 10% of the total cell protein. Since this molecular weight of the protein coincided with the estimated value, the protein having about 40 kilodaltons is identified to be the protein encoded by TTM-1 and named TTMG-1.

Furthermore, the gel thus applied on SDS-PAGE was transferred onto a nitrocellulose membrane for Western blotting [Towbin et al., Proc. Natl. Acad. Sci. USA, 76, 4350 (1979)] and as a primary antibody, chicken

serum immunized with Mycoplasma gallisepticum was used, whereby a band of about 40 kd stained with Coomassie Brilliant Blue R-250 was reacted. It was thus confirmed that TTMG-1 was derived from Mycoplasma gallisepticum.

[Example 5]

Purification of TTMG-1

After E. coli collected in Example 4 were suspended in 10 ml of Dulbecco's PBS, the suspension was treated with French press (manufactured by Otake Seisakusho: 1500 kgf/cm^2). Then, centrifugation was performed at 60000G for 30 minutes and the precipitates were recovered. After washing 3 times with KPB (10 mM potassium phosphate buffer solution, pH 7.0) supplemented with 1% NP-40, the precipitates were suspended in PBS containing 7.5 M urea. After centrifugation at 60000G for 30 minutes, the supernatant was recovered. The supernatant was fractionated by linear density gradient from 0M to 1M of NaCl concentration using QAE-TOYO PEARL COLUMN (manufactured by TOSO Co., Ltd.) which had been equilibrated with KPB having pH of 7.8 and containing 6M urea. The fraction containing TMG-1 was thus recovered at 0M of NaCl concentration. This fraction was further fractionated by linear density gradient from 0M to 1M of NaCl concentration using Red-TOYO PEARL COLUMN (manufactured by TOSO Co., Ltd.) which had been equilibrated with the same KPB as used for QAE-TOYO PEARL COLUMN. The fraction containing TMG-1 (about

200 µg) was thus recovered at 0.5M to 0.7M of NaCl concentration.

The thus obtained TTMG-1 was subjected to SDS-PAGE in a manner similar to Example 4. After staining with Brilliant Blue R-250, the purity was determined to be about 90% by TLC-scanner (TS-930: Shimadzu Seisakusho Ltd.).

From the culture broth of TG1, about 200 µg of TTMG-1 was purified.

[Example 6]

Growth inhibition of Mycoplasma gallisepticum

TTMG-1 obtained in Example 5 was dissolved in Dulbecco's PBS in a concentration of 200 µg/ml. After 1 ml of the solution was mixed with an equal volume of complete Freund adjuvant or aluminum hydroxide gel, the mixture was subcutaneously injected to chicken of 8 weeks age or older (line-M, SPF: Nihon Seibutsu Kagaku Kenkyusho) at the right thigh. Further 2 weeks after, 1 ml each of TTMG-1 described above was subcutaneously administered for the second immunization to chicken as in the first immunization. A week after, anti-TTMG-1 serum was collected from the heart of chicken.

On the other hand, Mycoplasma gallisepticum S6 strain inoculated by 10% on PPLO liquid medium (modified Chanock's medium). After incubation at 37°C for 3 days, the culture broth was passed through a membrane filter of 0.45 µm to remove the agglutinated cells. The

filtrate was diluted to a cell count of 10^3 CFU/ml with PPLO liquid medium, which was used for determination of the activity.

The cell solution was separately charged by 400 µl each in a sterilized polypropylene tube. To the cell solution was added 100 µl each of standard chicken serum, TMG-1 immunized serum (Japanese Patent Application Laid-Open No. 2-111795) and TTMG-1 immunized serum. By culturing at 37°C for 2 to 5 days, growth inhibition test was carried out.

On Days 0, 1, 2, 3 and 4 of the incubation, 10 µl each was collected from each culture broth for growth inhibition test of Mycoplasma gallisepticum. Each collected culture broth harvested was spread over a plate of PPLO agar medium followed by culturing at 37°C for 7 days. The cell count in the corresponding culture broth was deduced from the number of colonies formed. The results of cell count on Day 3 are shown in Table 1.

Table 1

Sample	Cell Count on Day 3 the number of cells
Standard chicken serum	1.3×10^8
Anti-TMG-1 chicken serum	3.4×10^6
Anti-TTMG-1 chicken serum	1.8×10^5

When the added sample was standard chicken serum or the culture broth supplemented with horse

serum, no difference was noted in the growth rate of Mycoplasma gallisepticum and the cell count reached the saturation on Day 3 of the incubation. In the culture broth supplemented with anti-TTMG-1 immunized chicken serum Mycoplasma gallisepticum immunized chicken serum or with Mycoplasma gallisepticum infected chicken serum, the growth of Mycoplasma gallisepticum was clearly inhibited on Day 3. The results indicate that TTMG-1 protein is an antigen which can induce the antibody capable of effectively inhibiting the growth of Mycoplasma gallisepticum.

[Example 7]

Effect of preventing infection of TTMG-1 immunized chicken with Mycoplasma gallisepticum

Mycoplasma gallisepticum KP-13 strain was cultured in PPLO liquid medium to reach a concentration of 1 x 10^6 CFU/ml. Two weeks after the second booster in the chicken immunized in Example 6, the cell solution was infranasaly inoculated in the nasal cavities by 0.5 ml each. Four days after the chicken was sacrificed and the infraorbital sinuses and the air sac were wiped with sterilized cotton applicators, respectively. The applicators were immersed in PPLO liquid medium (containing 1% penicillin and 0.05% thallium acetate), respectively, followed by stationary culture at 37°C for 168 hours. After stationary culture of further 20 µl in 2 ml PPLO medium (containing 1% penicillin and 0.05% thallium

acetate), the presence or absence of the bacteria was detected as in Example 6 to determine the effect of preventing infection.

The effect of preventing infection is shown in Table 2. The chicken inoculated with TTMG-1 of the present invention shows a marked effect of preventing infection as compared to non-immunized chicken, indicating that TTMG-1 of the present invention exhibits a remarkable vaccine effect.

Table 2

Immune Antigen	Recovery of *Mycoplasma gallinopticum*
	Infraorbital sinuses
TTMG-1	5/10
none	4/5

(1) General Information

(i) Applicants: USA

SAITO Shuji

OKAWA Setsuko

FUJISAWA Ayumi

IRITANI Yoshikazu

AOYAMA Shigemi

other than USA

NIPPON ZEON CO., LTD.

SHIONOGI & CO., LTD.

(ii) Title of the Invention:

POULTRY MYCOPLASMA ANTIGENS, GENE THEREOF AND RECOMBINANT VECTORS CONTAINING THE GENE AS WELL AS VACCINES UTILIZING THE SAME

(iii) Number of sequences: 3

(2) Information on Sequence No. 1

(i) Characteristic of Sequence

(A) Length of sequence: 1387 base pairs

(B) Type of sequence: nucleic acid

(C) Number of strand: double strand

(D) Topology: circular

(E) Kind of sequence: DNA

(xi) Indication of sequence: Sequence No. 1

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AAA AAC ATC AGA TTG TTA ATC TGA TAT CTT TGC TTA AAA AAA CAC AAA      48

ATC TTC TAA CAA AAT CCT AAA TAA ATA AGC CGT TAA ATT AAC TAA AAA      96

ATT AAA AAA ATG GTT TTT CTT ATC AAC CAA AAT TCT CTA GTA ATA AAC     144

GCT TAT TTA TTT TTA TTT TTA GTC ATC TTT TAA GAT ATA AAT ATA TCT     192

TAA TAT TCT ATG AAT AAG AAA AGA ATC ATC TTA AAG ACT ATT AGT TTG     240
          Met Asn Lys Lys Arg Ile Ile Leu Lys Thr Ile Ser Leu        13

TTA GGT ACA ACA TCC TTT CTT AGC ATT GGG ATT TCT AGC TGT ATG TCT     288
Leu Gly Thr Thr Ser Phe Leu Ser Ile Gly Ile Ser Ser Cys Met Ser     29
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Ile Thr Lys Lys Asp Ala Asn Pro Asn Asn Gly Gln Thr Gln Leu Gln    45

GCA GCG CGA ATG GAG TTA ACT GAT CTA ATC AAT GCT AAA GCA AGG ACA   384
Ala Ala Arg Met Glu Leu Thr Asp Leu Ile Asn Ala Lys Ala Arg Thr    61

TTA GCT TCA CTA CAA GAC TAT GCT AAG ATT GAA GCT AGT TTA TCA TCT   432
Leu Ala Ser Leu Gln Asp Tyr Ala Lys Ile Glu Ala Ser Leu Ser Ser    77

GCT TAT AGT GAA GCT GAA ACA GTT AAC AAT AAC CTT AAT GCA ACA CTA   480
Ala Tyr Ser Glu Ala Glu Thr Val Asn Asn Asn Leu Asn Ala Thr Leu    93

GAA CAA CTA AAA ATG GCT AAA ACT AAT TTA GAA TCA GCC ATC AAC CAA   528
Glu Gln Leu Lys Met Ala Lys Thr Asn Leu Glu Ser Ala Ile Asn Gln   109

GCT AAT ACG GAT AAA ACG ACT TTT GAT AAT GAA CAT CCA AAT TTA GTT   576
Ala Asn Thr Asp Lys Thr Thr Phe Asp Asn Glu His Pro Asn Leu Val   125

GAA GCA TAC AAA GCA CTA AAA AGC ACT TTA GAA CAA CGT GCT ACT AAC   624
Glu Ala Tyr Lys Ala Leu Lys Thr Thr Leu Glu Gln Arg Ala Thr Asn   141

CTT GAA GGT TTA GCT TCA ACT GCT TAT AAT CAG ATT CGT AAT AAT TTA   672
Leu Glu Gly Leu Ala Ser Thr Ala Tyr Asn Gln Ile Arg Asn Asn Leu   157

GTG GAT CTA TAC AAT AAT GCT AGT AGT TTA ATA ACT AAA ACA CTA GAT   720
Val Asp Leu Tyr Asn Asn Ala Ser Ser Leu Ile Thr Lys Thr Leu Asp   173

CCA CTA AAT GGG GGA ATG CTT TTA GAT TCT AAT GAG ATT ACT ACA GTT   768
Pro Leu Asn Gly Gly Met Leu Leu Asp Ser Asn Glu Ile Thr Thr Val   189
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TCA AAC TAC AGT TTT GTT GCT TTT AGT GCT GAT GTA ACA CCC GTC AAT     960
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TAT AAA TAT GCA AGA AGG ACC GTT NNN AAT GGT GAT GAA CCT TCA AGT    1008
Tyr Lys Tyr Ala Arg Arg Thr Val Xaa Asn Gly Asp Glu Pro Ser Ser    269

AGA ATT CTT GCA AAC ACG AAT AGT ATC ACA GAT GTT TCT NNN ATT TAT    1056
Arg Ile Leu Ala Asn Thr Asn Ser Ile Thr Asp Val Ser Xaa Ile Tyr    285

AGT TTA GCT GGA ACA AAC ACG AAG TAC CAA TTT AGT TTT AGC AAC TAT    1104
Ser Leu Ala Gly Thr Asn Thr Lys Tyr Gln Phe Ser Phe Ser Asn Tyr    301

GGT CCA TCA ACT GGT TAT TTA TAT TTC CCT TAT AAG TTG GTT AAA GCA    1152
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GTT CAA CAA GTT GAG TTT GCC ACT TCA ACT AGT GCA AAT AAT ACT ACA    1248
Val Gln Gln Val Glu Phe Ala Thr Ser Thr Ser Ala Asn Asn Thr Thr    349

GCT AAT CCA ACT CAG CAG TTG ATG AGA TTA AAG TTG CTA AAA TCG TTT    1296
Ala Asn Pro Thr Gln Gln Leu Met Arg Leu Lys Leu Leu Lys Ser Phe    365
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TAT CAG GTT TAA GAT TTG GCC AAA ACA CAA TCG AAT TAA GTG TTC CAA 1344
Tyr Gln Val *** 369

CGG GTG AAG GAA ATA TGA ATA AAG TTG CGC CAA TGA TTG GCA A 1387

(2) Information on Sequence No. 2

(i) Characteristic of Sequence

(A) Length of sequence: 32 base pairs

(B) Type of sequence: nucleic acid

(C) Number of strand: single strand

(D) Topology: linear

(xi) Indication of sequence: Sequence No. 2

TACGTTCTTCCTGGCAAACCTTACCACTACTT

(3) Information on Sequence No. 3

(i) Characteristic of Sequence

(A) Length of sequence: 21 base pairs

(B) Type of sequence: nucleic acid

(C) Number of strand: single strand

(D) Topology: linear

(xi) Indication of sequence: Sequence No. 3

CTACAAAGAACCTAAATATCA

WHAT IS CLAIMED IS:

1. A substantially pure protein capable of reacting with Mycoplasma gallisepticum immunized serum or Mycoplasma gallisepticum infected serum, having a molecular weight of about 40 kilodaltons encoded by DNA sequence derived from Mycoplasma gallisepticum and having restriction enzyme map shown in Fig. 1, or a protein functionally equivalent thereto.

2. A protein according to claim 1 which has an amino acid sequence shown by Sequence No. 1, or a protein functionally equivalent thereto.

3. DNA sequence encoding substantially the entire protein according to claim 2.

4. A recombinant vector in which DNA fragment according to claim 3 is incorporated.

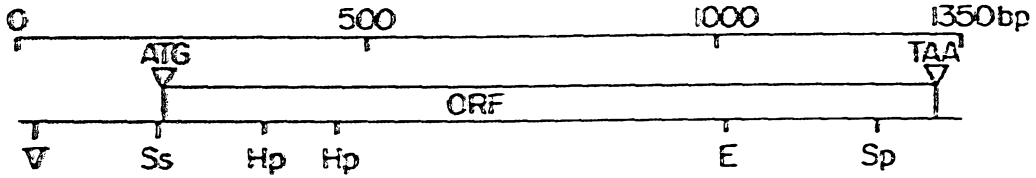
5. A host transformed by a recombinant vector according to claim 4.

6. A vaccine for Mycoplasma gallisepticum infection comprising as an effective ingredient a protein according to claim 1 or 2.

ABSTRACT

A highly effective vaccine for Mycoplasma gallisepticum infection utilizing a substantially pure protein capable of reacting with Mycoplasma gallisepticum immunized serum or Mycoplasma gallisepticum infected serum, having a molecular weight of about 40 kilodaltons encoded by DNA sequence derived from Mycoplasma gallisepticum and having a specific restriction enzyme map, or a protein functionally equivalent thereto.

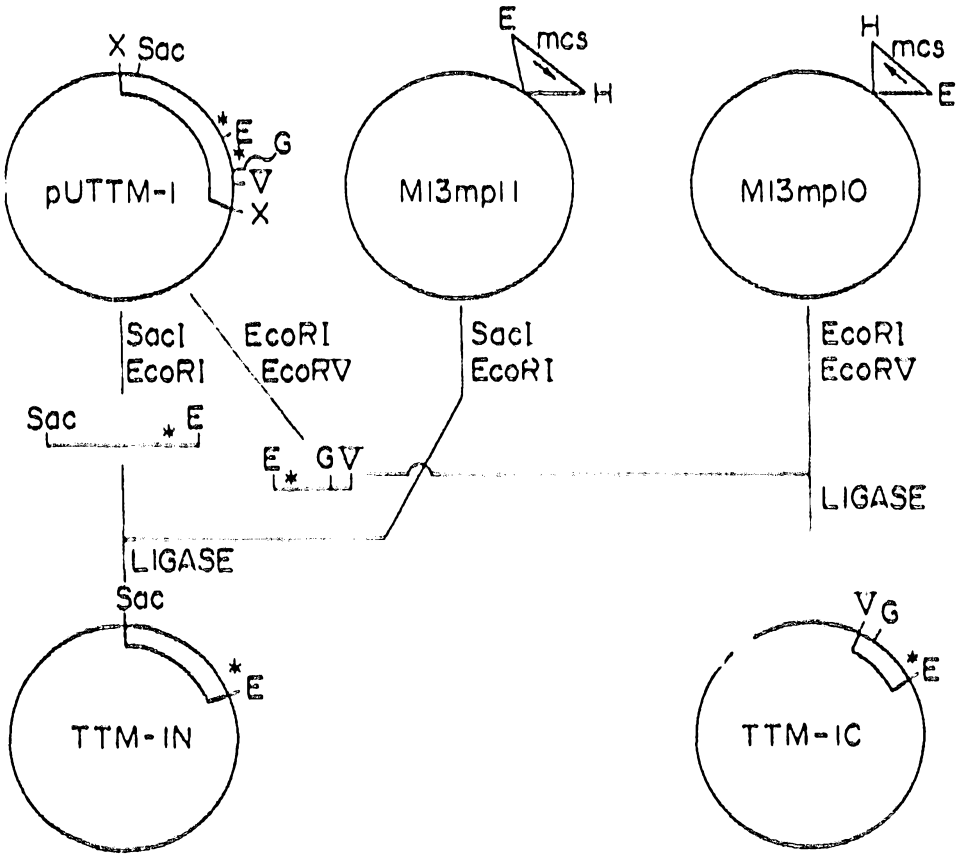
FIG. 1



100bp

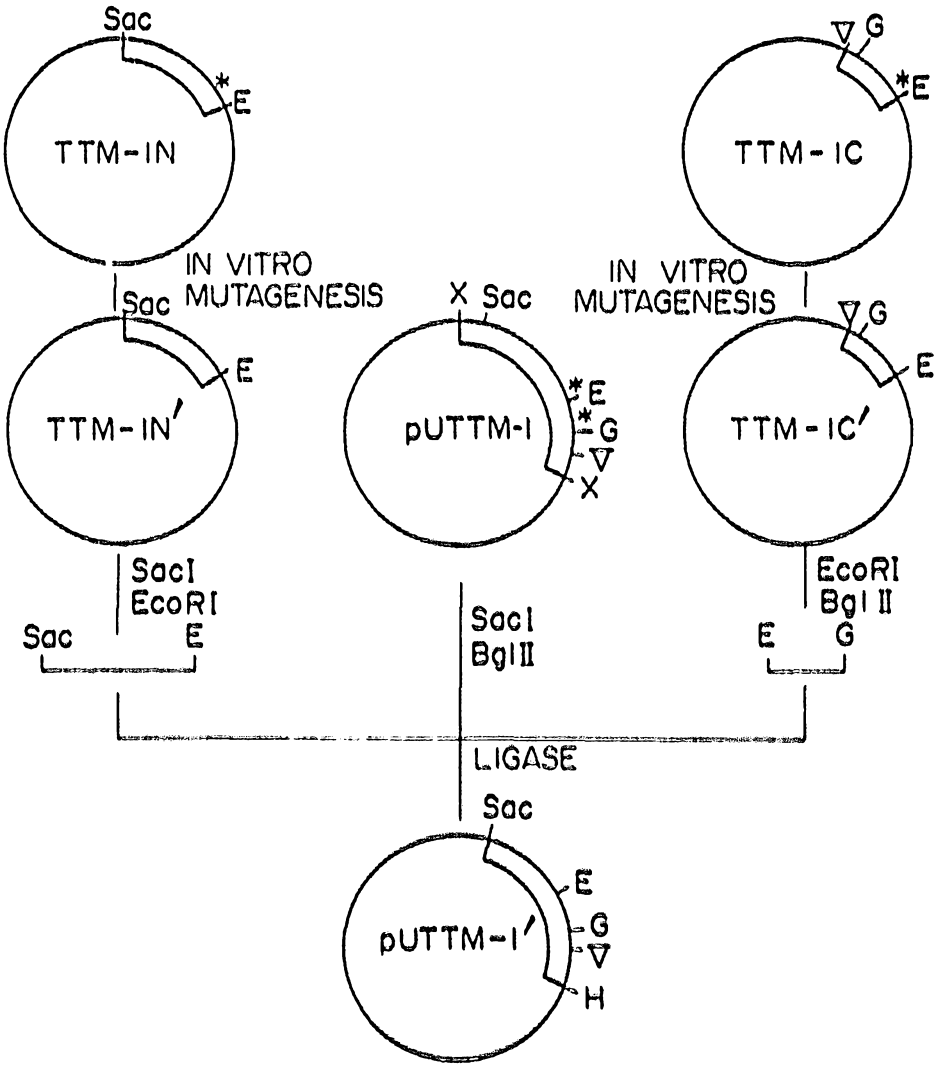
Hp HpaI
Ss SspI
E EcoRI
Sp SpeI
V EcoRV
ATG TRANSLATION INITIATION CODON
TAA TRANSLATION TERMINATION CODON

FIG. 2



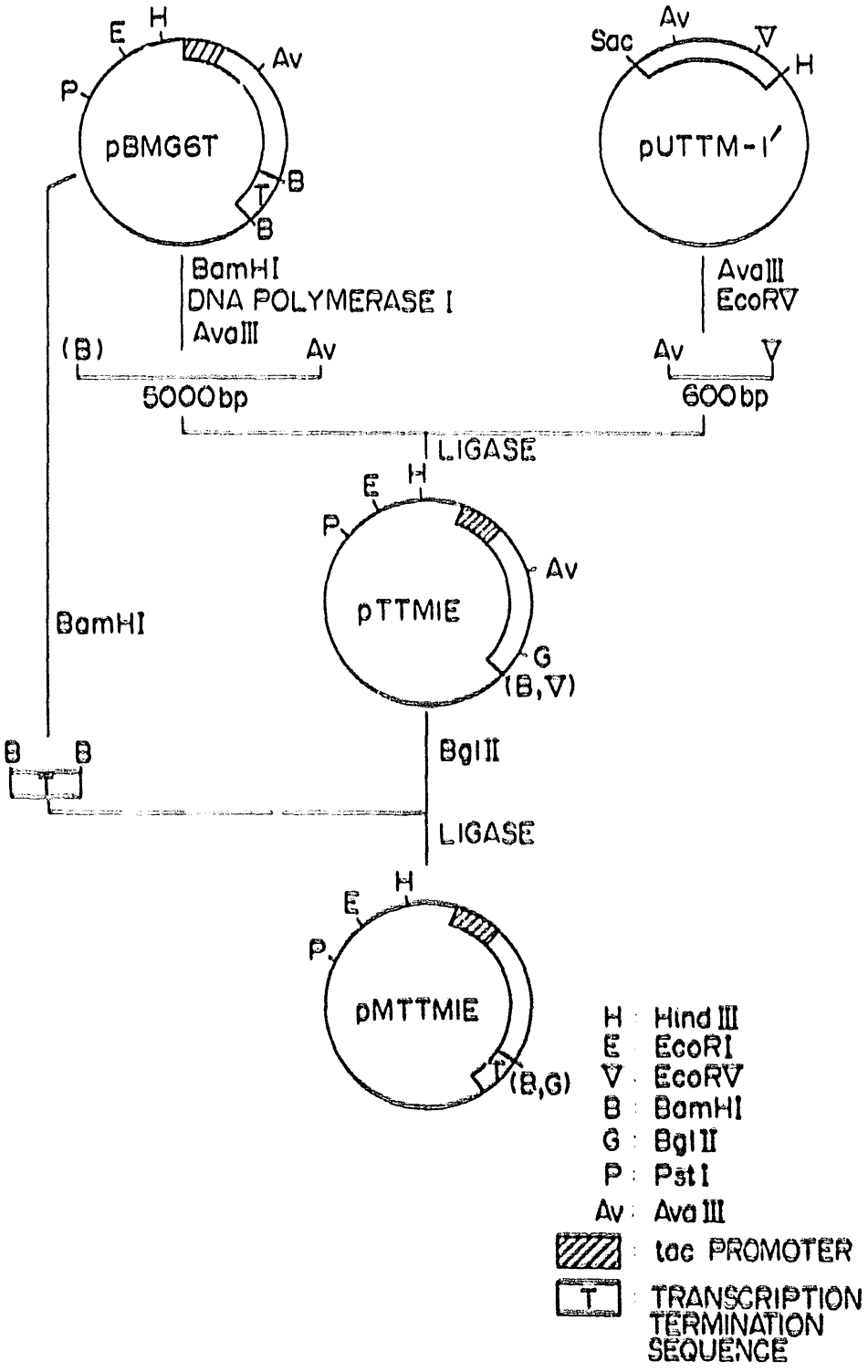
E : EcoRI
V : EcoRV
G : BglII
Sac : SacI
X : XbaI
Ss : SstI
Sp : SpeI
* SITE OF NUCLEOTIDE TO BE MUTATED

FIG. 3



E : EcoRI
∇ : EcoR∇
G : BglII
Sac : SacI
X : XbaI
Ss : SspI
Sp : SpeI
* SITE OF NUCLEOTIDE TO BE MUTATED

FIG. 4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H
Av
P
pBMG6T
T
B
B
BamHI
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AvaIII
(B)
Av
5000bp
Sac
Av
V
H
pUTTM-1'
AvaIII
EcoRV
Av
V
600bp
LIGASE
E
H
P
Av
pTTMIE
G
(B,V)
BamHI
B
B
BglII
LIGASE
E
H
P
pMTTMIE
V
(B,G)
H Hind III
E EcoRI
V EcoRV
B BamHI
G BglII
P PstI
Av Ava III
tac PROMOTER
T
TRANSCRIPTION
TERMINATION
SEQUENCE

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP93/00715

A. CLASSIFICATION OF SUBJECT MATTER
Int. Cl^5 C12P21/02

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)
Int. Cl^5 C12P21/00, C12N15/00

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 practicable, search terms used)
CAS. WPI ; mycoplasma

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP, A, 2-111795 (Nippon Zoon Co., Ltd.), April 24, 1990 (24. 04. 90), Fig. 2 (Family: none)	1-6
A	JP, A, 63-84484 (Shin Han Lii), April 15, 1988 (15. 04. 88), & EP, A, 260358 & US, A, 4666851	1-6
A	JP, A, 2-167079 (Nippon Flour Mills, Co., Ltd.), June 27, 1990 (27. 06. 90), Claim (Family: none)	1-6

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
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"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

Date of the actual completion of the international search	Date of mailing of the international search report
August 4, 1993 (04. 08. 93)	August 24, 1993 (24. 08. 93)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

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

国際調査報告

国際出願番号 PCT/JP 93/00715

A. 発明の属する分野の分類（国際特許分類（IPC））

Int. Cl⁵ C12P21/02

B. 調査を行った分野

調査を行った最小限資料（国際特許分類（IPC））

Int. Cl⁵ C12P21/00, C12N15/00

最小限資料以外の資料で調査を行った分野に含まれるもの

国際調査で使用した電子データベース（データベースの名称、調査に使用した用語）

CAS, WPI : mycoplasma

C. 関連すると認められる文献

引用文献のカテゴリー*	引用文献名 及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求の範囲の番号
A	JP, A, 2-111795（日本ゼオン株式会社） 24. 4月. 1990（24. 04. 90） 第2図（ファミリーなし）	1-6
A	JP, A, 63-84484（シン・ハン・リー） 15. 4月. 1988（15. 04. 88） &EP, A, 260358 &US, A, 4666851	1-6
A	JP, A, 2-167079（日本製粉株式会社）	1-6

☑ C欄の続きにも文献が列挙されている。

☐ パテントファミリーに関する別紙を参照。

* 引用文献のカテゴリー
「A」特に関連のある文献ではなく、一般的技術水準を示すもの
「E」先行文献ではあるが、国際出願日以後に公表されたもの
「L」優先権主張に疑義を提起する文献又は他の文献の発行日若しくは他の特別な理由を確立するために引用する文献（理由を付す）
「O」口頭による開示、使用、展示等に言及する文献
「P」国際出願日前で、かつ優先権の主張の基礎となる出願の日の後に公表された文献
「T」国際出願日又は優先日後に公表された文献であって出願と矛盾するものではなく、発明の原理又は理論の理解のために引用するもの
「X」特に関連のある文献であって、当該文献のみで発明の新規性又は進歩性がないと考えられるもの
「Y」特に関連のある文献であって、当該文献と他の1以上の文献との、当業者にとって自明である組合せによって進歩性がないと考えられるもの
「&」同一パテントファミリー文献

国際調査を完了した日	国際調査報告の発送日
04. 08. 93	24.08.93

名称及びあて先	特許庁審査官（権限のある職員）
日本国特許庁（ISA/JP） 郵便番号100 東京都千代田区霞が関三丁目4番3号	植野浩志 4B 9162 電話番号 03-3581-1101 内線 3449

様式PCT/ISA/210（第2ページ）（1992年7月）

C（続き）．　関連すると認められる文献

引用文献のカテゴリー*	引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求の範囲の番号
	27，6月，1990（27．06．90） 特許請求の範囲（ファミリーなし）	