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

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(56) Prior Art Documents
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(57) Fig. 1(a)-1 to 1(f)-2 shows amino acid sequences of polypeptides MG-1, MG-2, MG-3, MG-7, MG-8 and MG-9 exhibiting antigenicity of Mycoplasma gallisepticum and base sequences of M-1, M-2, M-3, M-7, M-8 and M-9 encoding the polypeptides. Fig. 2(a) and 2(b) similarly shows an amino acid sequence of polypeptide TMG-1 and a base sequence of TM-1 encoding the polypeptide. Fig. 3-1 to 3-4 indicate restriction enzyme cleavage maps of DNA fragments encoding the polypeptides in accordance with the present invention. Figs. 4, 5 and 6 indicate procedures for producing antigen protein plasmids in accordance with the present invention.

Claim

1. A polypeptide capable of reacting with anti-Mycoplasma gallisepticum poultry sera through an antigen-antibody reaction and having an amino acid sequence coded by a DNA fragment derived from Mycoplasma gallisepticum, said DNA having a restriction enzyme site map which corresponds to the map shown in any one of Figures 3(a) to 3(v).
2. A polypeptide as claimed in Claim 1, wherein said DNA has a base sequence which corresponds to one of the sequence shown in any one of Fig. 1(a) to Fig. 1(f).
3. A polypeptide as claimed in Claim 1 or Claim 2, wherein said amino acid sequence coded by said DNA fragment is one of the sequence shown in any one of Fig. 1(a) to Fig. 1(f).
4. A polypeptide as claimed in Claim 1, 2 or 3 wherein said amino acid sequence is the same sequence as that of polypeptide expressed in nature in Mycoplasma gallisepticum.
18. A poultry vaccine for Mycoplasma gallisepticum infection comprising a polypeptide as claimed in any one of claims 1 through 10 as an effective ingredient.
6. A polypeptide as claimed in claim 1, wherein said polypeptide is MG-1 whose amino acid sequence is described in Fig. 1(a)-1 and Fig. 1(a)-2, MG-2 whose amino acid sequence is described in Fig. 1(b), MG-3 whose amino acid sequence is described in Fig. 1(c), MG-4 whose amino acid sequence is described in Fig. 1(d), MG-5 whose amino acid sequence is described in Fig. 1(e), or MG-6 whose amino acid sequence is described in Fig. 1(f)-1 and Fig. 1(f)-2.

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COMPLETE SPECIFICATION

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COMPLETE SPECIFICATION FOR THE INVENTION ENTITLED:

Poultry mycoplasma antigens and recombinant vectors containing the gene as well as diagnostics and vaccines utilizing the same

The following statement is a full description of this invention, including the best method of performing it known to me/us:-

The present invention relates to antigen proteins of Mycoplasma gallisepticum that are infections
5 to poultry, genes encoding the antigen proteins, recombinant vectors integrated with the genes encoding the antigen proteins, hosts transformed by the vectors, as well as poultry diagnostics and vaccines for Mycoplasma gallisepticum infections utilizing the
10 antigen proteins produced by the hosts.

Mycoplasma gallisepticum infectious disease that is one of the most serious infections on poultry such as chickens, is characterized by chronic respiratory
15 disturbance accompanied by inflammation of the air sac in a chicken. A chicken infected with Mycoplasma gallisepticum as a pathogen develops very slight symptoms and rarely comes to death. However, when infected with Mycoplasma gallisepticum, an egg-laying
20 rate and a hatching rate of eggs produced by infected chickens are markedly reduced. As the result, shipping of eggs and egg-laying chickens are decreased, resulting in the considerable economic loss. In addition, Mycoplasma gallisepticum infection induces the reduction
25 in immunity so that chickens are liable to suffer from



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

5 Efforts have been hitherto made to prevent
poultry Mycoplasma gallisepticum infections using
antibiotics or by vaccination and to establish pathogen-
free poultry. However, administration of antibiotics
involves defects that bacilli resistant to Mycoplasma
10 would appear and Mycoplasma would readily proliferate
after discontinuation of administration of antibiotics.
On the other hand, prophylaxis by vaccination using
attenuated Mycoplasma might rather cause opportunistic
infections with other pathogens, or Mycoplasma
15 gallisepticum infections. As stated above, it is highly
difficult to establish and maintain a group of poultry
free of infections with Mycoplasma.

 Turning to determination whether or not
poultry is infected with Mycoplasma gallisepticum, it
20 has been judged by taking as a measure if serum collected
from poultry could inhibit proliferation of Mycoplasma
to form a characteristic inhibition zone on PPLO agar
plate including glucose and donor horse serum. However,
the method involves shortcomings that 3 to 7 days are
25 required to judge whether or not a clear inhibition
zone is formed; during the period, infection tends to
be spread over the whole group of poultry.

As a result of extensive investigations to

1 solve the drawbacks in the prior art, the present
inventors have discovered polypeptides derived from
Mycoplasma gallisepticum and having antigenicity of
Mycoplasma gallisepticum and further found that antisera,
5 derived from the polypeptides using said polypeptides
as antigens, can prevent growth of Mycoplasma
gallisepticum and, the polypeptides are expected to be
useful as poultry vaccines capable of preventing
Mycoplasma gallisepticum infections and are useful as
10 poultry diagnostics for Mycoplasma gallisepticum
infections. The present invention has thus come to be
accomplished.

According to a first aspect of the present
15 invention, there is provided polypeptides which causes
an antigen-antibody reaction with anti-Mycoplasma
gallisepticum poultry sera.

According to a second aspect of the present
invention, there is provided a gene encoding the
20 polypeptides.

According to a third aspect of the present
invention, there is provided a recombinant vector in
which the gene has been integrated.

According to a forth aspect of the present
25 invention, there is provided a host transformed or
transfected by the recombinant vector.

According to a fifth aspect of the present



1 invention, there is provided a poultry vaccine for
Mycoplasma gallisepticum infections comprising the
polypeptides as an effective ingredient.

Further according to a sixth aspect of the
5 present invention, there is provided a poultry
diagnostic for Mycoplasma gallisepticum infection com-
prising the polypeptides as an effective ingredient.

IN THE DRAWINGS:

10 Fig. 1(a)-1 to 1(f)-2 shows amino acid
sequences of polypeptides MG-1, MG-2, MG-3, MG-7, MG-8
and MG-9 exhibiting antigenicity of Mycoplasma
gallisepticum and base sequences of M-1, M-2, M-3, M-7,
M-8 and M-9 encoding the polypeptides. Fig. 2(a) and
15 2(b) similarly shows an amino acid sequence of
polypeptide TMG-1 and a base sequence of TM-1 encoding
the polypeptide. Fig. 3-1 to 3-4 indicate restriction
enzyme cleavage maps of DNA fragments encoding the
polypeptides in accordance with the present invention.
20 Figs. 4, 5 and 6 indicate procedures for producing
antigen protein plasmids in accordance with the present
invention.

25 In the first aspect of the present invention,
the polypeptides are the one that cause an antigen-
antibody reaction with poultry sera infected with
Mycoplasma gallisepticum.



1 Specific examples include DNA fragments
derived from Mycoplasma gallisepticum and polypeptides
that can be produced by fragments having restriction
enzyme cleavage maps shown in Fig. 3, (a) through (v).
5 More specifically, the polypeptides are as shown in
Table 1 and exemplified by about 30 kilodaltons of
polypeptide MG-1 derived from cloned antigen DNA M-1,
about 1 kilodalton of polypeptide MG-2 derived from
cloned antigen DNA M-2, about 30 kilodaltons of
10 polypeptide MG-3 derived from cloned antigen DNA M-3,
about 55 kilodaltons of polypeptide MG-4 derived from
cloned antigen DNA M-4, about 1 kilodalton of
polypeptide MG-5 derived from cloned antigen DNA M-5,
about 32 kilodaltons of polypeptide MG-6 derived from
15 cloned antigen DNA M-6, about 35 kilodaltons of
polypeptide MG-7 derived from cloned antigen DNA M-7,
about 35 kilodaltons of polypeptide MG-8 derived from
cloned antigen DNA M-8, about 35 kilodaltons of
polypeptide MG-9 derived from cloned antigen DNA M-9,
20 about 55 kilodaltons of polypeptide MG-10 derived from
cloned antigen DNA M-10, about 46 kilodaltons of
polypeptide MG-11 derived from cloned antigen DNA M-11,
about 15 kilodaltons of polypeptide MG-12 derived from
cloned antigen DNA M-12, about 29 kilodaltons of
25 polypeptide MG-13 derived from cloned antigen DNA M-13,
about 15 kilodaltons of polypeptide MG-14 derived from
cloned antigen DNA M-14, about 79 kilodaltons of
polypeptide MG-15 derived from cloned antigen DNA M-15,

1 about 15 kilodaltons of polypeptide MG-16 derived from
cloned antigen DNA M-16, about 55 kilodaltons of
polypeptide MG-17 derived from cloned antigen DNA M-17,
about 49 kilodaltons of polypeptide MG-18 derived from
5 cloned antigen DNA M-18, about 32 kilodaltons of
polypeptide MG-19 derived from cloned antigen DNA M-19,
about 35 kilodaltons of polypeptide MG-20 derived
from cloned antigen DNA M-20, about 9 kilodaltons of
polypeptide MG-21 derived from cloned antigen DNA M-21,
10 about 38 kilodaltons of polypeptide MG-22 derived from
cloned antigen DNA M-22, etc. The polypeptide is
further exemplified by a polypeptide having amino acid
sequence containing the amino acid sequence from one
of these polypeptides and having the same amino acid
15 sequence as the polypeptide expressed in Mycoplasma
gallisepticum. The polypeptide is also exemplified by
fused proteins having as C-terminus an amino acid
sequence of MG-1, MG-2, MG-3, MG-7, MG-8, MG-9 shown
in Fig. 1 (a)-1 through (f)-2 or TMG-1 shown in Fig.
20 2(a) and 2(b) and containing, as stabilizing protein,
bacteria-derived enzyme proteins such as β -galactosidase,
 β -lactamase, etc. at the M-terminus thereof. Other
polypeptides can also be converted into fused proteins
with bacteria-derived enzyme proteins.

25 The polypeptides which are concerned with
the first aspect of the present invention can be obtained
by using the host (relating to the forth aspect of the
invention) transformed or transfected by the recombinant

1 vector that is concerned with the third aspect of the
invention.

The recombinant vector described above can be
obtained by integrating a Mycoplasma gallisepticum-
5 derived DNA fragment into an expression vector in a
conventional manner.

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

Specific examples of the DNA fragment used
for recombination are DNA fragment encoding the amino
acid sequence shown in Fig. 1(a)-1 and Fig. 1(a)-2
(for example, 705 base pairs from 1 to 705 in Fig.
15 1(a)-1 and Fig. 1(a)-2), DNA fragment encoding the
amino acid sequence shown in Fig. 1(b) (for example, 30
base pairs from 1 to 30 in Fig. 1(b)), DNA fragment
encoding the amino acid sequence shown in Fig. 1(c)
(for example, 657 base pairs from 1 to 657 in Fig.
20 1(c)), DNA fragment encoding the amino acid sequence
shown in Fig. 1(d) (for example, 549 base pairs from 1
to 549 in Fig. 1(d)), DNA fragment encoding the amino
acid sequence shown in Fig. 1(e) (for example, 927 base
pairs from 1 to 927 in Fig. 1(e)), DNA fragment encoding
25 the amino acid sequence shown in Fig. 1(f)-1 and Fig.
1(f)-2 (for example, 531 base pairs from 1 to 531 in
Fig. 1(f)-1 and Fig. 1(f)-2); and DNA fragments added
at the upstream of the 5' end thereof with a DNA

1 fragment encoding enzyme protein, etc. of bacteria in
combination with the reading frame. In addition thereto,
the DNA fragment is also exemplified by DNA fragments
derived from Mycoplasma gallisepticum such as M-4, M-5,
5 M-6, M-10, M-11, M-12, M-13, M-14, M-15, M-16, M-17,
M-18, M-19, M-20, M-21, M-22, etc. Restriction enzyme
cleavage maps of these DNA fragments and their lengths
are shown in Fig. 3. Furthermore, the DNA fragment is
exemplified by the one, encoding the amino acid sequence
10 of that having the same amino acid sequence as that
of the polypeptide expressed in Mycoplasma gallisepticum
and containing the amino acid sequence of these antigen
polypeptide, etc. (for example, 783 base pairs from
40 to 822 in Fig. 2), given from genomic DNA of
15 Mycoplasma gallisepticum in such a manner well known
to one skilled in the art as the hybridization technique
using these DNA fragments as probes.

The vector which is used to construct the
recombinant vector is not particularly limited, however,
20 specific examples include plasmids such as pUC8, pUC9,
pUC10, pUC11, pUC18, pUC19, pBR322, pBR325, pBR327,
pDR540, pDR720, and the like; phages such as λ gt11,
 λ gt10, λ EMBL3, λ EMBL4, Charon 4A and the like.

The method for inserting the DNA fragment
25 described above into these vectors to produce recombinant
vectors may be performed in a manner conventional to
one skilled in the art. For example, the vector is
cleaved with a restriction enzyme and ligated with

1 the DNA fragments described above either directly or
via synthetic linker, under control of a suitable
expression regulatory sequence.

As the expression regulatory sequence used,
5 those may be mentioned; lac promoter operator, trp
promoter, tac promoter, lpp promoter, P_L promoter, amyE
promoter, Gal7 promoter, PGK promoter, ADH promoter,
etc.

In producing the recombinant vector for the
10 purpose of expressing these polypeptides derived from
Mycoplasma, techniques for producing a recombinant vector
by once integrating the aforesaid DNA fragment into
a suitable vector and then carrying out subcloning,
is well known to one skilled in the art. These subcloned
15 DNA fragments are excised with an appropriate restriction
enzyme and ligated under control of the expression
regulatory sequence described above. Thus, the re-
combinant vector capable of producing the polypeptide
can be produced.

20 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, YCp50, and the like.

25 Using the obtained recombinant vector, a
variety of appropriate hosts are transformed to micro-
organisms that can produce the polypeptides capable
of expressing antigenicity of Mycoplasma gallisepticum,

- 1 a part of the polypeptides or a fused protein containing
said polypeptides.

The appropriate host used herein can be chosen
taking into account adaptability to expression vector,
5 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, etc.
The host transformed with an appropriate expression
10 vector can be cultured and proliferated under suitable
culture conditions well known to one skilled in the
art.

Upon production of the polypeptide, conditions
for inducing the action of expression regulation sequence
15 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 solution.

The poultry vaccine for Mycoplasma
20 gallisepticum infections can be prepared in a manner
similar to conventional technique from the thus obtained
host which is concerned with the forth aspect of the
invention. The host can be cultured under conditions
ordinarily used for culturing microorganisms of this
25 type. In the case of E. coli, the bacteria can be
cultured in LB medium at 37°C under aerobic conditions.

After culturing, the polypeptide of the
present invention can be purified by means of

1 chromatography, precipitation by salting out, density
gradient centrifugation and the like which are well known
to one skilled in the art and may optionally be chosen.
The thus obtained polypeptide can be used as a vaccine.

5 Alternatively, the host can be inactivated
and the inactivated host can be used as a vaccine.
In this case, the inactivation is carried out in a
conventional manner after culture of the host is com-
pleted. The inactivation may be attained by heating
10 but it is simpler to add an inactivating agent to the
culture solution. As the inactivating agent, there
may be used Merzonin (trademark, thimerosal manufactured
by Takeda Pharmaceutical Co., Ltd.; hereinafter the
same), β -propiolactone, tyrosine, salicylic acid,
15 Crystal Violet, benzoic acid, benzetonium chloride,
polymyxin, gramicidin, formalin, phenol, etc. The
inactivated culture solution is added, if necessary and
desired, with a suitable quantity of adjuvant. The
inactivated product is then separated with a siphon or
20 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
25 necessary and desired, 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,

1 polymyxin, gramicidin, formalin, phenol, etc.

In order to further enhance immune activity, adjuvant may also be added to the obtained vaccine.

The adjuvant is generally used in a volume of 1 to 99
5 based on 100 volume of the vaccine.

When the vaccine is used, it can be mixed with diluents, filler, etc. in a conventional manner. The vaccine exhibits the effect with a dose of at least 1 μ g antigenic polypeptide per kg wt. The upper limit
10 is not critical unless the dose shows acute toxicity. The dose can be determined opportunely, for example, under such conditions that the counteractive antibody titer ($\log_{10}$) is 1.0 to 2.0. No acute toxicity was notable with a dose of 5 mg antigenic polypeptide per
15 kg wt. to chickens.

The poultry vaccine for Mycoplasma gallisepticum infection obtained in the present invention is inoculated to poultry intramuscularly, subcutaneously or intracutaneously, etc.

20 Likewise the vaccine, the polypeptide of the present invention obtained by purification and isolation can be used as diagnostics since the polypeptide can strongly bind to antibody in sera collected from poultry infected with Mycoplasma gallisepticum. A test sample
25 can be diagnosed with respect to Mycoplasma gallisepticum infections, by methods well known to one skilled in the art, such as by ELISA which comprises immobilizing the polypeptide onto a microtiter plate, reacting with

1 poultry serum which is a test sample, then reacting
with a secondary antibody labeled with peroxidase, etc.
and peroxidase substrate and, determining a change in
absorbancy of the reaction solution; etc.

5 According to the present invention, the
polypeptides having antigenicity derived from Mycoplasma
gallisepticum can be provided. The recombinant vectors
in which DNAs encoding these polypeptides can also be
provided. Furthermore, microorganisms such as bacteria,
10 yeast, etc. transformed (or transfected) by these
recombinant vectors can be provided. The polypeptides
produced by these microorganisms have the same anti-
genicity as that of the polypeptide derived from
Mycoplasma gallisepticum. Using the polypeptides, more
15 effective vaccines and poultry diagnostics for Mycoplasma
gallisepticum infection which are handled in more rapid
and simpler way can be provided.

Hereafter the present invention is described
in more detail by referring to the examples below. In
20 the examples and comparative examples as well as refer-
ence examples, parts and % are all by weight, unless
otherwise indicated.

Example 1

(1) Preparation of genomic DNA of Mycoplasma
25 gallisepticum

Mycoplasma gallisepticum S6 strain was cultured
at 37°C for 3 to 5 days in liquid medium obtained by

1 supplementing 20% donor 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
5 solution decreased. At the point of time when the
color of the pH indicator contained in the culture
solution changed from red to yellow, incubation was
terminated. The culture solution was centrifuged at
10,000 rpm for 20 minutes to collect the cells. The
10 collected cells were then suspended in 1/10 volume of
PBS based on the volume of culture solution. 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
15 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.
20 The extract was precipitated with ethanol to give 200
µg of genomic DNA of Mycoplasma gallisepticum.

(2) Construction of genomic DNA library

To 40 µg of the genomic DNA of Mycoplasma
gallisepticum obtained in (1) was added 4 units of
25 restriction enzyme A_{lu} I. The mixture was incubated at
37°C for 10 minutes to cause partial cleavage. The
partially cleaved genomic DNA was subjected to 0.8% low
melting point agarose gel electrophoresis. A DNA

1 fragment having a length of from about 1.0 kbp to about
4.0 kbp was recovered from the gel, treated with phenol
and precipitated with ethanol to give 4 μ g of the DNA
fragment partially cleaved with A λ u I.

5 S-Adenosyl-L-methionine was added to 1.2 μ g
of the DNA fragment partially cleaved with A λ u I in
a final concentration of 80 μ M, and 20 units of EcoR I
methylase was further added thereto to methylate the
deoxyadenosine site in EcoR I recognition sequence,
10 whereby the sequence was rendered insensitive to EcoR
I. EcoR I linker was ligated with the DNA fragment by
ligase and further mixed with a fragment of λ gt11 DNA
cleaved with EcoR I. Ligation was performed by ligase.
Using the reaction solution, in vitro packaging was
15 carried out in a conventional manner (DNA Cloning, vol.
1, A Practical Approach, edited by D.M. Glover). The
packaging product was transfected to E. coli Y1088
(Amersham Inc.), which was then cultured at 37°C for
12 hours in LB agar medium containing 0.003% of 5-bromo-
20 4-chloro-3-indolyl- β -D-galactopyranoside and 0.03 mM
of isopropylthio- β -D-galactopyranoside (IPTG). In the
formed plaques, a library size was estimated by the
number of white plaques and 10^6 pfu (plaque forming
unit) of DNA library was prepared.

25 (3) Immunoscreening of genomic DNA library

Phage obtained from the DNA library prepared
in (2) was added to a suspension of E. coli Y1090
(Amersham Inc.) in 10 mM MgSO $_4$ aqueous solution in such

1 a way that 500 to 1000 plaques were formed in a plate
of 8 cm ϕ , which was allowed to adsorb for 15 minutes.
Furthermore, 2.5 ml of LB soft agar medium heated
to 45°C was added and overlaid on the LB agar medium
5 to form layers. Incubation was conducted at 42°C for
3 to 4 hours. A nylon membrane filter was immersed in
10 mM IPTG aqueous solution. After air drying, the
filter was overlaid on the plate described above followed
by incubation at 37°C for further 2 to 3 hours. After
10 the incubation, the nylon membrane filter was stripped
off from the plate and washed with TBS (50 mM Tris-HCl,
pH 8.0, 150 mM NaCl). After further immersing in TBS
containing 2% of skimmed milk for 30 minutes, the filter
was immersed for an hour with anti-Mycoplasma chicken
15 serum diluted with TBS to 500-fold. Thereafter, the
filter was washed by immersing in TBS for 15 minutes.
The filter was further washed by immersing in TBS
containing 0.05% of surfactant (Tween 20) for 10 to 15
minutes. The washing procedure was repeated 4 or 5
20 times. Then, the filter was treated for 60 minutes with
biotinated antibody to chicken IgG. After treating
with a secondary antibody, the filter was washed 5 or 6
times with PBS containing 0.05% of Tween 20 and further
immersed in horse radish peroxidase-avidin D solution
25 to treat the same for 60 minutes. After the treatment,
the filter was washed 5 or 6 times with PBS containing
0.05% of Tween 20 and further washed with 10 mM Tris-
HCl showing pH of 8.0. Thereafter the filter was

1 immersed in buffer containing 4-chloronaphthol and
hydrogen peroxide. By this series of procedures, only
the plaque in which the antigen protein derived from
Mycoplasma gallisepticum had been expressed was colored
5 to purple.

By the immunoscreening of 5×10^4 plaques
described above, 50 positive plaques were obtained.

(4) Preparation of immunopositive recombinant λ gt11
phage DNA

10 E. coli Y 1090 strain was cultured at 37°C
for 12 hours in LB medium containing 50 μ g/ml of
ampicillin and the culture solution was added to 10-fold
volume of LB medium containing 10 mM MgSO_4 . Then,
recombinant λ gt11 phage obtained in (3) which had been
15 determined to be positive in the immunoscreening was
infected to E. coli Y1090 at moi of 0.05 followed by
culturing at 37°C for 5 to 10 hours. After E. coli was
lysed, centrifugation was performed at 8,000 rpm for 10
minutes to obtain the supernatant. An equal volume of
20 TM buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgSO_4) as well
as DNase I were so added to the supernatant to show
the concentration of 0.016 mg/ml followed by incubation
for 15 minutes. Furthermore, NaCl and polyethylene
glycol (PEG 6000) were so added thereto as to show the
25 concentration of 0.5 M and 0.1 g/ml. The mixture was
shaken at 0°C for 15 minutes. The resulting mixture
was centrifuged at 10,000 rpm for 10 minutes to remove
the supernatant. The obtained pellets were dissolved

1 in 1/100 volume of TM buffer and an equal volume of
chloroform was added to the mixture followed by vigorous
stirring. The mixture was centrifuged at 15,000 rpm
for 10 minutes and recombinant λ gt11 phage was collected
5 in the aqueous phase to obtain a phage solution.

To the phage solution were so added EDTA,
SDS and pronase E as to show the concentration of
0.025M, 1%, 1 mg/ml. After incubation at 37°C for 4
hours, the solution was extracted with phenol (3 to 5
10 times). The extract was precipitated with ethanol to
give λ gt11 phage DNA containing cloned antigen DNA
(M-1).

(5) Removal of the same clone from immunopositive
recombinant λ gt11 phage

15 After digesting 1 μ g of each λ gt11 phage DNA
obtained in (4) with EcoR I, Mycoplasma gallisepticum-
derived DNA was recovered by means of 0.8% low melting
point agarose gel electrophoresis. The DNA was used
as probe DNA.

20 E. coli Y1090 strain was cultured in LB
medium at 37°C for 12 hours. 100 μ l of the culture was
mixed with 3 ml of LB soft agar medium warmed at 45°C
and the mixture was overlaid on LB agar medium. On the
medium 10 μ l each of the respective λ gt11 clones was
25 dropped onto definite positions. When cultured at 37°C
for 12 hours, plaques due to phage proliferation were
formed at the dropped positions. A nylon membrane was
put on this plate and the phage was adsorbed thereto.

1 After air drying, the membrane was treated with a
denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 10
minutes and further treated with a neutralizing solution
(3 M sodium acetate, pH 5.5) for 10 minutes. After air
5 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% Na₄P₂O₇, 50 µg/ml
of denatured salmon sperm DNA and the Mycoplasma
gallisepticum-derived DNA probe which had been previously
10 prepared and labeled in a conventional manner were
added to cause hybridization at 68°C for 14 hours.
After washing, the nylon membrane was overlaid on an
X ray film. The presence of spot was confirmed by
autoradiography. A plaque in which a spot was formed
15 was removed as the same plaque. It was thus confirmed
that 22 clones were independent.

(6) Production of recombinant plasmid (pUml) (cf. Fig.
4)

After digesting the recombinant λgt11 phage
20 DNA obtained in (5) with restriction enzyme EcoR I, the
digestion product was subjected to 0.8% low melting point
agarose gel electrophoresis. Genomic DNA fragment of
Mycoplasma gallisepticum integrated into cloning site
of the genomic DNA of λgt11 phage showed a length of
25 about 0.8 kbp. The DNA fragment was extracted from the
agarose gel and further extracted with phenol-chloroform
(1 : 1). The extract was precipitated with ethanol and
the precipitates were recovered. On the other hand,

1 plasmid pUC18 was similarly digested with EcoR I. Then,
the digestion product was extracted with phenol-
chloroform. The extract was precipitated with ethanol
and cleaved pUC18 was recovered. Then 5' end phosphate
5 was removed by treating with alkaline phosphatase.
After pUC18 DNA was again extracted with phenol-
chloroform, DNA was recovered by ethanol precipitation.

The cleaved pUC18 was ligated with the
Mycoplasma gallisepticum genome-derived EcoR I digestion
10 product (about 0.8 kbp) by ligase and competent E. coli
TG1 strain was transformed. The transformants were
cultured at 37°C for 15 hours in LB agar medium contain-
ing 0.003% of 5-bromo-4-chloro-3-indolyl- β -D-
galactopyranoside, 0.03 mM of isopropylthio- β -D-
15 galactopyranoside and 40 μ g/ml of ampicillin. Among
transformed E. coli grown on the agar medium, white
colonies were cultured at 37°C for 15 hours in LB liquid
medium containing 40 μ g/ml of ampicillin and plasmids
were extracted by the method of Birnboim & Doly [Nucleic
20 Acid Research, 7, 1513 (1979)]. After digesting with
EcoR I, recombinant plasmid containing a DNA fragment
having the same length of the original EcoR I fragment
derived from Mycoplasma gallisepticum was detected by
0.8% low melting point agarose gel electrophoresis and
25 named pUM1. The plasmid pUM1 is the recombinant vector
of the present invention.

(7) Sequence analysis of pUM1 insert DNA

Sequence of insert DNA fragment was determined

1 by the Dideoxy method of Sanger et al. [Proc. Natl.
Acad. Sci., USA, 74, 5463 (1977)] using pUM1 prepared
in (6). A restriction enzyme cleavage map of the cloned
DNA fragment is shown in Fig. 3 (a) and the nucleotide
5 sequences of the DNA fragment are shown in Fig. 1(a)-1
and Fig. 1(a)-2.

From the facts that a molecular weight of the
fused protein with β -galactosidase produced in (9) later
described was about 145 kilodaltons and translation
10 termination codon (TGA) was present in bases of 706 to
708 in Fig. 1(a)-2, it is noted that the fragment
encoding the polypeptide exhibiting antigenicity of
Mycoplasma gallisepticum is 705 bp from 1 to 705 in
Fig. 1(a)-1 and Fig. 1(a)-2 and the amino acid sequence
15 deduced from the sequence is as shown in Fig. 1(a)-1
and Fig. 1(a)-2.

(8) Production of plasmid capable of expressing antigen
protein (cf. Fig. 5)

The recombinant plasmid (pUM1) obtained in
20 (6) was digested with EcoR I. The digestion product
was subjected to 0.8% low melting point agarose gel
electrophoresis. Insert DNA having a length of about
0.8 kbp was recovered from the gel and extracted with
phenol-chloform. The extract was precipitated with
25 ethanol to recover DNA.

On the other hand, plasmid pMA001 [Gene, 28,
127-132 (1984)] harboring λ ac promoter-operator and λ ac
Z gene was digested with EcoR I. After the digestion

1 product was extracted with phenol-chloroform, the
extract was precipitated with ethanol and cleaved
pMA001 was recovered. Then, 5' end phosphate was
removed by treating with alkaline phosphatase. After
5 again extracting with phenol-chloroform, pMA001 DNA was
recovered by ethanol precipitation.

The cleaved pMA001 was ligated with the EcoR
I digestion product (about 0.8 kbp) of insert DNA by
ligase and competent E. coli TGl strain was transformed.
10 A recombinant plasmid capable of expressing as a fused
protein with β -galactosidase in which about 0.8 kbp of
genomic DNA of Mycoplasma gallisepticum had been ligated
in the correct direction at the downstream of lac Z
gene of pMA001 was selected in a manner similar to (6).
15 The recombinant plasmid was named pMAD1. This plasmid
is the recombinant vector of the present invention.

Then, pMAD1 was partially digested with EcoR
I. The partial digestion product was subjected to 0.8%
low melting point agarose gel electrophoresis. About
20 7.2 kbp of fragment obtained by cleaving pMAD1 with EcoR
I at one site was recovered from the agarose gel.
After treating with phenol-chloroform, cleaved pMAD1 was
recovered by ethanol precipitation.

On the other hand, pSP11 [Journal of
25 Bacteriology, June 1986, 937-944] was doubly cleaved
with Hinc II and EcoR V. The cleavage product was
subjected to 1.0% low melting point agarose gel
electrophoresis. About 700 bp of fragment containing

1 transcription termination sequence was recovered. After
similarly treating with phenol-chloroform, the DNA
fragment was recovered by ethanol precipitation. The
cleaved pMAD1 and about 700 bp of DNA fragment containing
5 transcription termination sequence were combined and
cohesive ends of the respective DNA fragments were
rendered blunt ends by DNA-polymerase I (Klenow's
fragment). They were further ligated with ligase and
competent E. coli TGI strain was transformed. A
10 recombinant plasmid in which the transcription termina-
tion sequence had been inserted at the downstream of
genomic DNA of Mycoplasma gallisepticum of pMAD1 was
selected in a manner similar to (6). The recombinant
plasmid was named pMAH1. This plasmid is the recombinant
15 vector of the present invention.

(9) Expression and detection of antigen protein
(β -galactosidase fused protein)

(9-a) E. coli (MC169 strain) transformed with the
recombinant plasmid obtained in (8) was precultured
20 at 37°C overnight in LB medium containing 50 μ g/ml of
ampicillin. 1 ml of the preculture was taken and
added to 100 ml of LB liquid medium likewise containing
50 μ g/ml of ampicillin followed by culturing at 37°C.
Two hours later, isopropylthio- β -D-galactopyranoside
25 was so added thereto as to show the concentration of
1 mM followed by culturing at 37°C for further 5 hours.
After the incubation, centrifugation was performed at
8,000 rpm for 10 minutes to collect E. coli. By adding

1 1.0 ml of PBS, E. coli was resuspended. The suspension
was subjected to freezing and thawing. The cells were
further sonicated and then centrifuged at 15,000 rpm
for 30 minutes to recover the supernatant.

5 (9-b) Then, the supernatant was subjected to 8%
SDS polyacrylamide gel electrophoresis (SDS-PAGE) at
50 mA for 2 hours. After the electrophoresis, the gel
was stained with Coomassie Brilliant Blue R-250 to
newly detect a band of about 145 daltons. pMA001-
10 derived β -galactosidase (in part) is approximately 115
kilotdaltons. It is thus considered that the newly
formed polypeptide would correspond to fused protein
MGg-1 in which about 30 kilodaltons of polypeptide
MG-1 having Mycoplasma gallisepticum-derived amino acid
15 sequence shown in Fig. 1(a) is jointed at the C-terminus
of β -galactosidase.

(9-c) On the other hand, the supernatant described
above was subjected to 8% SDS-PAGE in a manner similar
to (9-b) and a protein band separated in the gel was
20 then transferred onto a nylon membrane filter. After
the transfer, the filter was reacted with anti-
Mycoplasma serum by procedures as in (3) to perform
Western blotting. As the result, only the band corre-
sponding to the protein of about 145 kilodaltons newly
25 found in (9-b) was detected. It was made clear that
the protein was fused protein MGg-1 of Mycoplasma
gallisepticum-derived antigen protein MG-1 and β -
galactosidase.

1 Example 2

A recombinant plasmid capable of expressing Mycoplasma gallisepticum-derived antigen protein was produced from immunopositive plaque to anti-sera of

5 Mycoplasma gallisepticum obtained in Example 1 (3), by procedures similar to Example 1 (4), (5), (6) and (8). Cloning λ phage vector used and subcloning vector and expression vector used are shown in Table 1. Restriction enzyme cleavage maps and lengths of cloned

10 Mycoplasma gallisepticum gene are shown in Fig. 3 (b) through (v).

Then, the expression vector described above was allowed to express in a manner similar to Example

1 (9), whereby a part of β -galactosidase and a fused

15 protein were obtained. Molecular weights of fused proteins MGg-1 to MGg-22 and antigen proteins MG-1 to MG-22 derived from Mycoplasma gallisepticum are shown in Table 1, respectively.

Table 1

Run No.	Cloning λ Vector	Subcloning Vector	Expression Vector	Fused Protein		Mycoplasma-derived polypeptide	
				Name	kd	Name	kd
2-1	M-1	pUM1	pMAH-1	MGg-1	145	MG-1	30
2-2	M-2	pUM2	pMAH-2	MGg-2	116	MG-2	1
2-3	M-3	pUM3	pMAH-3	MGg-3	145	MG-3	30
2-4	M-4	pUM4	pMAH-4	MGg-4	170	MG-4	55
2-5	M-5	pUM5	pMAH-5	MGg-5	116	MG-5	1
2-6	M-6	pUM6	pMAH-6	MGg-6	147	MG-6	32
2-7	M-7	pUM7	pMAH-7	MGg-7	150	MG-7	35
2-8	M-8	pUM8	pMAH-8	MGg-8	150	MG-8	35
2-9	M-9	pUM9	pMAH-9	MGg-9	150	MG-9	35
2-10	M-10	pUM10	pMAH-10	MGg-10	170	MG-10	55
2-11	M-11	pUM11	pMAH-11	MGg-11	161	MG-11	46
2-12	M-12	pUM12	pMAH-12	MGg-12	130	MG-12	15
2-13	M-13	pUM13	pMAH-13	MGg-13	144	MG-13	29
2-14	M-14	pUM14	pMAH-14	MGg-14	130	MG-14	15

- to be continued -

- continued -

<u>Run No.</u>	<u>Cloning λ Vector</u>	<u>Subcloning Vector</u>	<u>Expression Vector</u>	<u>Fused Name</u>	<u>Protein kd</u>	<u>Mycoplasma-derived polypeptide</u>	
						<u>Name</u>	<u>kd</u>
2-15	M-15	pUM15	pMAH-15	MGg-15	194	MG-15	79
2-16	M-16	pUM16	pMAH-16	MGg-16	130	MG-16	15
2-17	M-17	pUM17	pMAH-17	MGg-17	170	MG-17	55
2-18	M-18	pUM18	pMAH-18	MGg-18	164	MG-18	49
2-19	M-19	pUM19	pMAH-19	MGg-19	147	MG-19	32
2-20	M-20	pUM20	pMAH-20	MGg-20	150	MG-20	35
2-21	M-21	pUM21	pMAH-21	MGg-21	124	MG-21	9
2-22	M-22	pUM22	pMAH-22	MGg-22	153	MG-22	38

1 Example 3

Harvest of polypeptide gene TM-1 containing pUM1 polypeptide in which Mycoplasma gallisepticum has been expressed in nature

5 (1) Genomic Southern Hybridization of Mycoplasma gallisepticum using pUM1 insert DNA as a probe

After 1 µg of Mycoplasma gallisepticum DNA obtained in Example 1 (1) was digested with restriction enzyme EcoR I, the digestion product was subjected to
10 0.6% low melting point agarose gel electrophoresis. After the electrophoresis, the gel was immersed in a denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 10 minutes and further immersed in a neutralizing solution (3 M sodium acetate, pH 5.5) for 10 minutes to denature
15 DNA. 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,
20 pH 7.8), 10-fold Denhardt, 0.1% SDS, 0.1% Na₄P₂O₇, 50 µg/ml of denatured salmon sperm DNA and pUM1 insert DNA which had been labeled 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
25 revealed that hybridization occurred on a fragment of about 1.7 kbp.

(2) Cloning of EcoR I-digested fragment of about 1.7 kbp into pUC18 and colony hybridization

1 After 4 μ g of Mycoplasma gallisepticum DNA
obtained in Example 1 (1) was digested with restriction
enzyme EcoR I, the digestion product was subjected to
0.6% low melting point agarose gel electrophoresis.
5 After the electrophoresis, a fragment of about 1.7 kbp
was recovered. The fragment was ligated by ligase with
pUC18 cleaved through digestion with EcoR I and competent
E. coli TGI strain was transformed. The transformants
were cultured at 37°C for 15 hours in LB agar medium
10 containing 0.003% of 5-bromo-4-chloro-3-indolyl- β -D-
galactopyranoside, 0.03 mM of isopropylthio- β -D-
galactopyranoside and 40 μ g/ml of ampicillin. White
colonies grown on the agar medium were transferred onto
a nylon membrane followed by hybridization in a manner
15 similar to (1). Autoradiography revealed that cloning
was effected and, the plasmid was named pUMGT64.

(3) Determination of the entire base sequence of TM-1

The entire base sequence of TM-1 was analyzed
in a manner similar to Example 1 (7) to determine the
20 sequence. The entire base sequence is as shown in Fig.
2(a) and 2(b).

Example 4

Production of expression plasmid pBMG6T of polypeptide
TMG-1 encoded by TM-1 (Fig. 6)

25 Firstly, pDR540 was digested with restriction
enzymes BamH I and Pst I; and the digestion product
was then subjected to 0.8% low melting point agarose

1 gel electrophoresis. A fragment was recovered from
the gel. By treating with phenol-chloroform and
precipitating with ethanol, a fragment of 1140 bp
harboring tac promoter was recovered. Next, pUMGT64
5 obtained in (2) was fully digested with BamH I and
then partially digested with restriction enzyme Ssp I.
After 0.8% low melting point agarose gel electrophoresis,
a fragment was recovered from the gel. By treating with
phenol-chloroform and precipitating with ethanol, a
10 fragment of about 840 bp containing TM-1 gene of the
full length was recovered. On the other hand, pBR322
was digested with BamH I and Pst I. After 0.8% low
melting point agarose gel electrophoresis, a fragment
was recovered from the gel. By treating with phenol-
15 chloroform and precipitating with ethanol, a fragment of
3240 bp was recovered. These 3 fragments were ligated
with ligase and transformed competent E. coli TG1 strain.
A plasmid was selected in a manner similar to Example 1
(6) and named pBMG6. This pBMG6 plasmid was cleaved
20 with BamH I. By treating with phenol-chloroform and
precipitating with ethanol, a fragment of 3240 bp was
recovered. Next, 8 mer of BamH I linker was ligated
with EcoR V-Hinc II fragment of about 700 bp containing
the transcription termination sequence shown in Example
25 1 (8). By treating with phenol-chloroform and
precipitating with ethanol, a DNA fragment was recovered.
After the DNA fragment was digested with BamH I, the
digestion product was subjected to 0.8% low melting

1 point agarose gel electrophoresis. A fragment was
recovered from the gel and treated with phenol-
chloroform. By ethanol precipitation, a fragment of
about 700 bp containing the transcription termination
5 sequence was recovered. The cleaved pBMG6 and the
fragment of about 700 bp were ligated with ligase. A
plasmid was selected in a manner similar to Example 1
(6) and named pBMG6T.

Example 5

10 Expression of TMG-1

After E. coli TGl strain transformed with
pBMG6T was cultured at 37°C for 12 hours in LB medium
containing 50 µg/ml ampicillin, 1 ml of the culture
was collected and added to 100 ml of LB medium contain-
15 ing 50 µg/ml of ampicillin followed by culturing at
37°C. Two hours later, isopropylthio-β-D-
galactopyranoside was so added as to show the concentra-
tion of 1 mM and culturing was continued at 37°C for
further 12 hours. After culturing, E. coli was
20 centrifuged at 8,000 rpm for 10 minutes. After the
cells were collected, the cells 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 to newly detect a band of about
25 29 kilodaltons, amounting to about 10% of the total
cell protein. Since this molecular weight of the
protein is equal to the estimated value, said protein

- 1 having about 29 kilodaltons is identified with the
one encoded by TM-1 and named TMG-1.

Example 6

Purification of TMG-1

- 5 After E. coli collected in Example 5 were
suspended in 10 ml of PBS, the suspension was treated
by freezing and thawing and then sonicated. Then,
centrifugation was performed at 8,000 rpm for 10 minutes
and the supernatant was recovered. The supernatant was
10 subjected to ion exchange chromatography (Pharmacia
Fine Chemicals Inc., FPLC anion exchange column MONO Q
10/10, 20 mM triethanolamine, pH 7.3, NaCl density
gradient 0 M to 1 M [60 minutes], sample amount of 20
mg, flow rate of 4 ml/min, fraction size of 4 ml). From
15 each of the collected fractions, 10 µl was adsorbed
onto a nitrocellulose membrane. Immuno dot blotting
(primary antibody; Mycoplasma gallisepticum infected
chicken serum, secondary antibody; biotininated anti-chicken
IgG rabbit serum, color forming system; chicken
20 peroxidase complex, substrate; 4-chloro-1-naphthol) was
carried out, whereby TM-1 polypeptide could be detected
around 0.6 M of NaCl concentration. The detected
fraction was subjected to 8% SDS-PAGE and stained with
Coomassie Brilliant Blue R-250. It was confirmed that
25 about 90% of the total protein was TMG-1. By the
procedures, about 200 µg of TMG-1 could be purified
from the culture solution of TGI transformed by pBMG6T.

1 Example 7

Test on growth inhibition of Mycoplasma gallisepticum

Polypeptide MGg-1 encoded by pMAH1 which was obtained in a manner similar to Example 2 and TMG-1 obtained in Example 6 were respectively dissolved in Dulbecco's PBS buffer so that each shows the concentration of 500 µg/ml. Each solution, 1 ml, was subcutaneously injected to each Japanese white rabbit weighing about 2 kg, together with an equal volume of complete Freund adjuvant respectively. Further 4 weeks later, each of MGg-1 solution, 0.5 ml, and TMG-1 solution, 0.5 ml, described above was respectively administered to each rabbit subcutaneously as well as intravenously into the ear vein for the second immunization. Seven days later, it was confirmed in a conventional manner that antibody titer was increased and, anti-MGg-1 serum and anti-TMG-1 serum were collected from the ear artery of the rabbit.

On the other hand, Mycoplasma gallisepticum was liquid cultured by the method described in Example 1 (1). The culture solution, which color changed from red to yellow by a pH indicator, was diluted to 128-fold with medium for Mycoplasma gallisepticum. To 500 µl of the diluted culture solution was aseptically added 25 µl each of 6 samples: Mycoplasma gallisepticum infected chicken serum, the MGg-1 rabbit serum and anti-TMG-1 rabbit serum, standard rabbit serum and standard chicken serum which were quite free of antibody to

1 Mycoplasma gallisepticum and medium for Mycoplasma
5 gallisepticum. By culturing at 37°C, growth inhibition
test was carried out.

On Days 0, 3 and 4 of the incubation, 10 µl
5 each was collected from each of the culture solutions
for growth inhibition test of Mycoplasma gallisepticum.
Each of the collected culture solutions was spread on
a plate of 1.2% agar medium for Mycoplasma gallisepticum
followed by culturing at 37°C in a 5% CO₂ incubator.
10 The number of cells in the corresponding culture
solution was deduced from the number of colonies of
Mycoplasma gallisepticum formed, 7 days later. The
results are shown in Table 2.

When the added sample was the culture solution
15 of standard rabbit serum, standard chicken serum or
medium, there was no difference in a proliferation rate
of Mycoplasma gallisepticum and, the cell number reached
the saturation on Day 3 of the incubation. In the
culture solution added with anti-MGg-1 rabbit serum,
20 anti-TMG-1 rabbit serum and infected chicken serum,
proliferation of Mycoplasma gallisepticum was not
proliferated at all up to Day 3. The results reveal
that TMG-1 and fused proteins MGg-1 are antigens capable
of inducing antibodies which effectively inhibit the
25 growth of Mycoplasma gallisepticum.

Table 2

<u>Sample</u>	<u>Day 0*</u>	<u>Day 3</u>	<u>Day 4</u>
Anti-MG-1 rabbit serum	9.8×10^3	1.1×10^4	1.9×10^4
Anti-TMG-1 rabbit serum	9.8×10^3	1.0×10^4	9.0×10^3
Standard rabbit serum	9.8×10^3	2.8×10^6	3.3×10^6
Infected chicken serum	9.8×10^3	1.0×10^4	1.3×10^4
Standard chicken serum	9.8×10^3	2.9×10^6	3.2×10^6
Culture medium	9.8×10^3	3.0×10^6	3.2×10^6

* The day when the incubation was started.

1 Numerical values in the table indicate the
number of cells in 1 ml of the culture solution.

Example 8

E. coli transformed by pMAD1 was cultured in
5 LB medium at 37°C for 24 hours. The culture solution,
10 ml, was added to 1 l of LB medium charged in Sakaguchi
flask followed by culturing at 37°C for 2 hours.
Isopropylthio-β-D-galactopyranoside was added thereto
in a concentration of 1 mM followed by culturing for 24
10 hours. The culture solution was centrifuged at 5,000
rpm for 10 minutes while keeping at 2 to 5°C. After
the precipitates were centrifuged and washed with
phosphate buffered saline (pH 7.0) 3 times, 10 ml of
phosphate buffered saline containing 0.01% of thimerosal
15 was added to the precipitates. After sonication was
performed for 30 seconds 5 times, the supernatant was

1 collected and made crude antigen. To 10 volume of the
crude antigen were added 10 volume of aluminum hydroxide
gel containing 0.01% of thimerosal and 30 volume of
sterilized phosphate buffered saline. The resulting
5 mixture was made a vaccine.

The vaccine described above was intramuscularly
inoculated to 3 SPF chickens at the age of 17 days in
a dose of 0.5 ml. For supplemental immunization, the
vaccine was intramuscularly inoculated at the age of
10 39 days. At the age of 66 days, agglutination reaction
test of Mycoplasma gallicepticum and hemagglutination
inhibition (HI) test were carried out to determine
immune effect.

In the agglutination reaction test, 0.05 ml
15 of Mycoplasma antigen manufactured by Nippon Pharmacy
Co., Ltd. and 0.05 ml of vaccinized or non-vaccinized
chicken serum were mixed on a glass plate and those
forming a clear agglutination mass within 2 minutes
were determined positive.

20 The HI test was performed by adding an equal
volume of culture solution of 4 units of Mycoplasma
gallisepticum S6 strain to each of diluted sera, mixing
them, settling the mixture for 10 minutes to sufficiently
sensitize and then adding blood cells thereto.

25 The results of the cell agglutination reaction
test and HI test are shown in Table 3. The vaccinized
chicken serum according to the present invention showed
a markedly increased agglutination effect, indicating

- 1 that the vaccine of the present invention exhibits highly immunizing effect.

Table 3

<u>Group</u>	<u>No. of Chickens</u>	<u>Agglutination Antibody Titer*</u>	<u>HI Antibody Titer</u>
Vaccinized Group	1	+++	< 1 : 2
	2	+++	< 1 : 2
	3	-	< 1 : 2
Non-vaccinized Group	4	-	< 1 : 2
	5	-	< 1 : 2

* - : negative +++ : strongly positive

Example 9

Diagnosis of poultry Mycoplasma infection

- 5 Polypeptide obtained in Run No. (2-1) of Example 2 and TMG-1 obtained in Example 6 were dissolved in bicarbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃), respectively, in concentrations of 10 to 100 µg/ml.

On the other hand, bicarbonate buffer in which
 10 50 µl/well of the polypeptide had been dissolved was charged in a 96 well microtiter plate. After allowing to stand at 4°C overnight, incubation was carried out at 37°C for an hour to immobilize. After the immobilization, the plate was washed 5 times with 1% bovine serum
 15 albumin-containing PBS. To each well of the microtiter plate was added 100 µl each of sera collected from chickens infected with Mycoplasma gallisepticum as

1 a positive control and sera collected from test
chickens. Then incubation was performed at 37°C for an
hour. Each well was washed 5 times with PBS. A 1000-
fold diluted solution of rabbit IgG which bound horse
5 radish peroxidase to chicken IgG was added to each well
in an amount of 100 µl each per well followed by incuba-
tion at 37°C for an hour. After again washing with
PBS 5 times, 2,2'-azino-di-[3-ethyl-benzthiazoline
sulfonate] which was substrate of horse radish peroxidase
10 was added followed by incubation at 37°C for 30 minutes.
Absorbance was measured at a wavelength of 405 nm using
an immunoreader. As the result, only the serum
collected from the chicken infected with Mycoplasma
gallisepticum selectively absorbed the wavelength of
15 405 nm. The results reveal that the polypeptide can be
utilized as a poultry diagnostic for Mycoplasma
gallisepticum infection.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A polypeptide capable of reacting with anti-Mycoplasma gallisepticum poultry sera through an antigen-antibody reaction and having an amino acid sequence coded by a DNA fragment derived from Mycoplasma gallisepticum, said DNA having a restriction enzyme site map which corresponds to the map shown in any one of Figures 3(a) to 3(v).
2. A polypeptide as claimed in Claim 1, wherein said DNA has a base sequence which corresponds to one of the sequence shown in any one of Fig. 1(a) to Fig. 1(f).
3. A polypeptide as claimed in Claim 1 or Claim 2, wherein said amino acid sequence coded by said DNA fragment is one of the sequence shown in any one of Fig. 1(a) to Fig. 1(f).
4. A polypeptide as claimed in Claim 1, 2 or 3 wherein said amino acid sequence is the same sequence as that of polypeptide expressed in nature in Mycoplasma gallisepticum.
5. A polypeptide as claimed in any one of Claims 1 to 4, wherein said amino acid sequence is one shown in Fig. 2.
6. A polypeptide as claimed in claim 1, wherein said polypeptide is MG-1 whose amino acid sequence is described in Fig. 1(a)-1 and Fig. 1(a)-2, MG-2 whose amino acid sequence is described in Fig. 1(b), MG-3 whose amino acid sequence is described in Fig. 1(c), MG-4 whose amino acid sequence is described in Fig. 1(d), MG-5 whose amino acid sequence is described in Fig. 1(e), or MG-6 whose amino acid sequence is described in Fig. 1(f)-1 and Fig. 1(f)-2.



7. A polypeptide as claimed in claim 1, wherein said polypeptide has the same amino acid sequence as that of a polypeptide expressed in Mycoplasma gallisepticum in nature and the amino acid sequence includes the amino acid sequence of a polypeptide of claim 6.

8. A polypeptide as claimed in claim 7, wherein said polypeptide is TMG-1 having an amino acid sequence shown in Fig. 2.

9. A polypeptide as claimed in claim 1, wherein said polypeptide is a fused protein whose C-terminus is MG-1, MG-2, MG-3, MG-4, MG-5, MG-6, MG-7, MG-8, MG-9, MG-10, MG-11, MG-12, MG-13, MG-14, MG-15, MG-16, MG-17, MG-18, MG-19, MG-20, MG-21, or MG-22, each as hereinbefore described, and whose N-terminus is an enzyme protein derived from bacteria.

10. A polypeptide as claimed in claim 9, wherein said enzyme protein derived from bacteria is β -galactosidase or β -lactamase.

11. A DNA fragment encoding a polypeptide as claimed in any one of claims 1 through 8.

12. A DNA fragment according to claim 11 which is derived from Mycoplasma gallisepticum having a restriction enzyme site map and length shown in any one of Fig. (3) (a) through (v).

13. A DNA fragment according to claim 11 which base sequence is selected from the sequences described in Fig. 1(a)-1 through Fig. 1(f)-2.

14. A DNA fragment according to claim 11, wherein the base sequence is described in Fig. 2.



15. A recombinant vector having integrated therein a DNA fragment according to any one of claims 11 through 14.

16. A host transformed by a recombinant vector of claim 15.

17. A poultry diagnostic for Mycoplasma gallisepticum infection comprising a polypeptide as claimed in any one of claims 1 through 10 as an effective ingredient.

18. A poultry vaccine for Mycoplasma gallisepticum infection comprising a polypeptide as claimed in any one of claims 1 through 10 as an effective ingredient.

19. A poultry vaccine for Mycoplasma gallisepticum infection according to claim 18, wherein said polypeptide is selected from MG-1, a polypeptide having substantially the same function as that of MG-1 and TMG-1.

Dated this 19th day of November, 1991.

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NIPPON ZEON CO. LTD.
By Its Patent Attorneys
DAVIES COLLISON CAVE

