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WO-A2-2005/095591
JP-A- 2004 173 537
US-A- 5 240 858
KOJI YANAI: "The kanamycin biosynthetic gene cluster from streptomyces kanamyceticus", THE JOURNAL OF ANTIBIOTICS, vol. 57, no. 5, 1 May 2004 (2004-05-01), pages 351-354, XP55049065,
LAXMI PRASAD THAPA ET AL: "Heterologous expression of the kanamycin biosynthetic gene cluster (pSKC2) in Streptomyces venezuelae YJ003", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER, BERLIN, DE, vol. 76, no. 6, 28 July 2007 (2007-07-28), pages 1357-1364, XP019538757, ISSN: 1432-0614, DOI: 10.1007/S00253-007-1096-4
YANAI, K. ET AL.: 'Amplification of the entire kanamycin biosynthetic gene cluster during empirical strain improvement of Streptomyces kanamyceticus' PROC. NATL. ACAD. SCI. U.S.A. vol. 103, no. 25, 2006, pages 9661 - 9666, XP008140670
KOJI YANAI: 'Kanamycin Kogyo Seisan Kabu ni Okeru Seigosei Idenshi Cluster no Zofuku' BIOSCIENCE &

Fortsættes ...

INDUSTRY vol. 65, no. 5, 2007, pages 232 - 236, XP008140777

FIEPRO, F. ET AL.: 'The penicillin gene cluster is amplified in tandem repeats linked by conserved hexanucleotide sequences' PROC. NATL ACAD. SCI. USA vol. 92, 1995, pages 6200 - 6204, XP002042978

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Description

REFERENCE OF RELATED APPLICATIONS

- 5 [0001] The present application claims the benefit of priority from Japanese Patent Application No. 2008-147927 filed on June 5, 2008.

FIELD OF THE INVENTION

- 10 [0002] The present invention relates to a process for amplifying DNA useful for obtaining a microorganism which produces an objective substance in a high yield within a short period of time.

BACKGROUND OF THE INVENTION

- 15 [0003] High production strains used for fermentation industry are obtained by repeated sorting and breeding improvements over several decades together with mutation processes in order to obtain a good strain having special genetic variations. Thus, these high producing mutants are undeniably the lifeline of companies and regarded as the intensive products of very important techniques. However, strain improvement by mutation process has defects such as a lot of labor and time being required, poor reproducibility, and low probability of obtaining good strains. Therefore, strain improvement has recently been advanced increasingly with gene manipulation technology as a theoretical reproducible technology.

- 20 [0004] Processes for improving the productivity of an objective substance include the increase of copy number per cell of a gene relating to the biosynthesis of the substance for enhancing its expression amount. Biosynthesis of secondary metabolites such as antibiotics requires many genes, which form a cluster on chromosome having a length extending to several ten kb. In this case, the development of technology for increasing the gene copy number of the whole cluster will produce a lot of achievements. A process for increasing the gene copy number relating to the biosynthesis of an objective substance includes cloning in a plasmid which may retain a high copy number, but high-copy type plasmids has a defect of maintaining stability, which makes it difficult to clone DNA of a long region. In addition, cosmid vector and BAC vector which have been developed for the purpose of cloning the long region DNA are currently in limited copy number for improving the stability.

- 30 [0005] It has been described in US Patent No. 5240858 that a certain gene region can be amplified in tandem on chromosome in *Streptomyces achromogenes*. However, this technology is described only as a technique which can amplify the DNA region whose size is 10 kb or less and could not be applied to the tandem amplification of giant size gene regions on a genome.

- 35 [0006] On the other hand, it has been described that a kanamycin biosynthetic gene cluster has been first cloned on 2002 (Japanese Patent Laid-Open Publication No. 2004-173537). It has been further described in the gene analysis of kanamycin high production strains used in the fermentation industry that the copy number of the kanamycin biosynthetic gene cluster has been increased (Yanai, K. & Murakami, T., Journal of Antibiotics, (Japan), 2004, Vol. 57, p. 351 - 354). It has been then revealed that the amplification unit containing a kanamycin biosynthetic gene cluster has a size of 145 kb in a kanamycin high production strain, and the amplification unit has been amplified to 36 or more copies (Yanai, K. et al., Proceedings of the National Academy of Sciences of the United States of America, (USA), 2006, Vol. 103, p. 9661-9666). However, the high production strain exhibiting these phenomena is the one obtained as a result of mutation processes over a long period of time and repeated sortings in order to improving productivity of kanamycin (Yanai, K. et al., Proceedings of the National Academy of Sciences of the United States of America, (USA), 2006, Vol. 103, p. 9661 - 9666). Thus, it has been believed impossible to reproduce the amplification phenomena in a giant size DNA region found out in a kanamycin high production strain and to find a key gene relating to it.

- 45 [0007] The use of a gene cluster of the biosynthetic genes of kanamycin synthesis in heterologous expression vectors has been described (Laxmi Prasad Thapa et al., Applied Microbiology and Biotechnology, vol. 76, no. 6, 28 July 2007). Further, expression vectors comprising genes from a biosynthesis gene cluster, for example, kanamycin, have been described (WO 2005/095591 A2).

- 50 [0008] On the basis of the background described above, there still exists a need for a process for tandemly amplifying a giant size DNA region on a genome which may be applicable to a gene cluster required for the biosynthesis of secondary metabolites such as antibiotics.

55 SUMMARY OF THE INVENTION

- [0009] The present inventors have now found that a DNA region of a giant size can be efficiently amplified in the presence of a polynucleotide coding for a specific protein in cells. The present invention is based on such information.

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[0010] Thus, the object of the present invention is to provide a process for amplifying the DNA region of a giant size efficiently in cells.

[0011] According to the present invention there is provided a process for amplifying DNA as claimed in claim 1 below.

[0012] The present invention provides a process for amplifying DNA, comprising: preparing a recombinant cell comprising any one of polynucleotides selected from the group consisting of the following (A) to (E) and a DNA unit disposed in a cell genome,

wherein the DNA unit has a size of 22 to 154 kb and at least comprises a first DNA fragment selected from the group consisting of the following (F) to (H), a target gene and a second DNA fragment selected from the following (I) to (K), the target gene or the polynucleotide being exogenous to a host,

culturing the recombinant cell under conditions for causing gene amplification to amplify the DNA unit:

(A) a polynucleotide encoding a protein consisting of the amino acid sequence of SEQ ID NO: 1,

(B) a polynucleotide encoding a protein which consists of an amino acid sequence having the deletion, substitution, insertion or addition of one of more of amino acids in the amino acid sequence of SEQ ID NO: 1 and which is functionally equivalent to that consisting of the amino acid sequence of SEQ ID NO: 1,

(C) a polynucleotide encoding a protein which consists of an amino acid sequence having an identity of 90% or more to the amino acid sequence of SEQ ID NO: 1, and which is functionally equivalent to that consisting of the amino acid sequence of SEQ ID NO: 1,

(D) a polynucleotide consisting of the nucleotide sequence of SEQ ID NO: 2,

(E) a polynucleotide which hybridizes to the polynucleotide consisting of the nucleotide sequence of SEQ ID NO: 2 under stringent conditions, and which encodes a protein functionally equivalent to that consisting of the amino acid sequence of SEQ ID NO: 1,

(F) DNA consisting of the nucleotide sequence of SEQ ID NO: 3,

(G) DNA hybridizing to the DNA consisting of the nucleotide sequence of SEQ ID NO: 3 under stringent conditions,

(H) DNA having an identity of 90% or more to the nucleotide sequence of SEQ ID NO: 3,

(I) DNA represented by the nucleotide sequence of SEQ ID NO: 4,

(J) DNA hybridizing to the DNA consisting of the nucleotide sequence of SEQ ID NO: 4 under stringent conditions, and

(K) DNA having an identity of 90% or more to the nucleotide sequence of SEQ ID NO: 4.

[0013] According to the process for amplifying DNA according to the present invention, the DNA region of a giant size can be efficiently amplified in cells.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014]

Figure 1 represents an insert fragment of cosmid AB501.

Figure 2 represents an insert fragment of cosmid pAB801.

DESCRIPTION OF EMBODIMENTS

Deposition

[0015] The cosmid AB501 (Escherichia coli JM109/cosmid AB501) according to the present invention has been deposited to International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology, No. 6, Chuo, 1-1, Higashi, Tsukuba, Ibaragi, Japan, Zip Code: 305-8566 under the deposition number of FERM BP-11114 on the original deposit date of May 14, Heisei 20 (2008).

[0016] Further, the cosmid pAB801 (Escherichia coli JM109/pAB801) according to the present invention has been deposited to International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology under the deposition number of FERM BP-11121 on the original deposit date of April 28, Heisei 21 (2009).

Definition

[0017] The term "functionally equivalent" proteins or polynucleotides used herein means as follows.

[0018] In proteins or polynucleotides, structural variation in their sequences may be caused by genetic polymorphism or mutation, modification reaction, and the like. However, it is known that some proteins or polynucleotides, although having these variations, have substantially equivalent physiological and biological activities to proteins and polynucleotides having no such variations. Thus, such proteins or polynucleotides, in which no large difference is observed in

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spite of their structural differences from those having no variations, are referred to as the "functionally equivalent" proteins or polynucleotides.

[0019] The phraseology "amino acid sequence in which one or more amino acids of amino acid sequence have been deleted, substituted, inserted or added" used herein means that alterations have been made by well known techniques including site-specific mutagenesis or by the substitution of a plurality of amino acids which is likely to occur in nature.

[0020] Furthermore, the term "identity" with respect to amino acid sequences or nucleotide sequences is used as the meaning that the nucleotide or amino acid residues constituting the respective sequences accord with each other between the sequences to be compared. The values of "identity" described herein may be the ones calculated with an identity retrieval program which is well known to a person skilled in the art, and may be readily calculated by using a default parameter of

[0021] BLAST and the like.

[0022] The term "stringent condition" used herein means that the washing operation of a membrane after hybridization is conducted in a low salt concentration solution at a high temperature, for example in the washing condition of 2 x SSC concentration (1 x SSC: 15 mM trisodium citrate, 150 mM sodium chloride) in a 0.5% SDS solution at 60°C for 20 minutes. In addition, hybridization may be conducted according to a well known method and thus may be conducted according to the attended instruction of a commercially available library.

[0023] The term "RsA region" used herein means the sequence of 94693 to 94726 in the nucleotide sequence filed as Accession No. AB254080 (total nucleotide number 205447 bp) in the database of Genbank. In addition, the term "RsB region" means the sequence of 6177 to 6210 in the nucleotide sequence filed as Accession No. AB254081 (total nucleotide number 15046 bp) in the database of Genbank.

POLYNUCLEOTIDE/PROTEIN OF THE INVENTION

[0024] The process for amplifying DNA of the present invention comprises amplifying DNA as the target of amplification in cells (also referred to hereinafter as "target gene") in the presence of a polynucleotide encoding a protein consisting of the amino acid sequence of SEQ ID NO: 1 or a protein functionally equivalent thereto (referred to hereinafter as "polynucleotide of the invention").

[0025] It is an unexpected fact that DNA in a giant size can be efficiently amplified in cells in the presence of the polynucleotide of the invention. The polynucleotide of the invention may be present in a cell genome or in a cell matrix as far as it does not inhibit the amplification of DNA in the target gene. Furthermore, according to one embodiment, the polynucleotide of the invention is present in the cell genome.

[0026] Moreover, the polynucleotide of the invention may be DNA or RNA, but preferably DNA.

[0027] According to one embodiment of the present invention, the polynucleotide described above is a DNA selected from the following (i), (ii) and (iii) (referred to hereinafter as the DNA of the invention):

(i) DNA encoding a protein selected from the following 1), 2) and 3):

- 1) a protein consisting of the amino acid sequence of SEQ ID NO: 1,
- 2) a protein comprising an amino acid sequence which has a deletion, substitution, insertion or addition of one or more amino acids in the amino acid sequence of SEQ ID NO: 1, and
- 3) a protein comprising an amino acid sequence which has an identity of 90% or more to the amino acid sequence of SEQ ID NO: 1,

(ii) DNA consisting of the nucleotide sequence of SEQ ID NO: 2,

(iii) DNA encoding a protein which hybridizes to the nucleotide sequence of SEQ ID NO: 2 under stringent conditions and has the function of amplifying DNA.

[0028] The DNA of the invention is preferably a DNA encoding a protein consisting of the amino acid sequence of SEQ ID NO: 1, and more preferably a DNA consisting of the nucleotide sequence of SEQ ID NO: 2. Furthermore, according to one embodiment, the DNA of the invention also contain a DNA contained in the cosmid AB501 deposited under Accession No. FERM BP-11114.

[0029] Furthermore, according to another preferred embodiment, the polynucleotide of the invention includes the following polynucleotides:

- (A) a polynucleotide encoding a protein consisting of the amino acid sequence of SEQ ID NO: 1,
- (B) a polynucleotide encoding a protein which consists of an amino acid sequence which has a deletion, substitution, insertion or addition of one or more amino acids in the amino acid sequence of SEQ ID NO: 1 and which is functionally equivalent to that consisting of the amino acid sequence of SEQ ID NO: 1,

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(C) a polynucleotide encoding a protein which consists of an amino acid sequence having an identity of 90% or more to the amino acid sequence of SEQ ID NO: 1, and which is functionally equivalent to that consisting of the amino acid sequence of SEQ ID NO: 1,

(D) a polynucleotide consisting of the nucleotide sequence of SEQ ID NO: 2, and

(E) a polynucleotide which hybridizes to the polynucleotide consisting of the nucleotide sequence of SEQ ID NO: 2 under stringent conditions, and which encodes a protein functionally equivalent to that consisting of the amino acid sequence of SEQ ID NO: 1.

[0030] Moreover, the protein consisting of the amino acid sequence of SEQ ID NO: 1 or a protein functionally equivalent to that consisting of the amino acid sequence of SEQ ID NO: 1 (referred to hereinafter as "the protein of the invention") has an excellent activity for amplifying DNA and can be advantageously used for the amplification of DNA in the cell genome.

[0031] The protein of the invention may also be added, for example, as a composition together with a desired buffer and the like to cells to be cultured in order to amplify the DNA region in a cell genome.

[0032] Thus, according to another embodiment of the present invention is provided a composition for amplifying DNA, comprising a protein selected from the following 1) to 3):

1) a protein consisting of the amino acid sequence of SEQ ID NO: 1,

2) a protein comprising an amino acid sequence which has a deletion, substitution, insertion or addition of one or more amino acids in the amino acid sequence of SEQ ID NO: 1, and

3) a protein comprising an amino acid sequence which has an identity of 90% or more to the amino acid sequence of SEQ ID NO: 1.

[0033] In the protein of the invention, the phraseology "one or more amino acids" means amino acids preferably in the range of 1 to 50, more preferably 1 to 30, further preferably 1 to 10, further preferably 1 to 5, and further preferably 1 to 2.

[0034] Furthermore, in the protein of the invention, the phraseology "an amino acid sequence which has an identity of 90% or more" means an amino acid sequence having an identity of preferably 95% or more, more preferably 98% or more, and further preferably 99% or more.

[0035] In addition, the proteins of 2) and 3) are preferably the ones which are functionally equivalent to the protein of 1). In this connection, the functional identity of the proteins 2) and 3) to the protein 1) can be confirmed, for example, by comparing the cases of applying these proteins or their polynucleotides to a Streptomyces strain with use of the amplification level of DNA between the RsA region and the RsB region in the strain as an index. Such a comparison experiment may be easily carried out by a person skilled in the art, for example, by referring Examples 8 to 10.

DNA region/DNA unit as the target of amplification

[0036] Furthermore, in the process for amplifying DNA of the present invention, the DNA region to be amplified is preferably the DNA region between the RsA region and the RsB region.

[0037] The RsA region and the RsB region are DNA regions containing a kanamycin biosynthetic gene cluster which is present on the chromosomal DNA of Streptomyces kanamyceticus. The RsA region and the RsB region have been described in detail by Yanai, K, et al. "Proceedings of the National Academy of Sciences of the United States of America", (USA), 2006, Vol. 103, p. 9661 - 9666, which is incorporated herein by reference.

[0038] The DNA region between the RsA region and the RsB region can be efficiently amplified as a DNA unit in the presence of the polynucleotide of the invention. The DNA unit between the RsA region and the RsB region is preferably in the range of 22 to 154 kb. According to the present invention, DNA units in such a giant size can also be amplified advantageously.

[0039] Furthermore, the RsA region is a DNA consisting of the nucleotide sequence of SEQ ID NO: 3, and the RsB region is a DNA consisting of the nucleotide sequence of SEQ ID NO: 4. Thus, according to one embodiment, the process for amplifying DNA according to the present invention is carried out in the presence of the following DNA of (a) and (b):

(a) DNA comprising the nucleotide sequence of SEQ ID NO: 3, and

(b) DNA comprising the nucleotide sequence of SEQ ID NO: 4.

[0040] The DNA comprising the nucleotide sequence of SEQ ID NO: 3 in (a) may only contain the nucleotide sequence of SEQ ID NO: 3, and one or more nucleotides may be deleted from the nucleotide sequence of SEQ ID NO: 3 as far as the process for amplifying DNA according to the present invention occurs on the basis of the specific recombination in the DNA consisting of the nucleotide sequence of SEQ ID NO: 3 and the DNA consisting of the nucleotide sequence of SEQ ID NO: 4. The DNA comprising the nucleotide sequence of SEQ ID NO: 3 preferably includes the DNA consisting

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of the nucleotide sequence of SEQ ID NO: 3.

[0041] Furthermore, the DNA comprising the nucleotide sequence of SEQ ID NO: 4 in (b) may only contain the nucleotide sequence of SEQ ID NO: 4, and one or more nucleotides may be deleted from the nucleotide sequence of SEQ ID NO: 4, as far as the process for amplifying DNA according to the present invention occurs on the basis of the specific recombination in the DNA consisting of the nucleotide sequence of SEQ ID NO: 3 and the DNA consisting of the nucleotide sequence of SEQ ID NO: 4. The DNA comprising the nucleotide sequence of SEQ ID NO: 4 preferably includes the DNA consisting of the nucleotide sequence of SEQ ID NO: 4.

[0042] Furthermore, in the process for amplifying DNA according to the present invention, DNA as the target of amplification may be the DNA unit inserted between DNAs which are functionally equivalent to the RsA region and the RsB region. In this connection, the term DNAs which are functionally equivalent to the RsA region and the RsB region means the ones which are amplified equivalently to the RsA region and the RsB region in the cell genome in the presence of the polynucleotide of the invention. The functional identity may be readily confirmed by a person skilled in the art, for example, by referring to Examples 8 to 10.

[0043] In addition, according to the preferred embodiment of the present invention, the DNA unit comprises a first DNA fragment selected from the group consisting of the following (F) to (H) and a second DNA fragment selected from the group consisting of the following (I) to (K):

(F) DNA consisting of the nucleotide sequence of SEQ ID NO: 3,

(G) DNA hybridizing to the DNA consisting of the nucleotide sequence of SEQ ID NO: 3 under stringent conditions,

(H) DNA having an identity of 90% or more to the nucleotide sequence of SEQ ID NO: 3,

(I) DNA represented by the nucleotide sequence of SEQ ID NO: 4,

(J) DNA hybridizing to the DNA consisting of the nucleotide sequence of SEQ ID NO: 4 under stringent conditions, and

(K) DNA having an identity of 90% or more to the nucleotide sequence of SEQ ID NO: 4.

[0044] Moreover, in (H) and (K) described above, the term "DNA having an identity of 90% or more" means the DNA having an identity of preferably 95% or more, more preferably 98% or more, further preferably 99% or more.

[0045] Besides, the target gene encoding the objective substance is preferably disposed in a DNA unit having the RsA region and the RsB region or DNAs functionally equivalent thereto. Thus, according to one embodiment of the present invention, the target gene is present between the DNAs (a) and (b). In addition, according to another embodiment of the present invention, the DNA unit comprises the first DNA fragment, the target gene, and the second DNA fragment in this order from the 5'-terminal.

[0046] Furthermore, the DNA unit is preferably disposed in a cell genome. In addition, according to one embodiment, both the DNA unit and the polypeptide of the present invention are disposed in the cell genome. In this case, the disposition and the distance between the polynucleotide of the present invention and the DNA unit in the cell genome are appropriately determined by a person skilled in the art in consideration of the expression level of the target gene.

Target gene/objective substance

[0047] The target gene of the present invention may be, but is not limited specifically to as far as it can be introduced into the DNA unit, a single gene or a gene group required for the biosynthesis of an objective substance.

[0048] Besides, the objective substance coded by the target gene is not specifically limited as far as it is a substance of which productivity may be improved by increasing the copy number of the target gene, but the preferred objective substances include medically and/or agriculturally useful antibiotics such as aminoglycoside antibiotics, physiologically active substances, enzymes, and the like.

[0049] Furthermore, it is preferable to insert a selection marker gene including a drug resistance gene in the DNA in consideration of selecting the DNA amplified cells. The drug resistance gene is not limited specifically as far as it may cause gene expression in an organism having DNA amplification caused therein and the gene product functions, but it is preferably a kanamycin resistance gene.

Transfer of target gene or polypeptide of the invention into host/vector

[0050] Moreover, the target gene or polypeptide of the invention may be either endogenous or exogenous to a host, but at least one of the target gene or the polypeptide is preferably exogenous to a host.

[0051] The target gene or polypeptide of the invention is suitably introduced into a host cell with a vector. In the case of introducing the polynucleotide of the invention into a host, a vector for DNA amplification which comprises a polynucleotide selected from the group consisting of (A) to (C) in the functional form is preferably used:

(A) a polynucleotide encoding a protein consisting of the amino acid sequence of SEQ ID NO: 1,

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(B) a polynucleotide encoding a protein which consists of an amino acid sequence having the deletion, substitution, insertion or addition of one or more of amino acids in the amino acid sequence of SEQ ID NO: 1 and which has DNA amplification activity, and

(C) a polynucleotide encoding a protein which consists of an amino acid sequence having an identity of 90% or more to the amino acid sequence of SEQ ID NO: 1, and which has DNA amplification activity.

[0052] The vector comprises the polynucleotide of the invention in a functional form and thus can express the protein of the invention in cells. In this connection, the phraseology "comprises in the functional form" means that the polynucleotide of the invention is inserted into the vector in such a manner that the protein of the invention can be expressed under the control of elements required for an appropriate expression described below.

[0053] Moreover, the target gene is preferably disposed, as described above, in a DNA unit inserted between the RsA region and the RsB region or between DNAs functionally equivalent thereto in the presence of the polypeptide of the invention. Thus, when the target gene is introduced into a host, a vector for amplifying DNA, comprising a DNA unit which comprises a first DNA fragment selected from the group consisting of (F) to (H), a target gene, and a second DNA fragment selected from the group consisting of (I) to (K), and capable of introducing the DNA unit into a cell genome, is preferably used.

[0054] Furthermore, the target gene and the polynucleotide of the invention are preferably introduced together with elements required for expression such as a promoter sequence and a transcription termination signal sequence into a host cell. The promoter and the transcription termination signal may be appropriately determined depending on the organism species of a host in order to promote the gene expression of high efficiency. In addition, The promoter and the transcription termination signal may be the original promoter and transcription termination signal of a gene containing the target gene and the polynucleotide of the invention.

[0055] The other elements required for expression in addition to the promoter sequence and the transcription termination signal sequence include, for example, an enhancer for efficiently expressing an objective gene and IRES (internal ribosome entry site) sequence. The elements required for expression can be disposed in an adequate site of a vector depending on their properties. In addition, the elements required for expression may be selected in consideration of the combination with a host and the productivity of the objective substance.

[0056] Also, in the case of introducing the polynucleotide of the invention or the DNA unit into the host genome by homologous recombination, a homologous DNA sequence having the identity capable of homologous recombination with a part of the host genome is disposed in the vector. The homologous DNA sequence contained in the vector may be single or plural as far as the efficient recombination and expression of the polypeptide of the present invention or the target gene are not prevented, but it is preferably two. In addition, these two homologous DNA sequences are preferably disposed in the 5'-terminal and 3'-terminal of the DNA unit to be introduced. Thus, according to one embodiment of the present invention, the vector comprises at least the homologous DNA sequence disposed in 5'-terminal, the expression unit of the objective protein gene and the homologous DNA sequence disposed in 3'-terminal.

[0057] Moreover, the homologous DNA sequence has the identity and length capable of homologous recombination with the host genome. In consideration of the readiness and probability of the homologous recombination reaction, the identity of the homologous DNA sequence and the host genome is preferably satisfactorily high, and the both are preferably the same sequence. Furthermore, the lengths of the two homologous DNA fragments are appropriately selected by a person skilled in the art respectively in consideration of the site to be introduced, the introduction efficiency, and the like.

[0058] The vectors used in the present invention are not limited specifically, provided that these vectors are capable of introducing the target gene or the polynucleotide of the invention into the cell genome, and include, for example, a plasmid vector, a cosmid vector, a phage vector and a BAC vector, preferably a cosmid vector.

[0059] The vectors described above can be constructed with use of the standard methods well known in the art, for example, the method described by Sambrook, J. et al., "Molecular Cloning: a laboratory manual", Cold Spring Harbor Laboratory Press, New York (1989).

Method for introducing vector into cells

[0060] The methods well known in the art may be used for the introduction of the vectors into cells and include, for example, an electroporation method, a microinjection method, a calcium phosphate method, a lipofection method and a conjugative transfer method. These transfer methods are appropriately selected by a person skilled in the art in consideration of host cells, vector sizes, transfer efficiencies, and the like.

Culture/selection of cells

[0061] In the method for amplifying DNA of the invention, cells into which the target gene and the polynucleotide of

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the invention have been introduced are cultured for amplifying a DNA unit under the condition of causing gene amplification.

[0062] When the cells have the selection marker gene described above, cells having an amplified DNA unit can be obtained by culturing the cells under a proper selection culture condition. For instance, when a strain containing both kanamycin biosynthetic genes and a kanamycin resistance gene is used as a host, a strain having an amplified DNA unit can be selected by subculturing the strain in a medium containing kanamycin for about three passages and by increasing serially the added amount of kanamycin during these passages.

[0063] Further, in the selection of a recombinant cell, the recombinant cell containing the plural copies of a DNA unit can be selected precisely by using genomic DNA sequencing, Southern blotting, and the like.

Recombinant cell/recombinant microorganism

[0064] Furthermore, the recombinant cell of the invention is manufactured by the technique described above and comprises the plural copies of the DNA unit as the target of amplification introduced into the genome. Thus, the microorganism obtained by the process of the present invention comprises an amplification DNA region into which the plural copies of the target gene have been introduced. Such recombinant cells and microorganisms may be used for efficiently producing the objective substance.

[0065] Moreover, in the cells described above, the DNA unit is preferably exogenous to the host. Besides, the copy number of the DNA unit is 2 or more. Also, according to the more preferred embodiment of the present invention, the target gene in the recombinant cell is incorporated in the genome as the DNA unit containing at least a promoter sequence and a transcription termination signal sequence. The target gene or the DNA unit in the recombinant cell described above may also be incorporated reiteratively preferably in tandem.

Host

[0066] Next, the host is not specifically limited as far as it does not prevent the practice of the DNA amplification of the invention, but it is preferably a microorganism, more preferably an antibiotic producing strain, and the like. More specifically, the host is preferably Actinomycetes, more preferably a Streptomyces derived strain, more preferably, Streptomyces kanamyceticus, Streptomyces coelicolor or Streptomyces lividans, more preferably Streptomyces kanamyceticus.

Production of objective substances

[0067] In the present invention, an objective substance can be produced by culturing a recombinant cell containing plural copies of the target gene obtained by the above described method in a medium. The detailed culturing condition of the recombinant cell is appropriately determined depending on the property and state of the cell by a person skilled in the art.

[0068] The objective substance may also be isolated by the well known techniques such as centrifugation, gel filtration and filtration through filter.

EXAMPLES

[0069] The present invention is now described specifically with reference to examples, but it is not limited thereto.

Example 1: Amplification of the kanamycin biosynthetic genes of Streptomyces kanamyceticus JCM 4775 strain

1) Subculture and improvement of productivity of kanamycin Streptomyces kanamyceticus

[0070] The lyophilized cell (L-tube) of the JCM4775 strain (RIKEN BioResource Center) was inoculated in a seed medium (corn steep liquor 3%, dry yeast 0.25%, CaCl₂ 0.1%, Staminol (marketed by Nippon Nogyo Shizai Kabushiki Kaisha, manufactured by Sapporo Breweries Ltd.) 0.1%, pH7.5 before sterilization, a volume of 40 ml being charged in a 250 ml Erlenmeyer flask). The medium was incubated on a rotary shaker at 220 rpm and 28°C for 48 hours to give a strain A (first generation). Next, a 1 ml portion of the culture was inoculated in two seed mediums containing no antibiotic and containing 250 µg/ml of kanamycin, respectively. These cultures were incubated on a rotary shaker at 220 rpm and 28°C for 48 hours to give a strain B containing no antibiotic and a strain C containing 250 µg/ml of kanamycin (second generations). A 1 ml portion of the culture fluid B was then inoculated in the seed medium containing no kanamycin and incubated on a rotary shaker at 220 rpm and 28°C for 48 hours to give a strain D (third generation). A 1 ml portion of the culture fluid C was inoculated in seed medium containing 500 µg/ml and 2000 µg/ml, respectively, of kanamycin

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and incubated on a rotary shaker at 220 rpm and 28°C for 48 hours to give a strain E containing 500 µg/ml of kanamycin and a strain F containing 2000 µg/ml of kanamycin (third generations), respectively.

[0071] In order to preserve the strains obtained above, the culture fluids of the first generation (A), the second generation (B, C), and the third generation (D, E, F), respectively, were mixed with the same volume of 20% skimmed milk on completing the incubation for 48 hours and cryopreserved at -80°C. A 0.5 ml portion of each strain A, B, C, D, E and F was inoculated in a seed medium containing no antibiotic and incubated for 48 hours. After completing the incubation, a 50 µl portion of each incubated culture fluid was spread on an agar medium for production (Starch 1%, Glucose 0.25%, Soybean meal 0.6%, Peptone 0.15%, KCl 0.0025%, MgSO₄·7H₂O 0.025%, K₂HPO₄ 0.05%, NaCl 0.15%, CaCO₃ 0.15%, pH7.0 before sterilization, 20ml/dish) and incubated at 28°C for 7 days. The agar medium on which *Streptomyces kanamyceticus* JCM4775 had been grown was punched out with a cork borer (diameter 5 mm), and the disk piece was placed on an agar plate containing *B. subtilis* ATCC6633 and cultured at 37°C for 18 hours to form an inhibitory zone by kanamycin. As the standard for examine production amounts, agar media for production to which 0 µg/ml, 10 µg/ml, 100 µg/ml and 500 µg/ml, respectively, of kanamycin had been added were prepared and punched out with a cork borer (diameter 5 mm) in the same way as described above. As a result of comparing the inhibitory zones formed by these disks as the inhibitory zone obtained above, the concentrations of kanamycin produced in the agar media for production were A; 10 µg/ml, B; 10 µg/ml, C; 150 µg/ml, D; 10 µg/ml, E; 200 µg/ml, and F; 250 µg/ml, respectively. Thus, the kanamycin producing capacity of the first generation (A) was increased by 20 to 25 times in the third generations (E, F).

2) Evidence of gene amplification: detection of recombination junction site (RsB/RsA) by PCR

[0072] After completing the incubation of the first generation, second generation and third generation, respectively, for 48 hours, a 30 ml portion of each culture fluid was subjected to centrifugation at 7500 rpm for 10 minutes. After decanting the supernatant thus obtained, cells were lyophilized in vacuo. The 1/10 portion of the dry cells was used for isolating chromosomal DNA in the following method. That is, the 1/10 amount of the dry cells was diluted with 1 ml of a TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH8) and 50 µl (concentration: 20 mg/ml) of a lysozyme solution. After lysis at 37°C for 30 minutes, 2 ml of a lysis-adsorption solution attended with a Genomic DNA Purification kit, MagExtracto™ -Genome- (TOYOBO) was added to the lysis solution. After agitation, 1 ml of the mixture was transferred to an Eppendorf tube and subjected to centrifugation at 12000 rpm for 5 minutes. An 850 µl portion of the supernatant was transferred to an Eppendorf tube without cap, and DNA was separated with a MFX-6000 system (TOYOBO) according to the instruction attended with the kit. Next, a gene amplification test by the PCR method was conducted with the chromosomal DNA of each strain of A, B, C, D, E and F obtained in 1). The synthetic primers used are KM-16' (5'-CCGGCACTTC-CGCTCCAA-3', SEQ ID NO: 5) and KM-17' (5'-GCGGGTTCGCCAACTCCA-3', SEQ ID NO: 6). The PCR reaction was carried out with TaKaRa LA TaqR with GC Buffer (Takara Bio Inc.) by the partial modification of the attended protocol. That is, the reaction solution comprises 0.5 µl of TaKaRa LA Taq™ (5 units/µl), 25 µl of 2 x GC Buffer II, 8 µl of dNTP solution (each 2.5 mM), 2.5 µl of dimethyl sulfoxide, 100 pmol (1 µl) of chromosomal DNA, 100 pmol (1 µl) of primer KM-16', 100 pmol (1 µl) of primer KM-17', and sterilized water (11 µl), and the final volume was adjusted to 50 µl. Annealing was carried out at a temperature of 50°C, amplification was conducted by 25 cycles, and extension was conducted at 72°C for 2 minutes. When DNA recombination occurs in the RsA region (5'-GAAGTGACGATACCTT-GGTCCTCTCAAATCAAGA-3', SEQ ID NO: 3) and the RsB region (5'-ACCACGACGACACCCTGGTCCGCGCGGAG-GAGGT-3'; SEQ ID NO: 4), it leads to the amplification of a 1.2kb DNA fragment (Yanai, K. et al., "Proceedings of the National Academy of Sciences of the United States of America (USA), 2006, Vol. 103, p. 9661 - 9666). As the result of the agarose gel electrophoresis of the reaction solution, no DNA fragments to be amplified were detected on using the chromosomal DNA of the A, B and D strains, respectively, while a 1.2 kb amplification fragment was obtained on using the chromosomal DNA of the C, E and F strains, respectively. The amplification band of C exhibited an about half strength of ethidium bromide staining compared with E and F. It has been revealed from this result that the DNA region between the RsA region and the RsB region has been amplified by subculture with the addition of kanamycin.

3) Evidence of gene amplification: detection of amplified DNA region by Southern blot analysis

[0073] The chromosomal DNA (5 µg) of A, B, C, D, E and F, respectively, was cut with *Bam*HI and subjected to agarose gel electrophoresis. The DNA in the agarose gel was blotted to Hybond™ -N+ (GE Healthcare Bioscience). Hybridization was conducted with ECL™ Direct Nucleic Acid Labelling and Detection System (GE Healthcare Bioscience) according to the attended instruction. As the probe was used a 4.95 kb *Sph*I fragment derived from pKM92 (Yanai, K. et al., Proceedings of the National Academy of Sciences of the United States of America (USA), 2006, Vo. 103, p. 9661 - 9666).

[0074] While a 9.6 kb *Bam*HI fragment is detected in wild strains, a 10.8 kb *Bam*HI fragment is further detected upon DNA recombination in the RsA region and the RsB region (Yanai, K. et al., Proceedings of the National Academy of Sciences of the United States of America (USA), 2006, Vol. 103, p. 9661 - 9666). As the result of hybridization, a 9.6 kb band was detected in all samples. A 10.8 kb band was also detected in the sample of F, and the 9.6kb and 10.8kb

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bands had an almost equal density. While the 10.8kb band was detected also in the sample of E, it had a very weak density. It has been revealed from the detection of the 10.8 kb BamHI fragment in the samples of E and F that the DNA recombination have occurred between the regions of RsA and RsB in the strains E and F and a DNA region between these regions has been amplified.

Example 2: Preparation of cosmid 203-7 having the chemically synthesized RsA region and transfer into a wild-type strain

[0075] In order to examine the presence of a key gene which causes DNA amplification in a 106.6 kb DNA region between the regions RsC and RsD (nucleotide sequence 28935-135581) among the nucleotide sequence of the kanamycin biosynthetic gene cluster derived from Streptomyces kanamyceticus which has been registered as Accession No. AB254080 (total nucleotide number 205447 bp) in the database of Genbank, a strain having the RsA region (nucleotide sequence 94693-94726, SEQ ID NO: 3) but having deleted almost of the DNA region between the regions RsC-RsD was prepared in the following method and DNA amplification capacity was examined.

1) Preparation of cosmid AB201

[0076] Cosmid AB201 having an apramycin resistance gene and a new restriction enzyme EcoRV site inserted into the right terminal of the insertion fragment of cosmid 4-5 (Yanai, K. et al., Proceedings of the National Academy of Sciences of the United States of America (USA), 2006, Vol. 103, p. 9661 - 9666) was prepared in the following method.

[0077] Plasmid pIJ773 (Gust, B. et al., Proceedings of the National Academy of Sciences of the United States of America, (USA), 2003, Vol. 100, p. 1541 - 1546) was double digested with HindIII and EcoRI and subjected to agarose gel electrophoresis to give a ca. 1.3 kb DNA fragment containing the apramycin resistance gene as the object, which was used as the template for the amplification of a ca. 1.4 kb DNA fragment by the PCR method with two synthetic primers (RsA1U, RsA1L) represented by the following nucleotide sequences.

[0078]

RsA1U:

5'-CACGGCACGGAATACCACTGCGTGCCCGTCGACGACGGTATTCCG
GGGATCCGTCGACC-3' (SEQ ID NO: 7)

RsA1L:

5'-CCAGGTCTGGGAAGGGTGCTCTCCGCGCGAGCGGAGGTGATATCTT
GATTGAGAGGACCAAGGTATCGTCACTTCTGTAGGCTGGAGCTGCTT
C-3' (SEQ ID NO: 8)

[0079] The PCR reaction was conducted with TaKaRa LA Taq™ with GC Buffer (Takara Bio Inc.) under the condition described in Example 1-2). The ca. 1.4 kb DNA fragment containing the pIJ773 derived apramycin resistance gene was purified from the total amount of the reaction fluid with a QIAquick PCR Purification Kit (QIAGEN) according to the attended protocol.

[0080] Cosmid 4-5 was next transferred into E. coli BW25113/pIJ790 (Gust, B. et al., Proceedings of the National Academy of Sciences of the United States of America, (USA), 2003, Vol. 100, p. 1541 - 1546) to give an E. coli BW25113/pIJ790/cosmid4-5 strain. This strain was inoculated into 100 ml of an LB liquid medium (1% bacto trypton, 0.5% yeast extract, 0.5% sodium chloride) containing chloramphenicol, kanamycin and ampicillin in a concentration of 25 µg/ml, 25 µg/ml and 50 µg/ml, respectively, and incubated at 30°C overnight. To a test tube having a volume of 65 ml was charged 10ml of a SOB medium (2% bacto trypton, 0.5% yeast extract, 0.05% sodium chloride, 0.0186% potassium chloride), and chloramphenicol, kanamycin, ampicillin and L-arabinose were added in a concentration of 25 µg/ml, 25 µg/ml, 50 µg/ml and 10 mM, respectively. To the medium was inoculated 100 µl of the culture fluid of E.coli BW25113/pIJ790/cosmid4-5 strain which had been incubated overnight, and the mixture was shake incubated at 30°C for 4 hours. Total amount of the culture fluid was centrifuged at 4°C and 3000 rpm for 5 minutes to collect cells, which were then suspended in 10 ml of an ice-cooled 10% glycerol solution. After reiterating the procedure, the suspension was suspended again in 100 µl of the cooled 10% glycerol solution. Next, to 50 µl of the cell suspension in an Eppendorf tube was added 5 µl of a ca. 1.4 kb DNA fragment solution containing the pIJ773 derived apramycin resistance gene,

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and the mixture was placed into a preliminarily ice-cooled 2 mm gap electroporation cuvette (BM Equipment Co. Ltd.: BM6200). Electroporation was carried out with an Electro Cell Manipulator 600 (BM Equipment Co. Ltd.) under the condition of 12.5 kV, 25 μ F and 128 Q. To the treated cells was added 1 ml of a preliminarily ice-cooled LB liquid medium, and the mixture was statically cultured at 37°C for 1 hour. The culture was spread on an LB agar medium to which 50 μ g/ml of ampicillin and apramycin, respectively, had been added and cultured at 37°C overnight to give a strain which exhibited resistance to both ampicillin and apramycin. This strain was incubated in an LB liquid medium to which 50 μ g/ml of ampicillin and apramycin, respectively, had been added to isolate cosmid AB201.

2) Preparation of cosmid AB202

[0081] Cosmid AB202 having a streptomycin resistance gene and a new restriction enzyme Bsp1407I site inserted into the left terminal of the insertion fragment of cosmid 5-13 (Yanai, K. et al., Proceedings of the National Academy of Sciences of the United States of America (USA), 2006, Vol. 103, p. 9661 - 9666) was prepared in the following method.

[0082] Plasmid pIJ778 (Gust, B, et al., Proceedings of the National Academy of Sciences of the United States of America, (USA), 2003, Vol. 100, p. 1541 - 1546) was first double digested with HindIII and EcoRI and subjected to agarose gel electrophoresis to give a ca. 1.8 kb DNA fragment containing the streptomycin resistance gene as the object, which was used as the template for the amplification of a ca. 1.9 kb DNA fragment by the PCR method with two synthetic primers (RsA2U, RsA2L) represented by the following nucleotide sequences.

[0083]

RsA2U:

5'-CTCGCGCGGGAGCACCCCAGGCTGCCTGCAGAAACTGTACATTC
CGGGGATCCGTCGACC-3' (SEQ ID NO: 9)

RsA2L:

5'-AGTTCGCATCGCCCATCTAAGGAACTGGTGGGCCTTAGCTGTAGGC
TGGAGCTGCTTC-3' (SEQ ID NO: 10)

[0084] A ca. 1.9 kb DNA fragment containing a pIJ778 derived streptomycin resistance gene reaction was purified from the total amount of the reaction fluid with a QIAquick™ PCR Purification Kit (QIAGEN). This fragment was transferred into *E. coli* 25113/pIJ790/cosmid 5-13 by the electroporation method to give a strain which exhibited resistance to both ampicillin and streptomycin. This strain was incubated in an LB liquid medium to which 50 μ g/ml of ampicillin and streptomycin, respectively, had been added to isolate cosmid AB202.

3) Preparation of cosmid203-7

[0085] Cosmid203-7 was prepared by inserting a ca. 16 kb Bsp1407I-EcoRV fragment of cosmid AB201 containing an apramycin resistance gene into the Bsp1407I-EcoRV site of the cosmid AB202.

[0086] First, cosmid AB201 was triply digested with Bsp1407I, EcoRV and SphI and subjected to agarose gel electrophoresis, and a ca. 16 kb Bsp1407I-EcoRV fragment was purified from the agarose gel with a QIAquick™ Gel Extraction Kit (QIAGEN). Next, cosmid AB202 was double digested with Bsp1407I and EcoRV, extracted from the agarose gel in the same manner, and mixed with the purified cosmid AB201 derived vector fragment for ligation reaction.

[0087] The ligated DNA solution was subjected to *in vitro* packaging with a MaxPlax™ Lambda Packaging Extracts (EPICENTRE™ Biotechnologies), transmitted to an *E. coli* XL1-BlueMRA strain and spread on an LB agar medium containing ampicillin (50 μ g/ml) and apramycin (20 μ g/ml). The colonies thus produced were incubated in an LB liquid medium to which 50 μ g/ml of ampicillin and apramycin, respectively, had been added to isolate cosmid 203-7. As a result of analyzing the nucleotide sequences at both terminals of the insertion fragment of cosmid 203-7, it has been revealed that the Bsp1407I fragment (nucleotide sequence of 123007-123183, 177bp) derived from cosmid5-13 has also been inserted simultaneously with the insertion of a BstAUI-EcoRV fragment derived from cosmid4-5. Thus, the cosmid 203-7 is a cosmid which has a deletion of the nucleotide sequence 33306-128995 among the DNA region between RsC-RsD (nucleotide sequence 28935-135581) but contains a 34 bp (nucleotide sequence 94693-94726) and the Bsp1407I fragment (177bp) as the RsA region.

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4) Transfer of cosmid 203-7 into *Streptomyces kanamyceticus*

[0088] Cosmid 203-7 was transferred to an *E. coli* ET12567/pUZ8002 strain (Practical *Streptomyces* Genetics, The John Innes Foundation, (England), Norwick, 2000) according to the ordinary method to give *E. coli* ET12567/pUZ8002/cosmid203-7.

[0089] *Streptomyces kanamyceticus* JCM4775 was conjugated with *E. coli* ET12567/pUZ8002/cosmid203-7 as described in the following. First, a *Streptomyces kanamyceticus* JCM4775 strain was incubated in a seed medium at 28°C for 48 hours, and 100 µl of the culture fluid was spread on a modified R2 agar medium (Sucrose 10.3 g, K₂SO₄ 0.025 g, MgCl₂·6H₂O 1.01 g, Glucose 1 g, Difco Casaminoacids 0.01 g, agar 2.2 g, separately sterilized 10% yeast extract 5ml in 95ml of water). After culturing at 28°C for 7 days, 3ml of a 20% glycerol solution was added and mycelia on the agar medium were collected by scratching. After collecting the cells by centrifugation at 3000 rpm for 5 minutes, the cells were suspended in 3ml of a 20% glycerol solution. On the other hand, after the *E. coli* ET12567/pUZ8002/cosmid 203-7 strain was incubated in an LB liquid medium containing 50 µg/ml of ampicillin and apramycin, respectively, at 37°C for 18 hours, 1 ml of the culture fluid was transplanted in 100ml of an LB liquid medium (50 µg/ml of ampicillin and apramycin, respectively) for incubation at 37°C for 4 hours. The culture fluid (50 ml) was centrifuged at 3000 rpm for 5 minutes to collect the cells, which was suspended in 20 ml of an LB liquid medium. After reiterating twice the procedure, the cells were suspended in 2ml of an LB liquid medium.

[0090] 100 µl of the cell suspension of *Streptomyces kanamyceticus* JCM4775 and 100 µl of the cell suspension of *E. coli* ET12567/pUZ8002/cosmid203-7 were combined in a 1.5 ml volume tube and centrifuged to collect cells, which were suspended in 100 µl of a 20% glycerol solution and spread on a 20 ml volume MS agar medium (agar: 2%, mannitol: 2%, soybean powder: 2%, 10 mM MgCl₂). After culturing at 28°C for 18 hours, 1 ml of sterilized water containing 400 µg of apramycin and 1500 µg of nalidixic acid was layered. After culturing at 28°C for 5 days, 1 strain of the *Streptomyces kanamyceticus* colonies was picked up, homogenized with a glass homogenizer and spread on a Nutrient agar medium (Difco, Nutrient Broth, containing 2% agar) containing 20 µg/ml of apramycin and 10 µg/ml of nalidixic acid to culture at 28°C for 4 days. The developed colonies were inoculated in a seed medium, cultured at 28°C for 48 hours, mixed with the same amount of a 20% skimmed milk solution, and then lyophilized for storage (*Streptomyces kanamyceticus* RsAcos3 strain).

5) Detection of recombination site (RsB/RsA) by the PCR method

[0091] The lyophilized cells (L-tube) of the *Streptomyces kanamyceticus* RsAcos3 strain obtained in 4) described above were inoculated in a seed medium, cultured for 48 hours (1st generation). A 1 ml portion of the cells was then transplanted in a seed medium containing 250 µg/ml of kanamycin and cultured for 48 hours (2nd generation). Furthermore, 1 ml portion of the cells was transplanted in a seed medium containing 2000 µg/ml of kanamycin and cultured for 48 hours (3rd generation). After completing the incubation of the first, second and third generations, respectively, for 48 hours, the cells were collected by centrifuging a 30 ml portion of each culture fluid at 7500 rpm for 10 minutes and lyophilized. The 1/10 portion of the dry cells was used for preparing chromosomal DNA with an MFX-6000 system (TOYOBO) in the same manner as described in Example 1-2).

[0092] Next, an experiment for detecting recombination in the RsA region and the RsB region by the PCR method was carried out with the chromosomal DNAs in the 1st, 2nd and 3rd generations, respectively. The experiment was carried out in the same manner as described in Example 1-2), except synthetic primers used are KM-18' (5'-CTC-GACAAGGTCTGCAAGCC-3', SEQ ID NO: 11) and M19'L (5'-ATCTTGATTGAGAGGACCA-3', SEQ ID NO: 12). As a result, it has been revealed that the ca. 0.9kb DNA fragment as the object is not amplified with any chromosomal DNAs, and the *Streptomyces kanamyceticus* RsAcos3 strain has no capacity for amplifying the DNA region between the regions RsA and RsB. Thus, it has been shown that the gene required for DNA amplification is present in the nucleotide sequence 33306 to 128995 in the nucleotide sequence of Accession No. AB254080.

Example 3: Preparation of *Streptomyces kanamyceticus* AB305cure strain having a deletion of ca. 37kb DNA region between the RsC-RsA regions

[0093] A *Streptomyces kanamyceticus* AB305cure strain having a deletion of a DNA region between the RsC-RsA regions (nucleotide sequence 50603 to 87960 in the nucleotide sequence Accession No. AB254080) was prepared in the following method.

1) Construction of plasmid pAB305

[0094] A ca. 3.4 kb fragment A (nucleotide sequence 47230-50602) was amplified with cosmid 2-1 (Non-patent literature 2) as a template and AfrU: 5'-GGAGAAGCATGCGAGGACAAGTCGCGGCTTGAAC-3' (SEQ ID NO: 13) and AfrLRV:

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5'-CAGGCGGATCCCTGCGATATCCGTAGCGCGCATAAACGAAGAA-3' (SEQ ID NO: 14) as primers by the PCR method. The fragment was double digested with BamHI and SphI and inserted into the BamHI-SphI site of pUC118 to give a plasmid pAB301.

[0095] Next, a ca. 3.9 kb fragment B (nucleotide sequence 87961-91943) was amplified with cosmid1-3 (Yanai, K. et al., Proceedings of the National Academy of Sciences of the United States of America (USA), 2006, Vol. 103, p. 9661 - 9666) as a template and BfrU: 5'-GCAGATGGATCCAGAGTCTAGATTCAGCTCGTTGATCACCATGTC-3 (SEQ ID NO: 15) and BfrL: 5'-CAGGCGAATTCGCGTGGGAATCGCTCCGCATCTT-3' (SEQ ID NO: 16) as primers by the PCR method. The fragment was double digested with BamHI and EcoRI and inserted into the BamHI - EcoRI site of pUC118 to give a plasmid pAB302.

[0096] Next, the fragment B derived from pAB302 (BamHI - EcoRI fragment) was inserted into the BamHI - EcoRI site of pAB301 to give a plasmid pAB303. In addition, in order to transfer a thiostrepton resistance gene (tsr) to pAB303, the plasmid pIJ 702 (Practical Streptomyces Genetics, The John Innes Foundation, (England), Norwich, 2000) was digested with BclI, and the ca. 1 kb BclI fragment containing the tsr gene was inserted into the BamHI site of pUC118 to give a plasmid pUC118tsr. The plasmid was double digested with XbaI and SmaI, and the XbaI - SmaI fragment containing the tsr gene was inserted into the EcoRV - XbaI site of pAB303 to give a plasmid pAB304 containing the fragment A - tsr gene - fragment B as an insertion fragment. Furthermore, pAB304 was double digested with SphI and EcoRI, and the fragment A - tsr gene - fragment B was isolated as a ca. 8.5 kb SphI - EcoRI fragment and inserted into the SphI - EcoRI site of pSET152 (Practical Streptomyces Genetics, The John Innes Foundation, (England), Norwich, 2000) to give a plasmid pAB305.

2) Preparation of Streptomyces kanamyceticus AB305 cure strain and evaluation of DNA amplification capacity

[0097] Plasmid pAB305 was transferred into an E. coli ET12567/pUZ8002 strain according to the ordinary method to give an E. coli ET12567/pUZ8002/pAB305 strain.

[0098] Next, the conjugation of Streptomyces kanamyceticus JCM4775 and E. coli ET12567/pUZ8002/pAB305. was carried out in the same manner as described in Example 2-4). The apramycin resistance strain thus obtained was confirmed again thiostrepton resistance with a Nutrient agar medium containing 20 µg/ml of apramycin and 10 µ/ml of thiostrepton and referred to as the Streptomyces kanamyceticus AB305 strain. PCR carried out with a chromosomal DNA prepared from the Streptomyces kanamyceticus AB305 strain as a template and primers 4tsrU: 5'-ataagcgccctcgttctctcg-3' (SEQ ID NO: 17) and BfrLoutL: 5'-gactcaccctcagccagaat-3' (SEQ ID NO: 18) led to the amplification of a ca. 4 kb DNA fragment. It has been shown from the result that the plasmid pAB305 has been incorporated into the chromosomal DNA of Streptomyces kanamyceticus JCM4775 by the homologous recombination of the fragment B region.

[0099] Next, an apramycin sensitive and thiostrepton resistant strain was separated from the Streptomyces kanamyceticus AB305 strain in the following procedure. The Streptomyces kanamyceticus AB305 strain was cultured in a seed medium at 28°C for 48 hours (1st generation). A 1 ml portion of the culture fluid was inoculated in a fresh seed medium and further cultured at 28°C for 48 hours (2nd generation). The same operation was reiterated until the fifth generation, and on and after the third generation, five glass beads having a diameter of 5 mm were added to the seed medium so that mycelia can be readily disentangled. The culture fluid of the fifth generation was diluted so as to be separated as a single colony and spread on a Nutrient agar medium. After 72 hours, the grown-up colony was replicated on a Nutrient agar medium to which thiostrepton (10 µg/ml) and apramycin (20 µg/ml) had been added. As a result of examining the phenotypes of 5400 strains, 48 apramycin sensitive strains were obtained, and 7 strains of them showed thiostrepton resistance. Chromosomal DNA was prepared from these strains, which was referred to as the Streptomyces kanamyceticus AB305cure strain since it has been confirmed by PCR that the nucleotide sequence 50603 to 87960 among the nucleotide sequence of Accession No. AB254080 had been substituted by the tsr gene.

[0100] In order to examine the DNA amplification capacity of the Streptomyces kanamyceticus AB305cure strain, the strain was inoculated in a seed medium (40ml). It was cultured for 48 hours (1st generation), and a 1 ml portion of the culture fluid was transplanted in a seed medium containing 250 µg/ml of kanamycin and cultured for 48 hours (2nd generation). Furthermore, a 1 ml portion was inoculated in a seed medium containing 2000µg/ml of kanamycin and cultured for 48 hours (3rd generation). After completing the culture of the first and third generations, respectively, for 48 hours, the cells were collected by centrifuging a 30 ml portion of each culture fluid at 7500 rpm for 10 minutes and lyophilized. The 1/10 portion of the dry cells was used for preparing chromosomal DNA with an MFX-6000 system (TOYOBO) in the same manner as described in Example 1-2).

[0101] Next, an experiment for detecting recombination in the regions RsA and RsB was carried out with the chromosomal DNAs of the first and third generations by the PCR method in the same manner as described in Example 1-2). It has been revealed from the result that no amplifiable DNA fragments were detected with the chromosomal DNA of the first generation, whereas a 1.2kb amplification fragment was obtained with the chromosomal DNAs of the third generations, respectively, and thus the Streptomyces kanamyceticus AB305cure strain had an amplification capacity of the DNA region between the regions RsA and RsB. Thus, it has been indicated from comparing the deletion region of the

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Streptomyces kanamyceticus RsAcos3 strain which had no amplification capacity of the DNA region between the regions RsA and RsB described in Example 2 that the gene required for DNA amplification is present between the nucleotide sequences 33306 to 50602 and 87961 to 128995 of Accession No. AB254080.

Example 4: Preparation of a Streptomyces kanamyceticus M27 strain having deletions of ca. 37 kb and ca. 22 kb DNA regions between regions RsC-RsA and RsA-RsD respectively

[0102] A Streptomyces kanamyceticus M27 strain having deletions in the region RsC-RsA (nucleotide sequence 50603 to 87960 of the nucleotide sequence of Accession No. AB254080) and the region RsA-RsD (nucleotide sequence 97641 to 120061 of the nucleotide sequence of Accession No. AB254080) was prepared in the following method.

1) Preparation of cosmid 3-7::AB402

[0103] Cosmid 3-7 (Yanai, K. et al., Proceedings of the National Academy of Sciences of the United States of America (USA), 2006, Vol. 103, p. 9661 - 9666) was transferred into an E. coli BW25113/pIJ790 strain to give an E. coli BW25113/pIJ790/cosmid 3-7 strain. A DNA fragment containing a ca. 1.4 kb apramycin resistance gene was amplified by PCR with a ca. 1.3 kb "EcoRI - HindIII fragment described in Example 2-1) as a template and primers 97682U (5'-TCTTCTGTCGTCTCATCCATCGTGCTGGCCTTCGATGACATTCCGG GGATCCGTCGACC-3', SEQ ID NO: 19) and 120181L (5'-GGGAAAGTACGGGAAAGATCTCGGTTACTCGCGATCCATGTAGG CTGGATCTGCTTC-3', SEQ ID NO: 20). The competent cell of the E. coli BW25113/pIJ790/cosmid3-7 strain was prepared in the same manner as described in Example 2-1), and transformation was carried out by electroporation with the DNA fragment containing a ca. 1.4 kb apramycin resistance gene described above. A transformant having resistance to both apramycin and ampicillin (E. coli BW25113/cosmid 3-7::AB402 strain) was cultured to give cosmid 3-7::AB402.

2) Preparation of Streptomyces kanamyceticus M27 strain and evaluation of DNA amplification capacity

[0104] Next, the cosmid3-7::AB402 was transferred into an E. coli ET12567/pUZ8002 strain to give E. coli ET12567/pUZ8002/cosmid 3-7::AB402. The Streptomyces kanamyceticus AB305cure strain and the E. coli ET12567/pUZ8002/cosmid3-7::AB402 were conjugated in the same manner as described in Example 2-4), and the apramycin resistant strain thus obtained was referred to as the Streptomyces kanamyceticus M27 strain. The chromosomal DNA of the Streptomyces kanamyceticus M27 strain was prepared, and it has been confirmed by PCR with a variety of primers that the insertion fragment of cosmid 3-7::AB402 was incorporated into the chromosomal DNA of Streptomyces kanamyceticus by double crossover homologous recombination. Thus, it has been indicated that the Streptomyces kanamyceticus M27 strain has deleted the regions of the nucleotide sequences 50603 to 87960 and 97641 to 120061 in the nucleotide sequence of Accession No. AB254080.

[0105] In order to examine the DNA amplification capacity of the Streptomyces kanamyceticus M27 strain, the strain was inoculated in a seed medium (40ml) and cultured for 48 hours (1st generation). Next, a 1 ml portion of the culture fluid was transplanted in a seed medium containing 250 µg/ml of kanamycin and cultured for 48 hours (2nd generation). A 1 ml portion of the culture fluid was further transplanted in a seed medium containing 2000µg/ml of kanamycin and cultured for 48 hours (3rd generation).

[0106] After completing the culture of the first and third generations for 48 hours, the cells were collected by centrifuging a 30 ml portion of each culture fluid at 7500 rpm for 10 minutes and lyophilized. A 1/10 portion of the dry cells was used for preparing chromosomal DNA with an MFX-6000 system (TOYOBO) in the same manner as described in Example 1-2).

[0107] Next, an experiment for detecting recombination in the regions RsA and RsB by the PCR method was carried out with the chromosomal DNAs of the first and third generations in the same manner as described in Example 1-2). It has been revealed from the result that no amplifiable DNA fragments were detected with the chromosomal DNA of the first generation, whereas a 1.2kb amplification fragment was obtained with the chromosomal DNA of the third generation and thus the Streptomyces kanamyceticus M27 strain had an amplification capacity of the DNA region between the regions RsA and RsB. Thus, it has been indicated from comparing the deletion region of the Streptomyces kanamyceticus RsAcos3 strain which had no amplification capacity of the DNA region between the regions RsA and RsB described in Example 2 that the gene required for DNA amplification is present between the nucleotide sequences 33306 to 50602, 87961 to 97640 and 120062 to 128995 in the nucleotide sequence of Accession No. AB254080.

Example 5: Preparation of cosmid AB501 and M29 strain

[0108] Cosmid AB501 for preparing a strain which deleted all of the regions of the nucleotide sequences 29219 to 87960, 97641 to 120061 and 120621 to 139619 in the nucleotide sequence of Accession No. AB254080 was prepared by the method described below.

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1) Preparation and screening of cosmid library of chromosomal DNA of Streptomyces kanamyceticus M27 strain

[0109] After the Streptomyces kanamyceticus M27 strain prepared in Example 4 was cultured in a seed medium at 28°C for 48 hours, a 1 ml portion of the culture fluid was transplanted in 40 ml of a modified YEME liquid medium (0.3% Difco yeast extract, 0.5% Difco Bacto-peptone, 0.3% Oxoid malt extract, 0.1% Glucose, 3.4% Sucrose, 5 mM MgCl₂·6H₂O, 0.5% Glycine) charged in a 250 ml Erlenmeyer flask and cultured at 28°C for 24 hours, and the cells were collected by centrifugation. The cells for four flasks were suspended in 40 ml of a SET buffer (75 mM NaCl, 25 mM EDTA (pH8), 20 mM Tris-HCl (pH7.5)). To the suspension was added 800 µl of a 50mg/ml aqueous lysozyme solution, and the mixture was maintained at 37°C for 60 minutes. A 1120 µl portion of a 20mg/ml aqueous Proteinase K solution and 4.8 ml of a 10% SDS solution were added, and the mixture was warmed at 55°C for 2 hours. After the addition of 16 ml of a 5M NaCl solution and 40 ml of chloroform and enough mixing, the mixture was left standing at room temperature for 30 minutes and centrifuged at 4500 x g and room temperature for 15 minutes, and the aqueous layer was poured into a new tube. The mixture was diluted with a 0.6-fold volume of isopropanol, and after 3 minutes DNA was rolled around a Pasteur pipet with a closed tip, rinsed with 70% ethanol, subjected to natural drying and dissolved in 5ml of a TE buffer. DNA thus obtained had a concentration of 0.75mg/ml.

[0110] The chromosomal DNA thus prepared was partially digested with MboI and dephosphorylated with CIAP (calf intestinal alkaline phosphatase). On the other hand, SuperCos 1 (Stratagen) as a cosmid vector was digested with XbaI, then dephosphorylated with CIAP and further digested with BamHI. These were mixed and subjected to ligation reaction with Mighty Mix 6023 (Takara Bio Inc.) at 26°C for 10 minutes. The ligated DNA solution was subjected to in vitro packaging with a MaxPlax™ Lambda Packaging Extracts (EPICENTRE™ Biotechnologies), transmitted to an E.coli XL1-BlueMRA strain, and spread on an LB agar medium containing ampicillin (50 µg/ml) and apramycin (20 µg/ml). The colonies thus produced were incubated in an LB liquid medium containing 50 µg/ml of ampicillin and apramycin, respectively, at 37°C overnight to isolate the cosmid DNAs and analyze the nucleotide sequences at both terminals of the insertion fragment of each cosmid. Cosmid 1-10 was selected as a clone which contained NdeI at the position of 29213 and AflII at the position of 139611 on the nucleotide sequence of Accession No. AB254080.

2) Preparation of cosmid203-7::str

[0111] In order to substitute and insert the NdeI - AflII fragment (ca. 33 kb) of cosmid 1-10 into the NdeI - AflII site of cosmid 203-7 described in Example 2-3), the apramycin resistance gene of cosmid 203-7 was intended to be substituted with a streptomycin resistance gene. A ca. 1.9 kb DNA fragment containing the streptomycin resistance gene was amplified with the pJ778 derived HindIII - EcoRI fragment described in Example 2-2) as a template and primers RsA1Ussp (5'-CACGGCACGGAATACCACTGCGTGCCCGTCGACGACAATATTCCGGG GATCCGTCGACC-3', SEQ ID NO: 21) and RsA1LRV (5'-CAGACTCTGAGTGATATCTTGATTGAGAGGACCAAGGTTGTAGG CTGGAGCTGCTTC-3', SEQ ID NO: 22). E.coli BW25113/pJ790/cosmid 203-7 was transformed by the electroporation method with this DNA fragment. A cosmid DNA was prepared from a transformant which is sensitive to apramycin, but resistant to both ampicillin and streptomycin and referred to as cosmid 203-7: : str.

3) Preparation of cosmid AB501

[0112] The triple digestion product of cosmid 1-10 with NdeI, DraI and AflII and the double digestion product of cosmid 203-7::str with NdeI and AflII were mixed and subjected to ligation reaction with Mighty Mix 6023 (Takara Bio Inc.). The DNA solution after reaction was subjected to in vitro packaging with MaxPlax™ Lambda Packaging Extracts (EPICENTRE™ Biotechnologies), transmitted to an E.coli XL1-BlueMRA strain and spread on an LB agar medium containing ampicillin (50 µg/ml) and apramycin (20 µg/ml). The streptomycin sensitivity and apramycin resistance of the colonies were confirmed, and clones were selected by confirming the recombination at the NdeI site by PCR with KM37 (5'-TCTGCTCACC TCTGCGTCAG-3', SEQ ID NO: 23) and tsrL (5'-TGACGAATCGAGGTCGAGGA-3', SEQ ID NO: 24) derived from a thiostrepton resistance gene to prepare cosmid AB501.

[0113] It has been confirmed from the detection of a ca. 8 kb fragment not corresponding with the theoretical restriction enzyme map on digesting the cosmid AB501 with KpnI that a NdeI - AflII fragment derived from cosmid 1-10 as the insertion fragment was deleted during the course of the construction of cosmid 501. Thus, the ca. 4 kb XbaI - KpnI fragment which estimated to contain the deletion region was subcloned from the cosmid AB501 into pUC19 to analyze the nucleotide sequence. As a result, it has been revealed that the nucleotide (T) at 120620 and the nucleotide (T) at 139620 in the nucleotide sequence of Accession No. AB254080 were ligated and thus the nucleotide sequence 120621 to 139619 had been deleted. In consequence, the cosmid AB501 is composed of the nucleotide sequences 16650 to 29218, 87961 to 97640, 120062 to 120620 and 139620 to 146821 in the nucleotide sequence of Accession No. AB254080. In this connection, the nucleotide sequence 114645 to 114723 derived from cosmid 5-13, which does not affect the preparation of a Streptomyces kanamyceticus M29 strain described in the following, was added to the upstream of the

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nucleotide at 16650 position.

4) Preparation of *Streptomyces kanamyceticus* M29 strain and evaluation of DNA amplification capacity

[0114] Cosmid AB501 was transferred into an *E. coli* ET12567/pUZ8002 strain to give *E. coli* ET12567/pUZ8002/cosmid AB501. The *Streptomyces kanamyceticus* JCM4775 strain and the *E. coli* ET12567/pUZ8002/cosmid AB501 were conjugated in the same manner as described in Example 2-4), and 100 apramycin resistant strains thus obtained were spread on a Nutrient agar medium containing apramycin (20 µg/ml) and thiostrepton (10 µg/ml) to examine their sensitivities to thiostrepton. The 98 strains was sensitive to thiostrepton and estimated to be generated by double crossover homologous recombination in the nucleotide sequence regions 87961 to 97640 and 139620 to 146821, and thus it has been indicated that these strains are not the objective strain. On the other hand, the 2 strains are resistant to thiostrepton and estimated to be generated by double crossover homologous recombination in the nucleotide sequence regions 16650 to 29218 and 139620 to 146821. Furthermore, the PCR analysis of chromosomal DNAs of these two strains revealed that the vector part of the cosmid was not inserted into the chromosome and these strains were the double crossover homologous recombinant strains, so that this strain was referred to as the *Streptomyces kanamyceticus* M29 strain. The *Streptomyces kanamyceticus* M29 strain is a strain which has deleted the region of the nucleotide sequences 29219 to 87960, 97641 to 120061 and 120621 to 139619 in the nucleotide sequence of Accession No. AB254080.

[0115] In order to examine the DNA amplification capacity of the *Streptomyces kanamyceticus* M29 strain, the strain was inoculated in a seed medium (40ml) and cultured for 48 hours (1st generation). Next, a 1 ml portion of the culture fluid was transplanted in a seed medium containing 250 µg/ml of kanamycin and cultured for 48 hours (2nd generation). A 1 ml portion of the culture fluid was further transplanted in a seed medium containing 2000 µg/ml of kanamycin and cultured for 48 hours (3rd generation).

[0116] After completing the culture of the first and third generations for 48 hours, the cells were collected by centrifuging a 30 ml portion of each culture fluid at 7500 rpm for 10 minutes and lyophilized. A 1/10 portion of the dry cells was used for preparing chromosomal DNA with an MFX-6000 system (TOYOBO) in the same manner as described in Example 1-2).

[0117] Next, an experiment for detecting recombination in the regions RsA and RsB was carried out with the chromosomal DNAs of the first and third generations by the PCR method in the same manner as described in Example 1-2). It has been revealed from the result that no amplifiable DNA fragments were detected with the chromosomal DNA of the first generation, whereas a 1.2kb amplification fragment was obtained with the chromosomal DNA of the third generation and thus the *Streptomyces kanamyceticus* M29 strain had an amplification capacity of the DNA region between the regions RsA and RsB. Thus, it has been indicated from comparing the deletion region of the *Streptomyces kanamyceticus* RsAcos3 strain which had no amplification capacity of the DNA region between the regions RsA and RsB described in Example 2 that the gene required for DNA amplification is present between the nucleotide sequences 87961 to 97640 and 120062 to 120620 in the nucleotide sequence of Accession No. AB254080.

Example 6: Preparation of *Streptomyces kanamyceticus* AB113-2 strain and evaluation of DNA amplification capacity

1) Preparation of cosmid 5-13::AB113

[0118] A ca. 1.4 kb DNA fragment was amplified by PCR with a pIJ773 derived *Eco*RI - *Hind*III fragment as a template and primers M13U (5'-GGAGCACTTGCCGGTCTGGCCAGAACGCGGACGCCGTCATTCC GGGGATCCGTC-GACC-3', SEQ ID NO: 25) and M13L (5'-AGAGCAGTCAGGCTGGCAACCGCACATCCACGCGATCGTTGTAG GCT-GGAGCTGCTTC-3', SEQ ID NO: 26) according to the method described in Example 2-1). *E. coli* BW25113/pIJ790/cosmid5-13 was transformed by the electroporation method with this DNA fragment, and cosmid 5-13::AB113 was obtained from the apramycin resistant transformant thus produced.

2) Preparation of *Streptomyces kanamyceticus* AB113-2 strain and evaluation of DNA amplification capacity

[0119] The cosmid 5-13::AB113 was transferred into the *E. coli* ET12567/pUZ8002 strain to give *E. coli* ET12567/pUZ8002/cosmid 5-13::AB113. The *Streptomyces kanamyceticus* JCM4775 strain and *E. coli* ET12567/pUZ8002/cosmid 5-13::AB113 were conjugated in the same manner as described in Example 2-4) to give an apramycin resistant strain. The PCR analysis of the chromosomal DNA of the apramycin resistant strain thus obtained revealed that the cosmid5-13::AB113 was incorporated into the chromosomal DNA by the double crossover homologous recombination through the *Streptomyces kanamyceticus* derived DNA regions at both sides of the apramycin resistance gene, and this strain was referred to as the *Streptomyces kanamyceticus* AB113-2 strain. The *Streptomyces kanamyceticus* AB113-2 strain is the strain which have deleted the nucleotide sequence 118626 to 130558 in the nucleotide sequence of Accession No. AB254080.

[0120] In order to examine the DNA amplification capacity of the *Streptomyces kanamyceticus* AB113-2 strain, the

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strain was inoculated in a seed medium (40ml) and cultured for 48 hours (1st generation). Next, a 1 ml portion of the culture fluid was transplanted in a seed medium containing 250 µg/ml of kanamycin and cultured for 48 hours (2nd generation). A 1 ml portion of the culture fluid was further transplanted in a seed medium containing 2000 µg/ml of kanamycin and cultured for 48 hours (3rd generation). After completing the culture of the first and third generations for 48 hours, the cells were collected by centrifuging a 30 ml portion of each culture fluid at 7500 rpm for 10 minutes and lyophilized. A 1/10 portion of the dry cells was used for preparing chromosomal DNA with an MFX-6000 system (TOYOBO) in the same manner as described in Example 1-2).

[0121] Next, an experiment for detecting recombination in the regions RsA and RsB by the PCR method was carried out with the chromosomal DNAs of the first and third generations in the same manner as described in Example 1-2). It has been revealed from the result that no amplifiable DNA fragments were detected with the chromosomal DNA of the first generation, whereas a 1.2kb amplification fragment was obtained with the chromosomal DNA of the third generation and thus the *Streptomyces kanamyceticus* AB113-2 strain had an amplification capacity of the DNA region between the regions RsA and RsB. Thus, it has been indicated in combination with the results described in Examples 2 and 5 that the gene required for DNA amplification is present in the nucleotide sequence 87961 to 97640 in the nucleotide sequence of Accession No. AB254080.

Example 7: Preparation of orf1082 gene disruption strain and evaluation of DNA amplification capacity

[0122] Genes present in the nucleotide sequence 87961 to 97640 in the nucleotide sequence of Accession No. AB254080 are 8 genes of *orf1079* to *orf1086*. Among them, the *orf1082* gene product (SEQ ID NO: 1) exhibited identity with a DNA relevant protein, so that the *orf1082* gene disruptant was prepared in the manner described below and its DNA amplification capacity was evaluated.

[0123] A ca. 1.4 kb DNA fragment was amplified by PCR with a pIJ773 derived *EcoRI* - *HindIII* fragment as a template and primers M8U (5'-TCAAGACCTCCGATACGGGCTTCTGTGCCGTTTCAGTCGAATTCG GGGATCCGTCGACC-3', SEQ ID NO: 27) and M8L (5'-CAACGCCGTCGACCTCTACGCGAGGACACGGTGGAGAATGTAG GCTGGAGCT-GCTTC-3', SEQ ID NO: 28) in the same manner as described in Example 2-1). *E. coli* BW25113/pIJ790/cosmid 1-3 obtained by transferring cosmid 1-3 into the *E. coli* BW25113/pIJ790 strain was transformed by the electroporation method with this DNA fragment, and cosmid 1-3: AB108 was obtained from the apramycin resistant transformant thus produced.

[0124] Next, *E. coli* ET12567/pUZ8002/cosmid1-3:AB108 was obtained by transferring the cosmid 1-3: AB108 into a *E. coli* ET12567/pUZ8002 strain. The *Streptomyces kanamyceticus* JCM4775 strain and *E. coli* ET12567/pUZ8002/cosmid 1-3: AB108 were conjugated in the same manner as described in Example 2-4) to give an apramycin resistant strain. PCR analysis of the chromosomal DNA of the *Streptomyces kanamyceticus* AB1-3(8) strain among the apramycin resistant strains thus obtained has revealed that the cosmid 1-3: AB108 was incorporated into the chromosomal DNA by the double crossover homologous recombination through the *Streptomyces kanamyceticus* derived DNA regions at both sides of the apramycin resistance gene, and that the *Streptomyces kanamyceticus* AB1-3(8) strain was an *orf1082* gene disruptant.

[0125] The *Streptomyces kanamyceticus* AB1-3(8) strain was inoculated in a seed medium (40ml). After culture for 48 hours (1st generation), a 1 ml portion of the culture fluid was then transplanted in a seed medium containing 250 µg/ml of kanamycin and cultured for 48 hours (2nd generation). A 1 ml portion of the culture fluid was further transplanted in a seed medium containing 2000 µg/ml of kanamycin and cultured for 48 hours (3rd generation).

[0126] After completing the culture of the first, second and third generations for 48 hours, the cells were collected by centrifuging a 30 ml portion of each culture fluid at 7500 rpm for 10 minutes and lyophilized. A 1/10 portion of the dry cells was used for preparing chromosomal DNA with an MFX-6000 system (TOYOBO) in the same manner as described in Example 1-2).

[0127] Next, an experiment for detecting recombination in the RsA region and the RsB region by the PCR method was carried out with the chromosomal DNAs in the 1st, 2nd and 3rd generations, respectively. As a result, it has been revealed that the ca. 1.2 kb DNA fragment as the object is not amplified with any chromosomal DNAs and the *Streptomyces kanamyceticus* AB1-3(8) strain has no capacity for amplifying the DNA region between the regions RsA and RsB. Thus, it has been shown that the gene *orf1082* (SEQ ID NO: 2) is the gene essential to amplifying the DNA region between the regions RsA and RsB

Example 8: Expression of melanin gene inserted into kanamycin biosynthetic genes

[0128] Melanin biosynthetic genes consisting of the genes *melC1* and *melC2* as the genes derived from a heterologous strain (Berman, V. et al., Gene, 37, 101-110 (1985): The nucleotide sequence of the tyrosinase gene from *Streptomyces antibioticus* and characterization of the gene product) registered as Accession No. M11582 in the database of Genbank were inserted between the regions RsA and RsB which were present on the chromosomal DNA of *Streptomyces kan-*

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kanamyceticus JCM4775.

[0129] After plasmid pSET152 was triply digested with *Bam*HI, *Sph*I and *Hind*III and subjected to agarose gel electrophoresis, a ca. 2.8 kb *Bam*HI - *Sph*I fragment containing an apramycin resistance gene was extracted from the gel and purified. Also, in order to obtain an insertion fragment, after pKM95 (Yanai, K. & Murakami, T., Journal of Antibiotics, (Japan), 2004, Vol. 57, p. 351 - 354) was double digested with *Bam*HI and *Sph*I and subjected to agarose gel electrophoresis, a 3.25 kb *Bam*HI - *Sph*I fragment containing a kanamycin biosynthetic gene, *orf9* gene was extracted from the gel and purified. After both DNA fragments were mixed and ligated with a ligation kit (Takara Bio Inc.), *E. coli* DH5 α was transformed. A plasmid pAB101 was prepared from an apramycin resistant transformant.

[0130] Next, after plasmid pIJ702 was triply digested with *Bam*HI, *Eco*RV and *Nde*I and subjected to agarose gel electrophoresis, a 2.97 kb *Bam*HI - *Eco*RV fragment containing a *melC1* gene and a *melC2* gene was extracted from the gel and purified. This fragment was inserted into the *Bam*HI - *Eco*RV site of a plasmid pAB101 to give a plasmid pAB102 (9.02 kb).

[0131] The plasmid pAB102 was transferred to an *E. coli* ET12567/pUZ8002 strain to give *E. coli* ET12567/pUZ8002/AB102. The *Streptomyces kanamyceticus* JCM4775 strain and the *E. coli* ET12567/pUZ8002/AB102 were conjugated in the same manner as described in Example 2-4) to give apramycin resistant strains. The PCR analysis of the chromosomal DNA of the *Streptomyces kanamyceticus* JCM4775/AB102-4 strain among the apramycin resistant strains revealed that the plasmid pAB102 was incorporated in a chromosomal DNA by single crossover homologous recombination through a DNA region derived from the kanamycin biosynthetic genes.

[0132] The *Streptomyces kanamyceticus* JCM4775/AB102-4 strain was inoculated in a seed medium (40ml). After culture for 48 hours (1st generation), a 1 ml portion of the culture fluid was then transplanted in a seed medium containing 250 μ g/ml of kanamycin and cultured for 48 hours (2nd generation). A 1 ml portion of the culture fluid was further transplanted in a seed medium containing 2000 μ g/ml of kanamycin and cultured for 48 hours (3rd generation).

[0133] After completing the culture of the first, second and third generations for 48 hours, the cells were collected by centrifuging a 30 ml portion of each culture fluid at 7500 rpm for 10 minutes and lyophilized. A 1/10 portion of the dry cells was used for preparing chromosomal DNA with an MFX-6000 system (TOYOBO) in the same manner as described in Example 1-2).

[0134] Next, an experiment for detecting recombination in the RsA and RsB regions by the PCR method was carried out with the chromosomal DNAs in the 1st, 2nd and 3rd generations, respectively in the same manner as described in Example 1-2). It has been revealed from the result that no amplifiable DNA fragments were detected with the chromosomal DNA of the first generation, whereas a 1.2kb amplification fragment was obtained with the chromosomal DNAs of the second and third generations and that the DNA region between the regions RsA and RsB containing pAB102 was amplified in these strains.

[0135] Next, after the first and third generation strains of the *Streptomyces kanamyceticus* JCM4775/AB102-4 were cultured in a seed medium containing 1% casamino acid, 0.05% tyrosine and 0.0005% copper sulfate for 48 hours, and the production amount of melanin in the supernatant was examined. The examination was carried out according to the partial modification of the method described by Mun, Y. et al., Biological and Pharmaceutical Bulletin, (Japan), 2004, Vol. 27, p. 806 - 809. That is to say, after a 2N sodium hydroxide solution containing 20% dimethyl sulfoxide and the supernatant were mixed in an equivalent amount and heated at 80°C for 30 minutes, dark thick floaters were removed to measure the absorbance of the strains at 475 nm with Hitachi spectrophotometer (U-2810). The first generation strain showed an absorbance of 0.42, and the third generation strain showed an absorbance of 0.62. Thus, it has been revealed that the third generation strain produced a large amount of melanin with the increase in copies of a melanin producing gene as compared with the first generation strain.

Example 9: DNA amplification in *Streptomyces kanamyceticus* which deleted the DNA region between RsC-RsD

1) Preparation of plasmid pKM2003

[0136] *Sma*-Stu-1 (5'-GGGAGGCCTA-3', SEQ ID NO: 29) and *Sma*-Stu-2 (5'-AGCTTAGGCCTCCC-3', SEQ ID NO: 30) were annealed and subjected to ligation with plasmid pUC119 which had preliminarily been double digested with *Hind*III and *Sma*I to give plasmid pUC119-Stu.

[0137] Next, a ca. 6.6 kb *Sma*I fragment (nucleotide sequence 88479 to 95063 in the nucleotide sequence of Accession No. AB254080) containing the RsA region and the *orf1082* gene was prepared from cosmid 1-3 and inserted into the *Sma*I site of pUC119-Stu. The plasmid obtained was digested with *Kpn*I, and the orientation of an insert fragment was examined to select a plasmid that the *Kpn*I site (94889) present in the *Sma*I fragment was inserted in the side of the *Hind*III site of pUC119-Stu, which was designated pKM2001.

[0138] A ca. 4.1 kb *Stu*I fragment consisting of the nucleotide sequence 135493 to 139615 in the nucleotide sequence of Accession No. AB254080 was prepared from cosmid 5-13 (Yanai, K. et al., Proceedings of the National Academy of Sciences of the United States of America, (USA), 2006, Vol. 103, p. 9661 - 9666) and inserted into the *Stu*I site of

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pKM2001, and a plasmid in which the StuI fragment was inserted downstream of the SmaI fragment in the direction of the nucleotide sequence 135493 - 139615 was selected and designated pKM2002.

[0139] Actinomyces conjugal transfer plasmid pSET152 (Bierman, M. et al., Gene, (Holland), 1992, Vol. 116, p. 43 - 49) was digested with SphI, blunted with T4 DNA polymerase, and then ligated with a HindIII linker (Takara Shuzo Co., Ltd.) to construct pSET153. A ca. 2.8 kb HindIII - EcoRI fragment derived from pSET153 and a ca. 10.7 kb HindIII - EcoRI fragment derived from pKM2002 were ligated to construct a conjugal transferable plasmid pKM2003.

2) Transfer of pKM2003 to Streptomyces kanamyceticus and evaluation of DNA amplification capacity

[0140] The plasmid pKM2003 was transferred to an E. coli ET12567/pUZ8002 strain (Practical Streptomyces Genetics, The John Innes Foundation, (England), Norwich, 2000) according to the ordinary method to give E. coli ET12567/pUZ8002/pKM2003.

[0141] As the Streptomyces kanamyceticus strain to which pKM2003 was transferred was used strain 12-6-4 in which a 106.6 kb DNA region between the regions RsC and RsD (nucleotide sequence 28935-135581) in the nucleotide sequence of Accession No. AB254080 (Yanai, K. et al., Proceedings of the National Academy of Sciences of the United States of America, (USA), 2006, Vol. 103, p. 9661 - 9666). The Streptomyces kanamyceticus 12-6-4 strain is the strain which has deleted the RsA region and the DNA region between RsC and RsD containing the orf1082 gene and thus has no DNA amplification capacity. The Streptomyces kanamyceticus 12-6-4 strain and E. coli ET12567/pUZ8002/pKM2003 were conjugated in the same manner as described in Example 2-4). PCR was carried out with chromosomal DNA prepared from the resultant apramycin resistant strain as a template and KM-25: 5'-CCGCTCT-CATTCCGGTCAG-3' (SEQ ID NO: 31) and KM-202: 5'-CCCCTGACTTTCGTCGAG-3' (SEQ ID NO: 32) as primers to amplify a ca. 4.6 kb DNA fragment. It has been revealed from this result that the plasmid pKM2003 was incorporated in the chromosomal DNA of the Streptomyces kanamyceticus 12-6-4 strain by the homologous recombination of the StuI fragment region.

[0142] This strain was inoculated in a seed medium (40ml) in order to examine the DNA amplification capacity of the strain and cultured for 48 hours (1st generation), and then a 1 ml portion of the culture was transplanted into a seed medium containing 500 µg/ml of kanamycin and cultured for 48 hours (2nd generation). Subculture was further carried out in the same manner with kanamycin increased to concentrations of 2000 µg/ml, 4000 µg/ml and 6000 µg/ml to give culture fluids of the third, fourth and fifth generations, respectively. A 5 ml portion of the culture fluid of the first and fifth generations, respectively, was centrifuged at 7500 rpm for 10 minutes to collect the cells. The chromosomal DNA was prepared from the resultant cells by the salting out method (Practical Streptomyces Genetics, The John Innes Foundation, (England), (Norwich), 2000).

[0143] Next, an experiment for detecting recombination in the RsA and RsB regions by the PCR method was carried out with the chromosomal DNAs in the 1st and 5th generations, respectively in the same manner as described in Example 1-2). In this connection, KM-201: 5'-CCATCCCGTCGAAGAGCC-3' (SEQ ID NO: 33) was used in place of KM-17' as the recombination detection primer. As a result, no amplifiable DNA fragments were detected with the chromosomal DNA of the first generation, whereas a 1.0 kb amplification fragment was obtained with the chromosomal DNAs of the fifth generation. It has been confirmed from the nucleotide sequence analysis that the amplified DNA fragment was the DNA fragment consisting of the anticipated nucleotide sequence. It has been revealed from the result that the Streptomyces kanamyceticus 12-6-4/pKM2003 strain had a capacity of amplifying the DNA region between the regions RsA and RsB. Thus, it has been indicated that the gene required for DNA amplification is present in the SmaI fragment contained in pKM2003, that is, between the nucleotide sequence 88479 to 95063 in the nucleotide sequence of Accession No. AB254080.

Example 10: DNA amplification in Streptomyces coelicolor and Streptomyces lividans

1) Preparation of cosmid pAB801

[0144] The mixture of two oligonucleotides A (5'-AATTC CCTGCAGG TCTAGA ACTAGT A-3', SEQ ID NO: 34) and B (5'-AGCTT ACTAGT TCTAGA CCTGCAGG G-3', SEQ ID NO: 35) obtained by the chemical synthesis of modifying 5' terminal with a phosphate group were annealed and ligated with pUC19 which had preliminarily been double digested with EcoRI and HindIII to construct a plasmid pUC19-linker. The multiple cloning site of this plasmid is EcoRI-SbfI-XbaI-SpeI-HindIII.

[0145] A ca. 10 kb XbaI fragment (containing the nucleotide sequence 87961-97640 in the nucleotide sequence of Accession No. AB254080) which contains the RsA region obtained from cosmid AB501 described in Example 5 and an orf1082 gene was inserted into the XbaI site of the pUC19-linker to give plasmid pAB601. The XbaI fragment was inserted in the direction of EcoRI-SbfI-XbaI-RsA-orf1082-XbaI-SpeI-HindIII to the multiple cloning site of the pUC19-linker.

[0146] Next, a streptomycin resistance gene fragment having the SpeI and SbfI sites at both ends was transferred

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into a cosmid pKM7 (Yanai, K. et al., Proceedings of the National Academy of Sciences of the United States of America, (USA), 2006, Vol. 103, p. 9661 - 9666) in the same manner as described in Example 2. First, a ca. 1.9 kb DNA fragment was amplified by PCR with a HindIII-EcoRI fragment containing a streptomycin resistance gene obtained from plasmid pIJ778 as a template and pKM7Δ12U (5'-GATCCCCGTGCACACCGAGGGCGAGCTCGCCCCGACTAGTATTC

5 CGATCCGTCGACC-3', SEQ ID NO: 36) and pKM7Δ12L (5'-GTCGGTCACCGCCCGTACGACGGCCGGTCCGCCT-GCAGGTGTA GGCTGGAGCTGCTTC-3', SEQ ID NO: 37) as primers. This DNA fragment was used for transforming the *E. coli* BW25113/pIJ790/pKM7 strain obtained by transferring pKM7 to an *E. coli* BW25113/pIJ790 strain by electroporation to give a clone which is resistant to ampicillin and streptomycin. Cosmid pKM7::str was isolated from this clone.

10 [0147] Next, cosmid pAB701 in which a ca. 10 kb SbfI-SpeI fragment derived from plasmid pAB601 (containing the nucleotide sequence 87961-97640 in the nucleotide sequence of Accession No. AB254080) was inserted into the SbfI-SpeI site of cosmid pKM7::str was prepared in the following method. The plasmid pAB601 and the cosmid pKM7::str, respectively, were double digested with SpeI and SbfI, mixed and then subjected to ligation reaction with a Rapid DNA Dephos and ligation Kit (Roche: Cat No. 04898125001) according to the attended instruction. The ligated reactant was subjected to in vitro packaging with a kit (Packaging kits: *E. coli* XL1-BlueMRA and MaxPlax™ Lambda Packaging

15 Extracts (EPICENTRE™ Biotechnologies). The packaged reaction mixture was transmitted an *E. coli* XL1-Blue MRA strain, and spread on an LB agar medium containing ampicillin (100 µg/ml) and kanamycin (100 µg/ml). After culture at 37°C overnight, clones growing on the agar medium could be detected. These clones may contain two cosmids (1) containing pKM7::str and (2) containing pAB701. The clone containing the objective cosmid pAB701 is streptomycin sensitive. Thus, when each clone was replicated on an LB agar medium containing ampicillin (100 µg/ml) and kanamycin

20 (100 µg/ml) and on an LB agar medium containing ampicillin (100 µg/ml), kanamycin (100 µg/ml) and streptomycin (100 µg/ml), 293 strains among 369 clones exhibited streptomycin sensitivity. A cosmid was prepared from these clones, confirmed to give a ca. 10 kb DNA fragment by double digestion with SpeI and SbfI, and designated cosmid pAB701.

[0148] Next, the cosmid pAB701 was transferred into an *E. coli* BW25113/pIJ790 strain to give an *E. coli* BW25113/pIJ790/pAB701 strain. The BW25113/pIJ790/pAB701 strain was transformed with the 5.2 kb SspI fragment derived from plasmid pMJCOS1 (Yanai, K. et al., Proceedings of the National Academy of Sciences of the United States

25 of America, (USA), 2006, Vol. 103, p. 9661 - 9666) and cultured overnight on an LB agar medium containing ampicillin (50 µg/ml) and apramycin (50 µg/ml). A cosmid was prepared from the developed colonies, and designated pAB801 (Figure 2). The cosmid pAB801 has a ca. 10 kb SbfI-SpeI fragment derived from plasmid pAB601 (containing the nucleotide sequence 87961 to 97640 in the nucleotide sequence of Accession No. AB254080) and a ca. 27.8 kb DNA

30 fragment derived from cosmid pKM7 (containing the nucleotide sequence 7107 to 19547 in the nucleotide sequence of Accession No. AB164642 and a 15046 bp nucleotide sequence of Accession No. AB254081) and further the regions oriT, attP, int derived from plasmid pSET152 in the vector part, and thus is a conjugal transferable cosmid to Actinomycetes.

35 (2) Transfer of cosmid pAB801 to *Streptomyces coelicolor* and *Streptomyces lividans* and evaluation of DNA amplification

[0149] Cosmid pAB801 was transferred to an *E. coli* ET12567/pUZ8002 strain according to the method described in Example 2 to give an *E. coli* ET12567/pUZ8002/pAB801 strain.

40 [0150] A *Streptomyces lividans* 1326 strain and a *Streptomyces coelicolor* MT1110 strain were spread on an MS medium (Practical Streptomyces Genetics, The John Innes Foundation, (England), Norwich, 2000), cultured at 30°C for 5 days to form spores. The spores were collected and suspended in 3 ml of sterilized water for preservation. A 200 µl portion of the spore suspension was combined with 400 µl of a 2 x YT liquid medium and treated by heating at 50°C for 10 minutes. On the other hand, the *E. coli* ET12567/pUZ8002/pAB801 strain was inoculated in 50 ml of an LB liquid medium containing chloramphenicol (25 µg/ml), kanamycin (25 µg/ml), ampicillin (50 µg/ml) and apramycin (50 µg/ml)

45 and incubated at 37°C overnight. A 500 µl portion of this culture was transplanted to a fresh LB liquid medium containing chloramphenicol (25 µg/ml), kanamycin (25 µg/ml), ampicillin (50 µg/ml) and apramycin (50 µg/ml) and incubated at 37°C for 4 hours. The cells were collected from total amount of the culture, washed twice with an LB liquid medium containing no antibiotics and suspended into 1.5ml of an LB liquid medium. A 500 µl portion of the suspension was added to the heat treated spore suspension. After centrifugation of the mixture, 50 µl of LB and 50 µl of 2xYT were

50 added to form a suspension. 90 µl and 10 µl of the suspension were spread on MS mediums, respectively, and cultured at 30°C overnight, and 1 ml of sterilized water containing 0.5 mg of nalidixic acid and 1.25 mg of apramycin was layered on each dish. After culture at 30°C for 3 days, the resistant strain was developed over the whole surface in the 90 µl spread section and about 1000 of the resistant strains per dish were developed in the 10 µl spread section. These strains were replicated on MS media (containing 25 µg/ml nalidixic acid and 50 µg/ml apramycin) and cultured at 30°C for 3

55 days. The developed apramycin resistant strain was homogenized, spread on an MS medium (containing 25 µg/ml nalidixic acid and 50 µg/ml apramycin) and cultured at 37°C for 7 days in order to have spores adhered.

[0151] These spores were inoculated in a SOB liquid medium (containing 25 µg/ml nalidixic acid) and a SOB liquid medium (containing 25 µg/ml nalidixic acid) to which 250µg/ml of kanamycin was added and incubated at 30°C for 48

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hours. Cells were collected from each culture fluid, and chromosomal DNA was prepared by the salting out method (Practical Streptomyces Genetics, The John Innes Foundation, (England), Norwick, 2000). DNA recombination was detected in the regions RsA and RsB with these chromosomal DNA as templates according to the method described in Example 1. As a result, no 1.2 kb amplification fragments were detected in the culture section to which kanamycin was not added, whereas 1.2 kb amplification fragment could be detected in the culture section to which kanamycin was added. Thus, it has been revealed that the DNA region between the regions RsA and RsB was amplified in the Streptomyces lividans 1326 and the Streptomyces coelicolor MT1110 strains to which cosmid pAB801 was transferred.

SEQUENCE LISTING

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<120> A method for DNA amplification in cell

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<150> JP 2008-147927

<151> 2008-06-05

<160> 37

<170> PatentIn version 3.1

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50

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 gcagccaagc agctccgggc cgaggccatc aaggctcgca gcactgctca cggcgacgac 4260
 45 caccgcatgt ccggcctcca gaaggaggcg agtctgcgag ctgagcacgg gcgtcagcac 4320
 gtcgacgccg aagaggccgc ggcgccctca tctccgaaac ccgactcctc gtacgccaac 4380
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55 <213> Streptomyces kanamyceticus

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34

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 35
 40
 45
 50
 55

P A T E N T K R A V

1. Fremgangsmåde til amplificering af DNA omfattende at:

fremstille en rekombinant celle omfattende ét hvilket som helst af polynukleotider valgt fra gruppen bestående af de følgende (A) til (E) og en DNA-enhed anbragt i cellege-
5 nomet,

hvor DNA-enheden har en størrelse på 22 til 154 kb og mindst omfatter et første DNA-fragment valgt fra gruppen bestående af de følgende (F) til (H), et målgen og et andet DNA-fragment valgt fra gruppen bestående af de følgende (I) til (K),

hvor målgenet eller polynukleotidet, som er eksogent til værten;

10 at dyrke den rekombinante celle under betingelser til at forårsage genamplifikation for at amplificere DNA-enheden,

(A) et polynukleotid, der koder for et protein bestående af aminosyresekvensen ifølge SEQ ID NO: 1,

(B) et polynukleotid, der koder for et protein, som består af en aminosyresekvens,
15 der har deletionen, substitutionen, insertionen eller additionen af én eller flere af aminosyrer i aminosyresekvensen ifølge SEQ ID NO: 1, og som er funktionelt ækvivalent med den, der består af aminosyresekvensen ifølge SEQ ID NO: 1,

(C) et polynukleotid, der koder for et protein, som består af en aminosyresekvens, der har en identitet på 90% eller mere til aminosyresekvensen ifølge SEQ ID NO: 1, og
20 som er funktionelt ækvivalent med aminosyresekvensen ifølge SEQ ID NO: 1,

(D) et polynukleotid bestående af nukleotidsekvensen ifølge SEQ ID NO: 2 og

(E) et polynukleotid, som hybridiserer til polynukleotidet bestående af nukleotidsekvensen ifølge SEQ ID NO: 2 under stringente betingelser, og som koder for et protein funktionelt ækvivalent med aminosyresekvensen ifølge SEQ ID NO: 1,

25 (F) DNA bestående af nukleotidsekvensen ifølge SEQ ID NO: 3,

(G) DNA, der hybridiserer til DNAet bestående af nukleotidsekvensen ifølge SEQ ID NO: 3 under stringent betingelser og

(H) DNA, der har en identitet på 90% eller mere til nukleotidsekvensen ifølge SEQ ID NO: 3,

30 (I) DNA repræsenteret ved nukleotidsekvensen ifølge SEQ ID NO: 4,

(J) DNA, der hybridiserer til DNAet bestående af nukleotidsekvensen ifølge SEQ ID NO: 4 under stringente betingelser og

(K) DNA, der har en identitet på 90% eller mere til nukleotidsekvensen ifølge SEQ ID NO: 4.

35 2. Fremgangsmåde ifølge krav 1, hvor DNA-enheden omfatter i rækkefølgen fra 5'-terminalen det første DNA-fragment, målgenet og det andet DNA-fragment.

3. Fremgangsmåde ifølge krav 1, hvor målgenet består af et antibiotisk biosyntetisk gencluster.

40 4. Fremgangsmåde ifølge krav 1, hvor DNA-enheden yderligere omfatter et lægemiddel-resistensgen.

5. Fremgangsmåde ifølge krav 1, hvor polynukleotidet er DNA og er anbragt i genomet af den rekombinante celle.

6. Fremgangsmåde ifølge krav 1, hvor værten er en antibiotika-producerende stamme.

5 7. Rekombinant celle, hvor mange kopier af DNA-enheden er indført i et genom ved fremgangsmåden ifølge krav 1.

8. Rekombinant celle ifølge krav 7, hvor målgenet er eksogent til en vært.

9. Anvendelse af en sammensætning til amplificering af DNA ved fremgangsmåden ifølge krav 1, hvor sammensætningen omfattende et protein valgt blandt:

10 1) et protein bestående af en aminosyresekvens ifølge SEQ ID NO: 1,

2) et protein omfattende en aminosyresekvens, som har en deletion, substitution, insertion eller addition af én eller flere aminosyrer i aminosyresekvensen ifølge SEQ ID NO: 1, som er funktionelt ækvivalent med den, der består af aminosyresekvensen ifølge SEQ ID NO: 1 og

15 3) et protein omfattende en aminosyresekvens som har en identitet på 90% eller mere til aminosyresekvensen ifølge SEQ ID NO: 1, som er funktionelt ækvivalent med den, der består af aminosyresekvensen ifølge SEQ ID NO: 1.

10. Anvendelse af en vektor til at amplificere DNA ved fremgangsmåden ifølge krav 1, hvor vektoren omfatter et polynukleotid valgt fra gruppen bestående af (A) til (C) i en styret form:

(A) et polynukleotid, der koder for et protein bestående af aminosyresekvensen ifølge SEQ ID NO: 1,

25 (B) et polynukleotid, der koder for et protein, som består af en aminosyresekvens, der har en deletion, substitution, insertion eller addition af én eller flere af aminosyrer i aminosyresekvensen ifølge SEQ ID NO: 1, og som er funktionelt ækvivalent med den, der består af aminosyresekvensen ifølge SEQ ID NO: 1, og

30 (C) et polynukleotid, der koder for et protein som består af en aminosyresekvens, der har en identitet på 90% eller mere til aminosyresekvensen ifølge SEQ ID NO: 1, og som er funktionelt ækvivalent med den, der består af aminosyresekvensen ifølge SEQ ID NO: 1.

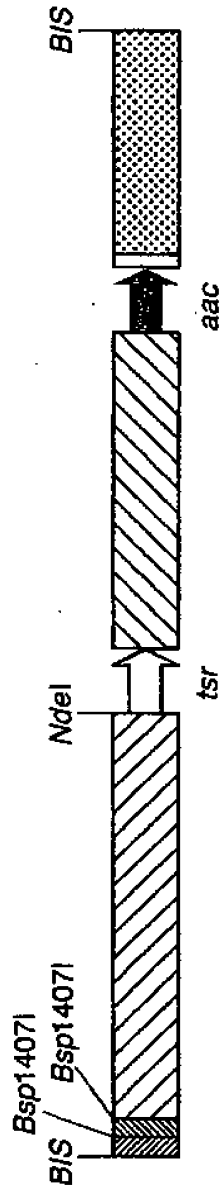
11. Anvendelse af en vektor til amplificering af DNA ved fremgangsmåden ifølge krav 1, vektoren omfattende en DNA-enhed, som har en størrelse på 22 til 154 kb og mindst omfatter et første DNA-fragment valgt fra gruppen bestående af den følgende (F) til (H), et målgen og et andet DNA-fragment valgt fra gruppen bestående af den følgende (I) til (K), og vektoren, som er i stand til at introducere DNA-enheden ind i et cellegenom:

(F) DNA bestående af en nukleotidsekvens ifølge SEQ ID NO: 3,

(G) DNA, der hybridiserer til DNAet bestående af nukleotidsekvensen ifølge SEQ ID NO: 3 under stringente betingelser, og

40 (H) DNA, der har en identitet på 90% eller mere til nukleotidsekvensen ifølge SEQ ID NO: 3,

- (I) DNA repræsenteret ved nukleotidsekvensen ifølge SEQ ID NO: 4,
 - (J) DNA, der hybridiserer til DNAet bestående af nukleotidsekvensen ifølge SEQ ID NO: 4 under stringente betingelser og
 - (K) DNA, der har en identitet på 90% eller mere til nukleotidsekvensen ifølge SEQ ID NO: 4.
- 5
12. Fremgangsmåde ifølge krav 1, hvor DNA-enheden er eksogen til værten.



B/S : Linking part of *Bam*HI and *Sau*3AI

tsr : Thiostrepton resistance gene

aac : Apramycin resistance gene

▨ : nucleotide sequence 114645 to 114726 of AB254080

▨ : nucleotide sequence 123007 to 123183 of AB254080

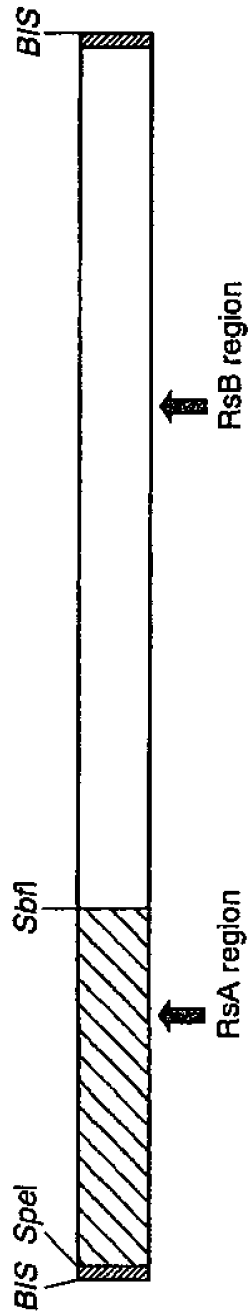
▨ : nucleotide sequence 16650 to 29218 of AB254080

▨ : nucleotide sequence 87961 to 97640 of AB254080

□ : nucleotide sequence 120062 to 120620 of AB254080

▤ : nucleotide sequence 139620 to 146821 of AB254080

FIG. 1



BIS : Linking part of *Bam*HI and *Sau*3AI

- ▨ : DNA region derived from pKM7
- ▧ : nucleotide sequence 87961 to 97640 of AB254080
- : nucleotide sequence 7107 to 19547 of AB164642 and nucleotide sequence 1 to 15046 of AB254081

FIG. 2