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PATENT REQUEST: CONVENTION PATENT

654326

We, being the person identified below as the Applicant, request the grant of a patent to the person identified below as the Nominated Person, for an invention described in the accompanying standard complete specification

Full application details follow:-

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Address:	3-6, Kyobashi 2-chome, Chuo-ku, Tokyo, 104, Japan
Invention Title:	NOVEL PLASMID pBUL1 DERIVED FROM A LACTOBACILLUS AND THE DERIVATIVES THEREOF
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Convention Details

Application Number	Country	Country Code	Date of Application
3-112674	Japan	JP	22 February 1991
3-87338	Japan	JP	28 March 1991
3-183922	Japan	JP	28 June 1991

Drawing number recommended to accompany the abstract Fig. 1.

D A T E D this 14th day of October, 1992.

MEIJI MILK PRODUCTS COMPANY LIMITED
By their Patent Attorneys:
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AUSTRALIA

PATENT

NOTICE OF ENTITLEMENT

We, MEIJI MILK PRODUCTS COMPANY LIMITED, of 3-6, Kyobashi 2-chome, Chuo-ku, Tokyo, 104, Japan, being the applicant and the person nominated for grant of patent in respect of the Application for an invention entitled NOVEL PLASMID pBUL1 DERIVED FROM A LACTOBACILLUS AND THE DERIVATIVES THEREOF
state the following:-

PCT-CONVENTION NATIONAL PHASE FILING

The person nominated for the grant of the patent has entitlement from the inventor(s) by virtue of an assignment of the invention from the actual inventor(s).

The person nominated for grant of the patent is the applicant of the basic application(s).

The basic application(s) listed on the request form and in the declaration made under Article 8 of the PCT are the first application(s) made in a Convention country in respect of the invention.

The person nominated for the grant of the patent is the depositor of the deposit listed hereinafter

Accession No.	Institution	Date
FERM BP-3758	Fermentation Research Institute Agency of Industrial Science and Technology	29 January 1991

14 October 1992

Michael J. Houlihan
Registered Patent Attorney

Date

To: The Commissioner of Patents

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NOVEL PLASMID PBUL1 DERIVED FROM LACTOBACILLUS AND DERIVATIVE THEREOF

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(57) Claim

1. Isolated plasmid pBUL1.

2. A pure culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* FERM BP-3758.

3. A DNA fragment designated by the thick line in Figure 1 which region is about 4 kbp between the position of the Eco47III site and the position about 1.1 kbp clockwise from the ScaI site and about 0.45 kbp counterclockwise from the NdeI site which region replicates pBUL1.

4. A plasmid into which the DNA fragment of claim 3 has been inserted.

6. A microorganism transformed with the plasmid of claim 4.

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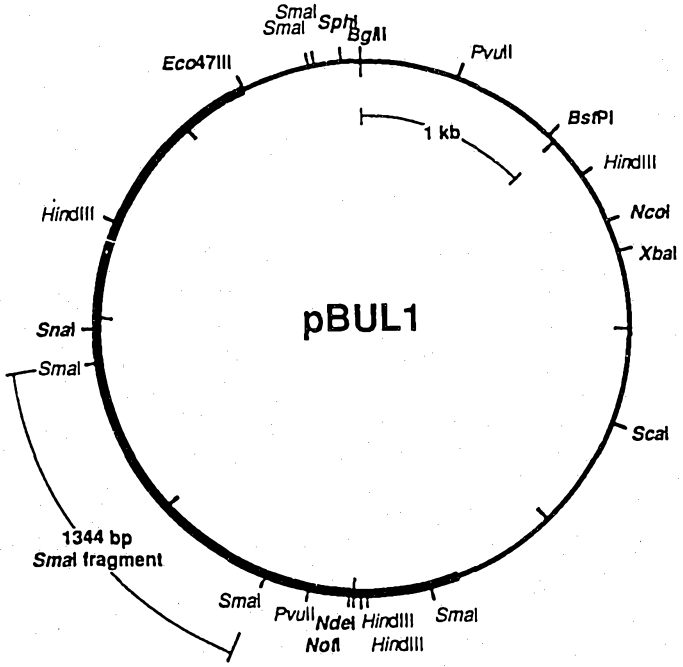
(54) Title: NOVEL PLASMID pBUL1 DERIVED FROM LACTOBACILLUS AND DERIVATIVE THEREOF

(54) 発明の名称 乳酸桿菌由来の新規なプラスミドpBUL1及びその誘導体

(57) Abstract

A plasmid pBUL1 having a length of about 7.9 kb and a restriction map, as shown, and its derivative. The plasmid is isolated from *Lactobacillus delbrueckii* subsp. *bulgaricus* M-878 strain and is useful as a vector for breeding various microorganisms such as lactic acid bacteria. The derivative is useful also as a shuttle vector (lactic acid bacterium-*Escherichia coli*).



(57) 要約

本発明は図１で示される制限酵素切断地図を有する長さ約７．９ｋｂのプラスミドｐＢＵＬ１とその誘導体に関する。

このプラスミドは、ラクトバチルス・デルブリュッキー・サブスピーシーズ・ブルガリカス（Ｌａｃｔｏｂａｃｉｌｌｕｓ　ｄｅｌｂｒｕｅｃｋｉｉ　ｓｕｂｓｐ．ｂｕｌｇａｒｉｃｕｓ）Ｍ－８７８株から分離された。

このプラスミドは、乳酸菌等各種微生物の育種用ベクターとして有用であり、その誘導体はシャトルベクター（乳酸菌－大腸菌）としても利用可能である。

SPECIFICATION

Title of the Invention:

NOVEL PLASMID pBUL1 DERIVED FROM A LACTOBACILLUS AND THE DERIVATIVES THEREOF

Field of the Invention:

The present invention relates to a novel circular double-stranded DNA plasmid pBUL1 derived from Lactobacillus delbrueckii subsp. bulgaricus, the utility and safety of which as a yogurt-producing bacterium have widely been admitted, and to the derivatives thereof, as well as to microorganisms as transformed with the plasmids.

Prior Art and Problems to be Solved by the Invention:

Lactic acid bacteria are very useful microorganisms which have been used in production of various fermented foods from old times. If a recombinant DNA technology which has rapidly been developed in recent years could be applied to lactic acid bacteria, much more enhancement of the utility of the bacteria would be expected. In fact, host-vector systems with a fairy high efficiency have already been reported for lactic acid bacteria of some species of, for example, Lactococcus lactis (reference 1), Streptococcus salivarius subsp. thermophilus (reference 2), Lactobacillus plantarum (reference 3) and Lactobacillus casei (reference 4), and the current stage of the technical improvement is to try industrial application of the host-vector systems.

However, despite of great efforts by many researchers, there has hitherto been no report of transformation of Lactobacillus delbrueckii subsp. bulgaricus and Lactobacillus delbrueckii subsp. lactis (hereinafter referred to as Lb. bulgaricus, and Lb. lactis, respectively), which are used extensively as bacteria for producing milk products such as yogurt. Application of broad-host-range plasmids, such as pNZ12 (reference 5), pGK12 (reference 6) and pIL253 (reference 7), with which transformants of lactic acid bacteria of some species have been obtained, to the above-mentioned two subspecies has been attempted, but transformation was not successful. Under the situation, establishment of host-vector systems of the above-mentioned two subspecies has been desired earnestly.

If a recombinant DNA technology is applied to microorganisms to be used in production of foods, the safety of vectors to be used for transformation of them must be established. As such vectors, those are desirable which exist naturally in microorganisms that have been used to produce foods as eaten from the past and the safety of those has been historically confirmed. On the other hand, fermented milk products such as yogurt are foods which have been eaten for a long period of time and the safety of which has been well confirmed. Therefore, the plasmids derived from microorganisms from fermented milk products, for example, those of the above-mentioned two subspecies, are useful vectors in order to

construct transformants of microorganims for food production. In addition they are also available vectors for transformation for the purpose of producing various physiologically active substances.

Means for Solving the Problems:

For the purpose of developing host-vector systems for Lb. bulgaricus and Lb. lactis, the present inventors variously investigated and studied plasmids of these subspecies. As a result, they are the first who have succeeded in isolating a plasmid from Lb. bulgaricus M-878 strain (FERM BP-3758) possessed by Meiji Institute of Health Science of Meiji Milk Products Co., Ltd., which has a length of about 7.9 kbp, which has a restriction endonuclease map as shown in Fig. 1, and which does not have recognition sites for BamHI, EcoRI, KpnI, PstI and SalI, the base sequence of the SmaI fragment with 1344 bp being represented as the Sequence Number 1 of the Sequence Table in Table 1 below. They named the plasmid pBUL1.

Table 1:

Sequence Table:

Sequence Number : 1

Length of Sequence : 1344

Type of Sequence : Nucleic Acid

Number of Strand : Double-stranded

Topology : Linear

Kind of Sequence : Other Nucleic Acid

Origin :

Name of Microorganism : Lactobacillus delbrueckii subsp. bulgaricus

Name of Strain : M-878

Sequence:

CCCGGGGCGA AACGACATGG GGCGCTCAAA CCATTGCTGA GGCGATCAAT TACGTGCAAG 60

CCCAGCATCC CGATCACGGC TATTTCCCAG CTCGCCAAAA TTCCGGCATG AGGGTTGTTG 120

AACCGGGTGA AAGAGCCACA GGCGAAACGC TTAGAATTAC GATTGACGGA CAGGAAAGAG 180

AATTTCCGTT CAACGGCTTT TTCTATAACC GGGATTATGA AATGACCGAG GTTGGGTTTG 240

CTAACAGGTT TGCCGATTGG TACGCCAAAG GGAAACTTGT TTATCACCCC GGCTTAAAGG 300

CGTGGCTTAT GTACAACCCA GAAACGGGGT CATGGATGCC GAATGAAGAC GACAGACTGG 360

GCAAGGATTT TAACCAGACC CCGGAAAAAC TCATCGATAA CTTGCGGATA AACCTCAAAT 420

TTGAACAACC GCTATGGAAA AAAGTTGGGT TTAATCCCCA AAAGCCTAAT AATCAAACAT 480

TCGGGGAAAA GGCTTATGCT AGTGGCTATA GCCGGATCAG CACGGCCGCT GGGCAAAAGG 540

CGACCCTTGA ATTAGCTCAG AGCCGTTTAA CCGTGCGTGC ATTTAACGAC TGTAAGACCG 600

AGCTTAACAC CCAGACAGGT TGGATTGACC TCAAAACGGG TGCTATTAGC CCTCACAACC 660

CGGCGAAACT TTTTGACAAG GTCACGGATG CTGGTTTGCC TAATAAAGCC ACAGAGGGTG 720

ATGGCGGCAA GCTCTGGGAT CGTTTCCTGA AAGAGACCTT TTGCGGCGAT CTTGATTTGA 780

TCGAGTTCGT ACAGGCCTGC ATAGGCTACA GCATTACGGG CAAAATCAAT GAACAGGTCA 840

TGTTTATCTG CAAAGGCAGT GGGGGCAACG GGAAGAGCAT TTTTCTTGAA TGCTTAAACG 900

AGGTGCTGGG CGATTATAGC TCTGTTATCC CAATAGAAAC GCTAACAGAC AACGGCAAGG 960

CTCAGCGTGA CGGATCAGCA CCAAGTCCAG ACCTTGCAAG CCTTGAGGGC AAGCGGTTCG 1020

TTATTACGAG CGAACCGAAA GAGCAGGTTA CAATCGATGC TGGGACGGTC AAAACGGTGA 1080

CGGGTGGCAC TAAGTTAAAA GTTAGAATGC TACACCAAAA CCCGATTGAG TTCCTGCCAC 1140

AGTTTAAAAT TTGGTGGCAA TCTAACGGCT TGCCAAACGT CAACTTTAAC GATTATGCTA 1200

TTTTACGGCG CTTGATCGTC ATCCCGTTTA AAAATGAGGT GCGAGAGGAT GCGGTAGATA 1260

TCAACCTCAA AAGCAAGCTA ATGAAAGAGA AAGAGTTTAT TTTAAAGTGG TGTGTTGAGG 1320

GCGTGGCTAA ATGGCAAGCC CGGG 1344

Brief Explanation of the Drawings:

Figure 1:

This is a restriction endonuclease map of pBUL1. The recognition site of each restriction enzyme is expressed by kbp unit, on the basis of BglII. pBUL1 does not have recognition sites for BamHI, EcoRI, KpnI, PstI and SalI. In the structure of pBUL1, the region necessary for replication of pBUL1 is shown by the thick line; and the region corresponding to the base sequence of the Sequence Number 1 (the third largest fragment of all SmaI fragments) is designated as "1344 bp SmaI fragment" in the outer periphery of the map.

Figure 2:

This shows a scheme of construction of the erythromycin-resistance cassette plasmid p8Em1, in which △ indicates the pUC118-derived multiple cloning site.

Figure 3:

This shows restriction endonuclease maps of the derivatives of pBUL1, i.e., pX3, pX4, pS3 and pS4 (Although all of these plasmids are circular, they are represented as linear profiles on the basis of BglII.). In the drawing, the fine lines each indicate the sequence of pBUL1, and the thick lines each indicate the pAMβ1-derived erythromycin-resistance gene.

Figure 4:

This shows restriction endonuclease maps of plasmids pU8ST8 and pU8ST9. In the drawing, the stripe arrows each indicate

the L-lactate dehydrogenase gene of Streptococcus salivarius subsp. thermophilus M-192 strain (ST-LDH gene); and the fine lines each indicate the sequence of plasmid pUC118. bla indicates the ampicillin-resistance gene.

Figure 5:

This shows restriction endonuclease maps of plasmids pXL38, pXL39, pXL48 and pXL49 (Although all of these plasmids are circular, they are represented as linear profiles on the basis of BglII.). In the drawing, the fine lines each indicate the sequence of pBUL1, the thick lines each indicate the pAMβ1-derived erythromycin-resistance gene, and the stripe arrows each indicate ST-LDH gene.

Figure 6:

This shows a scheme to presume the region indispensable for replication of pBUL1 by deletion method.

Figure 7:

This shows a restriction endonuclease map of pBR3Δ18E1 plasmid by ligation of pX3Δ18E plasmid with the E. coli plasmid (pBR322). In the drawing, the fine line indicates the sequence derived from plasmid pBR322, the thick line indicates the sequence derived from pX3Δ18E, the arrow (Em^r) indicates the erythromycin-resistance gene, and the arrow (Ap^r) indicates the ampicillin-resistance gene.

Figure 8:

This shows a restriction endonuclease map of pBRΔ18E2

plasmid. The symbols are the same as those shown in Fig. 7.

Figure 9:

This shows a restriction endonuclease map of p8X3Δ18E1 plasmid by ligation of pX3Δ18E plasmid with the E. coli plasmid (pUC118). In the drawing, the fine line indicates the sequence derived from plasmid pUC118, the thick line indicates the sequence derived from pX3Δ18E, the arrow (Em^r) indicates the erythromycin-resistance gene, and the arrow (Ap^r) indicates the ampicillin-resistance gene.

Figure 10:

This shows a restriction endonuclease map of p8X3Δ18E2 plasmid. The symbols are the same as those shown in Fig. 9.

Since the phenotype encoded by the pBUL1 was cryptic, an erythromycin-resistance gene was added to the plasmid as a selective marker in transformation experiments (Erythromycin may be referred to simply as "Em" hereunder.). As a result, the present inventors have succeeded in obtaining transformants expressing the above-mentioned selective marker in microorganisms of three genera, namely Bacillus subtilis, Lactococcus lactis subsp. lactis and Lactobacillus delbrueckii subsp. lactis. The fact indicates that the plasmid pBUL1 (hereinafter often referred to as "the plasmid of the invention") has a broad host range in Gram-positive bacteria. The present invention has been completed on the basis of these findings.

Since the gene which participates in self-replicatability of pBUL1 is considered to be encoded in a part of the plasmid DNA of the present invention as reported in other plasmids, any other plasmid derivatives derived either by deletion of unnecessary regions for replication from the plasmid of the invention, or by insertion or addition of any other DNA to pBUL1 are also considered to have the same function as the plasmid of the invention. For instance, as described in Example 6 followed hereinafter, any plasmid causes no hindrance in replication, if it contains, as a region necessary for replication, a region necessary for replication in about 4 kbp DNA fragment indicated by the thick line in Fig. 1 between

the recognition site for Eco47III and the position about 1.1 kbp apart clockwise from the ScaI site, namely about 0.45 kbp apart counterclockwise from the NdeI site. Therefore, the present invention is not restricted to only the plasmid pBUL1 itself but widely includes other derivative plasmids as obtained by modifying it as well as other recombinant plasmids as obtained by inserting other gene(s), for example, marker(s) such as Em-resistance gene or exogeneous gene(s) such as L-lactate dehydrogenase gene, or other promoter(s) or operator(s) into it.

As derivative plasmids of pBUL1 of the present invention, for example, there are mentioned plasmids containing necessary regions for replication ~~in plural parts~~ in the region of about 4 kbp shown as the thick line in Fig. 1 as mentioned above. As one example of them, there is the mentioned plasmid pX3Δ18E (Fig. 6, the bottom). This plasmid can be replicated not only in Gram-positive bacteria such as lactic acid bacteria but also in E. coli, as is obvious from Example 8 followed hereunder. This plasmid itself is nothing but a plasmid with a broad-host-range, which has been desired in this technical field. Since this plasmid is one which can be replicated not only in Gram-positive bacteria (e.g., lactic acid bacteria, Bacillus subtilis) but also in Gram-negative bacteria (e.g., E. coli), it is effective as a novel versatile shuttle vector which has extremely high practicability and can be used in both Gram-positive bacteria and Gram-negative bacteria.

Another examples of the pBUL1-derived plasmids can be obtained by ligation of other plasmids (fragments) with the region of pBUL1 necessary for replication, ~~such as SphI-EcoRI fragment of plasmid pX3Δ18E,~~ involved in the region of about 4 kbp shown as the thick line area in Fig. 1, or a DNA fragment of pBUL1 which contains this region. As the other plasmids to be ligated, various plasmids may be used widely. As some examples of them, there are exemplified E. coli-derived plasmids such as pBR series plasmids and pUC series plasmids.

Also the thus ligated plasmids have been identified to be replicable in both lactic acid bacteria and E. coli, like the above-mentioned plasmid pX3Δ18E, and they may alos be used as plasmids with a broad-host-range which have heretofore been strongly desired in this technical field or as shuttle vectors between Gram-positive bacteria and Gram-negative bacteria. As examples of such ligated plasmids, there are exemplified pBR3Δ18E1, pBR3Δ18E2, p8X3Δ18E1, p8X3Δ18E2 (Figs. 7 to 10), etc.

As mentioned above, plasmid pBUL1 and its derivative plasmids show a broad-host-range and are useful. Using them as a vector, expression of heterogeneous genes in lactic acid bacteria is possible.

For instance, where a recombinant plasmid was constructed by ligation of pBUL1 plasmid with an erythromycin-resistance gene as a selective marker gene and an L-lactate dehydrogenase

gene derived from a yogurt lactococcus, *Streptococcus salivarius subsp. thermophilus* (e.g., pXL38 in Fig. 5) as an exogenous gene, and the recombinant plasmid was introduced into *Lb. lactis* which does not naturally have L-lactic acid producibility, the resulting erythromycin-resistance transformant produced not only D-lactic acid but also almost the same amount of L-lactic acid as metabolic end-products.

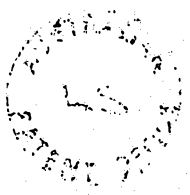
Thus, plasmid pBUL1 and its derivative plasmids are useful also as a vector for expression of heterogeneous genes in lactic acid bacteria.

For preparing the plasmid pBUL1 of the present invention, *Lb. bulgaricus* M-878 strain is first cultivated in a liquid medium which is used for cultivation of lactic acid bacteria, for example, LCM medium (reference 8), according to ordinary methods of cultivating lactic acid bacteria under ordinary incubation conditions. Next, the incubated cells are collected and are then subjected to lysis by known methods for lactic acid bacteria, for example, by using an enzyme such as lysozyme or mutanolysin, etc. From the resulting cell lysate, the intended plasmid can be isolated and purified by a usually employed method, such as phenol extraction and cesium chloride density gradient centrifugation in the presence of ethidium bromide. For construction of the derivative plasmids of pBUL1, which are other plasmids of the present invention, the plasmid pBUL1 may be treated for digestion, ligation and

others by known methods (reference 10).

In order to transform microorganisms by introducing thereinto a recombinant plasmid as obtained by inserting a selective marker gene to the plasmid pBUL1, a known method which is considered to be the best for the host may be selected from conventional methods, such as calcium chloride method, protoplast-polyethylene glycol method, electroporation method, etc., with no particular limitation, according to the characteristics of microorganisms to be transformed. Where lactic acid bacteria are used as a host, the electroporation method is preferred. A marker for selecting the transformants may be selected from various antibiotic-resistance genes known in this technical field. Where the transformed microorganisms are intended to be used in producing foods or medicines, use of markers which have been confirmed to have a high safety is desirable. If desired, other base sequences participating in control of expression, such as promoters, may also be inserted into the plasmids of the invention.

Recombinant plasmids are considered to be safe which are constructed from pBUL1 plasmid or its derivative, an enzyme gene derived from a microorganism used in food production and a safe selective marker gene. Therefore, the transformants of food-producing bacteria such as lactic acid bacteria with such a safe plasmid are also considered to be safe. Such safe transformants may be incubated by ordinary methods to produce



a large amount of enzymes and physiologically active substances, and they may be used in various food productions in ordinary ways to attain the intended objects. In addition, since the safety of the transformants is highly assured in any case, they do not cause by-production of any biohazards or harmful substances. Accordingly, it is expected that they could advantageously be utilized in industrial production of medicines and foods which especially need safety.

Next the present invention will be explained hereunder by way of the following examples.

Example 1 (Preparation of Plasmid pBUL1):

Lactobacillus delbrueckii subsp. bulgaricus M-878 strain (FERM BP-3758, as possessed by Meiji Institute of Health Science; herein often referred to simply as M-878 strain) was subcultivated with a skim milk medium (liquid medium as prepared by dissolving 10% skim milk powder and 0.1% yeast extract in distilled water and sterilized at 121°C for 7 minutes) and inoculated (0.5%) in 6 liters of LCMG medium (as prepared by adding 1% (w/v) glucose to LCM medium) and incubated at 37°C for 15 hours.

After the incubation, the cells were collected by centrifugation and washed twice with 20 mM Tris-HCl buffer (pH 7.0). The washed cells were suspended in 480 ml of a hypertonic buffer (20 mM Tris buffer containing 0.3 M raffinose, 5 mM magnesium chloride and 5 mM calcium chloride;

pH 7.0). To this were added mutanolysin and lysozyme in an amount of 5 μg/ml and 500 μg/ml, respectively, as the final concentrations, and were incubated at 37°C for 10 minutes. Then, 54 ml of 250 mM EDTA (pH 8.0) was added to the resulting solution, which was then subjected to centrifugation to collect the precipitates.

The precipitates were then suspended in 240 ml of 50 mM Tris buffer (pH 8.0) containing 6.7% (w/v) sucrose and 25 mM EDTA. The resulting suspension was then processed according to Anderson & Mckay method (reference 9), from lysis with SDS to rough purification of plasmid DNA.

The crude plasmid DNA preparation thus obtained was subjected to RNase treatment by an ordinary method (reference 10) and then to cesium chloride density gradient centrifugation in the presence of ethidium bromide to obtain about 1 μg of purified pBUL1 plasmid DNA.

The pBUL1 plasmid was cut with various commercially available restriction enzymes, and the length of each fragment obtained was calculated after agarose gel electrophoresis. As a result, pBUL1 was identified to be a circular double-stranded DNA plasmid having the restriction endonuclease map in Fig. 1 with a total length of about 7.9 kbp. pBUL1 did not have recognition sites for BamHI, EcoRI, KpnI, PstI and SalI. Of five fragments obtained by digestion of pBUL1 with SmaI, the third largest fragment (1344 bp; the position of which has been

designated in the outer periphery of the restriction endonuclease map in Fig. 1 as "1344 bp SmaI fragment") was analyzed with respect to the base sequence thereof, which is shown as Sequence Number 1 in Table 1.

Example 2 (Addition of Selective Marker Em-resistance Gene to pBUL1):

First, conjugatively transmissible plasmid pAMβ1 (reference 11) derived from Enterococcus faecalis was cut with HhaI and subjected to agarose gel electrophoresis to thereby cut out a gel fraction containing DNA fragments ranging about 1.1 kbp having the Em-resistance gene. DNAs were isolated from the thus cut-out gel using a GENECLEAN DNA purifying kit (product by BIO101 Co.). The DNA fragment was ligated to E. coli-derived pUC118 plasmid (product by Takara Shuzo Co.) according to the process shown in Fig. 2 to prepare "cassette plasmid" p8Eml in order to excise the Em-resistance gene successfully with various restriction enzymes.

Next, about 0.25 μg of p8Eml DNA was cut with XbaI and was ligated with a fragment as obtained by cutting about 0.025 μg of pBUL1 DNA with XbaI. A half of the reaction mixture after the ligation was used in transformation of Bacillus subtilis 207-25 strain (reference 13) by the method of Chang et al (reference 12). One third of the transformed cells was spread on a plate of DM3 medium containing 25 μg/ml of erythromycin and incubated for 2 days at 37°C to obtain

Em-resistant transformants.

Plasmid DNAs were prepared from the transformants obtained, and the restriction endonuclease cleavage pattern of the plasmids was analyzed. When pBUL1 and the Em-resistance gene were ligated at XbaI site, five transformants of all the nine analyzed contained a plasmid having the restriction endonuclease map of A in Fig. 3; and two of them contained a plasmid having the restriction endonuclease map of B in Fig. 3. The plasmid with the map A in Fig. 3 was named pX3; and that with the map B in the same was named pX4.

In the same manner as above, except that p8Em1 was cut with SmaI and pBUL1 was cut with ScaI, and the resulting fragments were subjected to blunt end ligation followed by transformation of Bacillus subtilis 207-25 strain with the ligated products, Em-resistant transformants were also obtained. Five of all the six transformants, analyzed with respect to plasmids therein, contained a plasmid having the restriction endonuclease map of C in Fig. 3; and one of them contained a plasmid having the restriction endonuclease map of D in Fig. 3. These plasmids were named pS3 and pS4, respectively.

As mentioned above, recombinant plasmids pX3, pX4, pS3 and pS4 (all having a length of about 9.0 kbp) were constructed by introducing the pAMβ1-derived Em-resistance gene (having a length of about 1.1 kb) to pBUL1. In addition, it was shown that pBUL1 could function as a plasmid replicon in

Bacillus subtilis.

Example 3 (Transformation of Lactococcus lactis subsp. lactis):

Lactococcus lactis subsp. lactis (hereinafter often referred to simply as "Lc. lactis") IL1403 strain (as obtained from Dr. Alain Chopin, INRA, France) was used. To this was introduced pBUL1 having the Em-resistance gene, whereby a transformant of Lc. lactis showing Em-resistance was successfully obtained. The details are mentioned below.

From the transformants of Bacillus subtilis obtained in Example 2, the recombinant plasmids, pX3 and pX4, constructed by inserting the Em-resistance gene into pBUL1 were prepared by the method of reference 14.

Next, pX3 and pX4 each were introduced into Lc. lactis IL1403 strain according to the method of reference 1. The transformants were obtained by selecting on agar plates of BL medium (product by Eiken Chemical Co.) containing 25 μg/ml of Em.

The transformants were incubated in LCMG medium, and plasmids were obtained from the cells after Anderson et al. (reference 9). The plasmids thus obtained were shown to have the same restriction enzyme recognizing sites as those of the plasmids used for the transformation. From the result, it was concluded that pBUL1 also functioned as a plasmid replicon in not only Bacillus subtilis but also Lc. lactis.

Example 4 (Transformation of Lactobacillus delbrueckii subsp. lactis):

From the transformants of Lactococcus lactis subsp. lactis IL1403 obtained in Example 3, plasmids pX3 and pX4 were prepared. Using them, Lb. lactis ATCC 12315 strain and M-908 strain, possessed by Meiji Institute of Health Science, were transformed by electroporation. There was no report of success in transformation of Lactobacillus delbrueckii species having a high industrial usefulness, despite of studies by many researchers. Using the plasmid of the invention, transformation of Lb. lactis was attained for the first time, as mentioned below.

Lb. lactis ATCC 12315 strain or M-908 strain as subcultivated in a skim milk medium was inoculated in LCMG medium in a concentration of 2% and incubated for 2 hours at 42°C. The cells were collected and washed, and then suspended in EP buffer (containing 0.4 M sucrose, 1 mM magnesium chloride and 7 mM potassium dihydrogenphosphate; pH 7.4) at a concentration of OD_{660} = 4.0 and cooled on ice. 0.8 ml of the cell suspension was put in an electroporation cuvette, about 0.1 to 2 μg of pX3 or pX4 plasmid was added thereto, and an electric pulse of 25 μF at 2.5 kV was discharged thereto with Gene Pulser (manufactured by Bio-Rad Co.).

Immediately after the discharge, the cells were suspended in 4 ml of an expression medium (LCMG medium to which 0.2 M raffinose, 5 mM magnesium chloride and 1% lactose had been added) and incubated statically for 2.5 hours at 37°C.

All the culture liquid thus incubated was poured into several plates and 10 to 15 ml of BL agar medium (sterilized and kept at 50°C) containing 25 μg/ml of Em was added thereto and mixed. After solidified, the plates were incubated anaerobically in Gaspak system (manufactured by Beckton-Dickinson Co.) at 37°C for 2 to 4 days, and the transformants were selected. According to the method, about 10 to 100 transformants per μg of the plasmid DNA were obtained.

The Em-resistant clones thus obtained showed a strong Em-resistance (>1 mg Em/ml) and had a plasmid having the same restriction endonuclease map as pX3 or pX4. From these results, they were confirmed to be transformants. Further, using the pX3 or pX4 plasmid DNA obtained from the transformants, the transformation frequency in Lb. lactis ATCC 12315 strain increased by about 10 times.

No transformants of Lb. lactis have heretofore been obtained by using other plasmids such as pGK12 or IL253 under the same conditions as above. From the fact, it is obvious that the pBUL1 of the present invention is useful as a vector for Lb. lactis.

Example 5 (Introduction and Expression in Lb. lactis of L-lactate Dehydrogenase Gene inserted in pBUL1):

A restriction enzyme SspI fragment (about 1.2 kbp) containing a gene (Japanese Patent Application No. 2-45976) coding for L-lactate dehydrogenase (hereinafter referred to as "ST-LDH")

derived from a lactic acid bacterium Streptococcus salivarius subsp. thermophilus M-192 strain (possessed by Meiji Institute of Health Science) was inserted into the SmaI recognition site of pUC118, to construct the recombinant plasmids pU8ST8 and pU8ST9 as shown in Fig. 4-A and Fig. 4-B, respectively.

These recombinant plasmids were cut with BamHI and KpnI and subjected to agarose gel electrophoresis. About 0.02 μg of DNA fragments containing the ST-LDH gene was cut out and isolated using a GENECLEAN DNA purification kit (manufactured by BIO101 Co.). These DNA fragments were ligated with about 0.3 μg of pX3 or pX4, obtained in Example 2, cut with BamHI and KpnI. Using this ligation mixture, Lc. lactis IL1403 strain was transformed, whereby transformants having the plasmids as shown in Fig. 5 were obtained. These plasmids were named pXL38, pXL39, pXL48 and pXL49, respectively.

These plasmids were prepared by the method of reference 9 and were used for transformation of Lactobacillus delbrueckii subsp. lactis ATCC 12315 strain by the same method as shown in Example 4. As a result, Em-resistant transformants were obtained. These carried plasmids each having the same restriction endonuclease map as the introduced plasmids.

These transformants were incubated in a skim milk medium. The culture was diluted with distilled water and subjected to centrifugation. The lactic acid in the resulting supernatant was measured by the use of a lactic acid measuring

kit (F Kit L-lactic Acid; manufactured by Boehringer Mannheir). As a result, L-lactic acid, which is not naturally produced at all by the host, was detected in an amount almost equivalent to D-lactic acid produced.

The cells of one of the transformants were disrupted and the cell extract was prepared. As a result, L-lactate dehydrogenase activity, which is not detected in the host cells, was detected in the cell extract of the transformant. The L-lactate dehydrogenase from the transformant was purified and the sequence of the 18 N-terminal amino acids thereof was examined. As a result, it was identical to that of ST-LDH. From the above-mentioned results, it was shown that a heterogeneous gene expression was possible in Lb. lactis with pBUL1 as a replicon.

Example 6 (Presumption of the Region in pBUL1 necessary for Replication by a Deletion Method):

pX3 was cut with BamHI and KpnI and subjected to deletion in the direction as indicated in Fig. 6, by the use of a DNA deletion kit for kilosequencing (manufactured by Takara Shuzo Co.). Each reaction mixture was applied to transform Lc. lactis IL1403 in the same method as in Example 3, and the transformants were selected in the presence of 25 μg/ml of Em.

Plasmids harboured by the Em-resistant transformants were prepared and their restriction enzyme recognition sites were

examined. As a result, the shortest deletant of pX3 was obtained whose deletion proceeded to the position in pX3 shown in Fig. 6, namely, the position about 1.1 kbp clockwise apart from ScaI site and about 0.45 kbp counterclockwise apart from NdeI site. The deleted plasmid was named pX3Δ18 and it was further cut with both PstI and either of BglII, SphI and Eco47III and then subjected to self-circularizing ligation. The reaction mixture was used in transformation of Lc. lactis IL1403 strain and the selection of the transformants was performed as in Example 3. As a result, transformants having plasmids of various sizes were obtained. The plasmid pX3Δ18E as shown in Fig. 6 is the shortest one, which was formed by cutting pX3Δ18 with PstI and Eco47III followed by self-ligation. The region necessary for replication of pBUL1 was found to be contained in about 4 kbp DNA fragment of pX3Δ18E.

Example 7 (Formation of Shuttle Vectors by Ligation of pX3Δ18E Plasmid with E. coli Plasmid):

pX3Δ18E plasmid obtained in Example 6 was prepared from Lc. lactis IL1403 strain by the method of reference 9. The thus obtained pX3Δ18E was cut with SphI in the multi-cloning site and was ligated with a DNA fragment obtained by cutting E. coli plasmid pBR322 (manufactured by Takara Shuzo Co.) or pUC118 with SphI. The reaction mixture was used to transform E. coli TG1 strain [Δ(lac-pro)supE thi hsdD5/F' traD36

proA+B+ lacI^q lacZΔM15] (manufactured by Amersham Co.) by the well-known calcium chloride method. The transformants were selected with 500 μg Em/ml. They had a recombinant plasmid composed of pX3Δ18E and pBR322 or pUC118, as shown in Fig. 7 to Fig. 10 (pBR3Δ18E1, pBR3Δ18E2; p8X3Δ18E1, p8X3Δ18E2).

These transformants showed ampicillin resistance (50 μg/ml) derived from E. coli pBR322 and pUC118 plasmids. These recombinant plasmids isolated and purified from E. coli were used to transform Lc. lactis IL1403 strain and transformants were selected with 25 μg/ml of Em as shown in Example 3. As a result, Em-resistant transformants were obtained. The plasmids possessed by the transformants were prepared and the restriction enzyme-recognition sites were examined. As a result, they each had the same restriction endonuclease map as the map of those prepared from E. coli. From these results, pX3Δ18E, one of the derivatives of pBUL1, was found to become a useful shuttle vector if it is ligated with an E. coli plasmid. For the transformants with such a recombinant plasmid could be selected with ampicillin and Em, or Em when the host was either E. coli or lactic acid, respectively. Thus, the usefulness of pBUL1 was further proved.

Example 8 (Replication of pX3Δ18E Plasmid in E. coli):

The plasmid pX3Δ18E, obtained in Example 6, was

prepared from the transformant of Lc. lactis IL1403 by the method of reference 9, and was used to transform E. coli TG1 strain in the same manner as in Example 7. The thus obtained Em-resistant (Em 500 μg/ml) transformant had a plasmid having the same restriction endonuclease map as that of pX3Δ18E used for the transformation. From the fact, it was found that the pX3Δ18E plasmid, a derivative of pBUL1, is replicable even in E. coli, that E. coli with pX3Δ18E may be selected on the basis of the Em resistance and that pX3Δ18E is useful as a shuttle vector between Gram-positive bacteria and Gram-negative bacteria. Thus, the usefulness of pBUL1 was further proved.

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Advantage of the Invention:

The species Lactobacillus delbrueckii is an industrially useful lactic acid bacterium, including subsp. bulgaricus and subsp. lactis which are important in production of yogurt and cheese and subsp. delbrueckii which is important in production of lactic acid.

However, with respect to Lactobacillus delbrueckii, neither transformation nor the presence of an autonomously replicable plasmid has heretofore been reported. The plasmid of the present invention is the first reported up to now. Using the plasmid of the present invention as a replicon, transformation of Lb. lactis which is one of this species has become possible for the first time. Accordingly, it is expected that various genes are inserted into the plasmid of the present invention to give recombinant plasmids which are introduced into subspecies of Lactobacillus delbrueckii to construct improved strains having useful characters, for example, a high lactose metablizing ability or an improved milk protein degrading activity. In addition, since the present invention provides a plasmid with a broad-host-range shuttle vector. Thus, the effectiveness of the present invention is further elevated.

Since the plasmid of the invention is one isolated from a strain of Lb. bulgaricus existing in yogurt, its safety has been confirmed historically. In addition, since the plasmid of the invention can replicate not only in species of Lactobacillus delbrueckii but also in other bacteria important in food industry, such as the genera Bacillus and Lactococcus, it is expected to be utilizable not only in the production of various foods but also in breeding of micro-organisms which produce various enzymes and physiologically active substances.

Reference to Deposited Microorganism under Rule 13-2:

1. Lactobacillus delbrueckii subsp. bulgaricus M-878

   a. Name and Address of the Institution for Deposition of the present Microorganism:

      Name: Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry

      Address: 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, 305, Japan

   b. Date of Deposition in the Institution stated in a: January 29, 1991

   c. Deposition Number rendered by the Institution stated in a: FERM BP-3758

The claims defining the invention are as follows:-

1. Isolated plasmid pBUL1.

2. A pure culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* FERM BP-3758.

3. A DNA fragment designated by the thick line in Figure 1 which region is about 4 kbp between the position of the Eco47III site and the position about 1.1 kbp clockwise from the ScaI site and about 0.45 kbp counterclockwise from the NdeI site which region replicates pBUL1.

4. A plasmid into which the DNA fragment of claim 3 has been inserted.

5. A plasmid into which the plasmid pBUL1-replication origin of claim 4 has been inserted.

6. A microorganism transformed with the plasmid of claim 4.

7. A microorganism transformed with the plasmid of claim 5.

8. The microorganism according to claim 6 which is *Lactobacillus delbrueckii*.

9. The microorganism according to claim 6 which is *Lactococcus lactis*.

10. The microorganism according to claim 6 which is *Bacillus subtilis*.

11. The microorganism according to claim 7 which is *Lactobacillus delbrueckii*.

12. The microorganism according to claim 7 which is *Lactococcus lactis*.

13. The microorganism according to claim 7 which is *Bacillus subtilis*.

DATED this 15th day of August 1994.

MEIJI MILK PRODUCTS COMPANY LIMITED
By their Patent Attorneys:
CALLINAN LAWRIE

Michael J. Hourihan.

ABSTRACT OF THE DISCLOSURE

Disclosed are the plasmid pBUL1 having a restriction endonuclease cleavage map as shown in Fig. 1 and having a length of about 7.9 kbp and its derivatives.

The plasmid was isolated from Lactobacillus delbrueckii subsp. bulgaricus M-878.

The plasmid is useful as a vector for breeding various microorganisms such as lactic acid bacteria, and the derivatives thereof are useful also as a shuttle vector (lactic acid bacteria - Escherichia coli).

FIG. 1

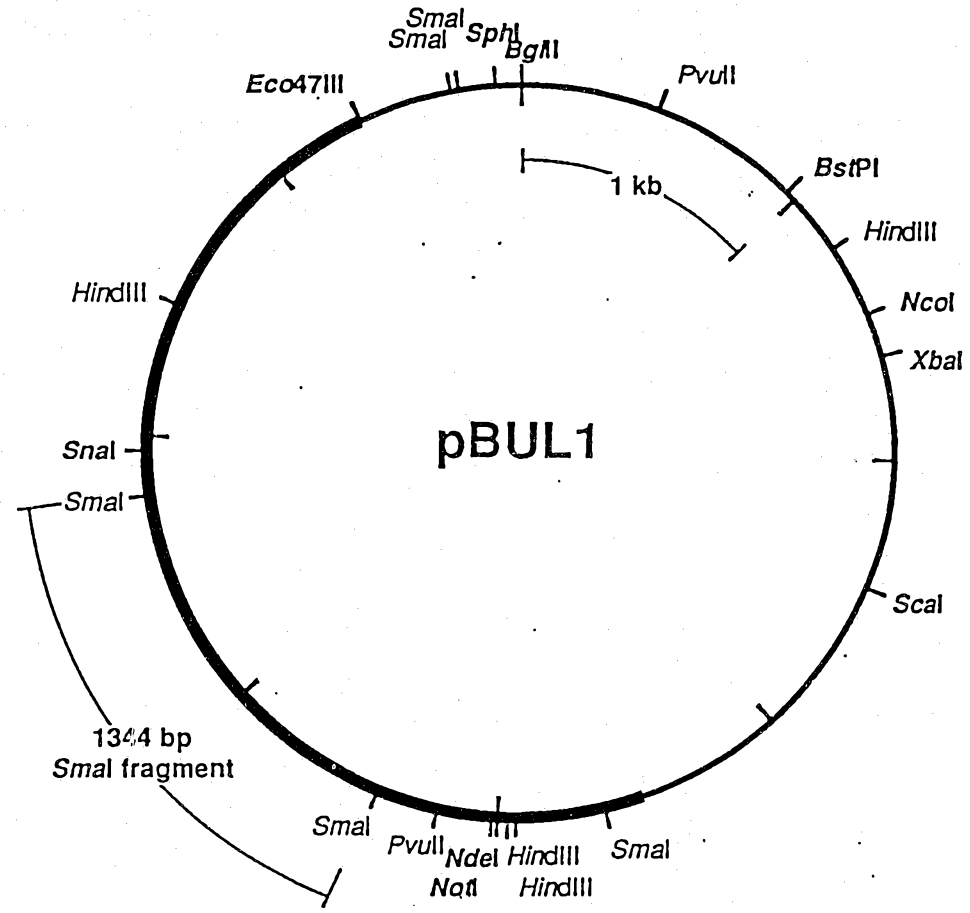

SmaI
SmaI
SphI
BglII
Eco47III
PvuII
1 kb
BstPI
HindIII
NcoI
XbaI
HindIII
pBUL1
SnaI
SmaI
ScaI
1364 bp
SmaI fragment
SmaI
PvuII
NdeI
NotI
HindIII
HindIII
SmaI

FIG. 2

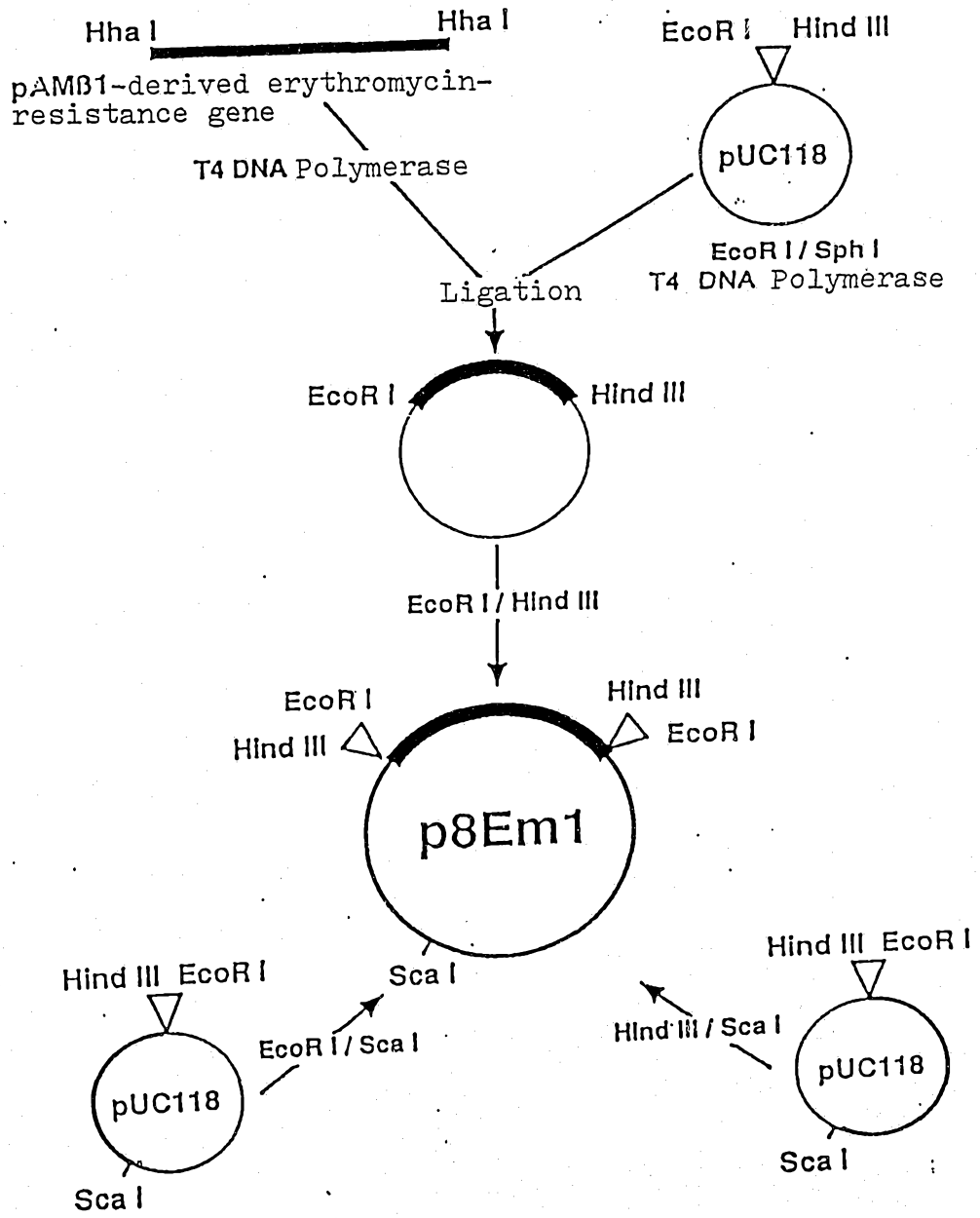

Hha I
Hha I
pAMß1-derived erythromycin-resistance gene
T4 DNA Polymerase
EcoR I
Hind III
pUC118
EcoR I / Sph I
T4 DNA Polymerase
Ligation
EcoR I
Hind III
EcoR I / Hind III
EcoR I
Hind III
Hind III
EcoR I
p8Em1
Hind III EcoR I
Sca I
EcoR I / Sca I
pUC118
Sca I
Hind III EcoR I
Hind III / Sca I
pUC118
Sca I

FIG. 3

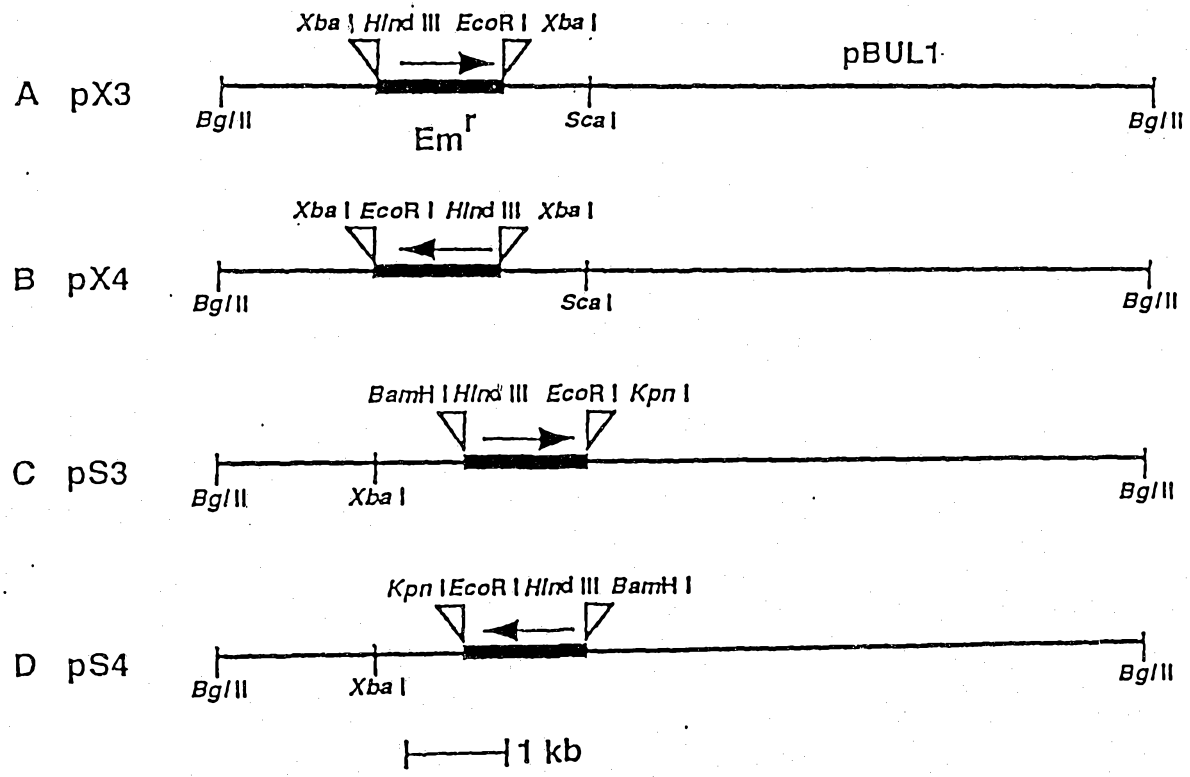
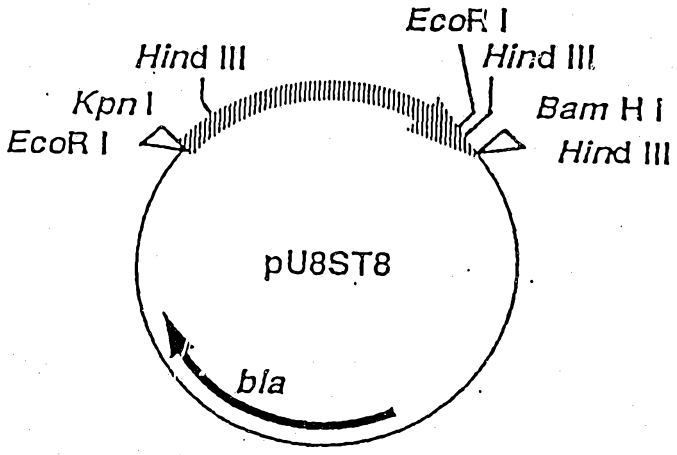

A pX3
Xba I Hind III EcoR I Xba I
pBUL1
Bgl II
Em r
Sca I
Bgl II
B pX4
Xba I EcoR I Hind III Xba I
Bgl II
Sca I
Bgl II
C pS3
BamH I Hind III EcoR I Kpn I
Bgl II
Xba I
Bgl II
D pS4
Kpn I EcoR I Hind III BamH I
Bgl II
Xba I
Bgl II
1 kb

FIG. 4

A pU8ST8



B pU8ST9

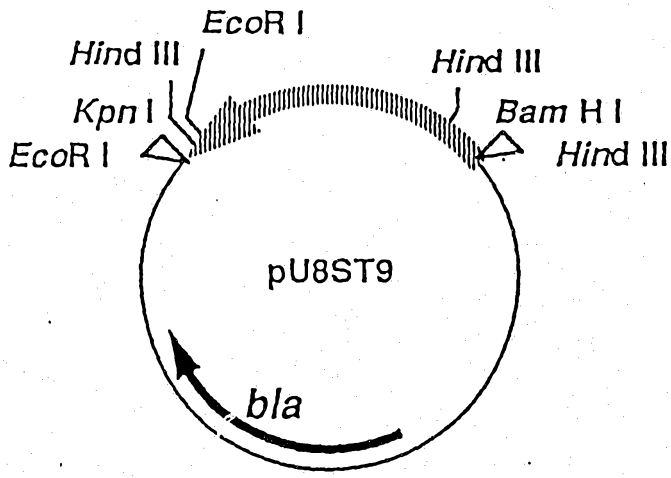


FIG. 5

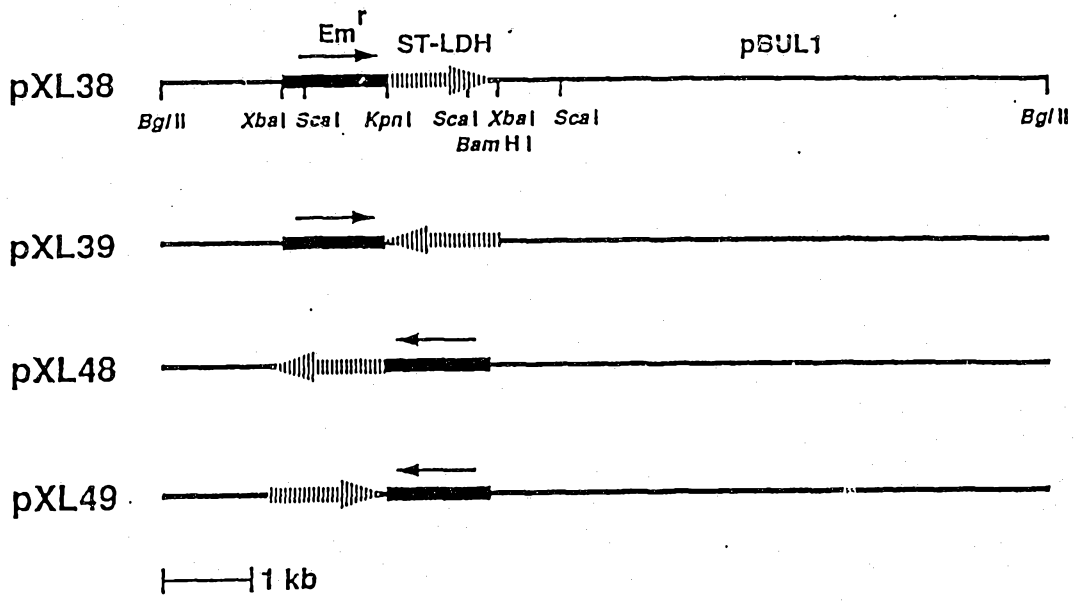
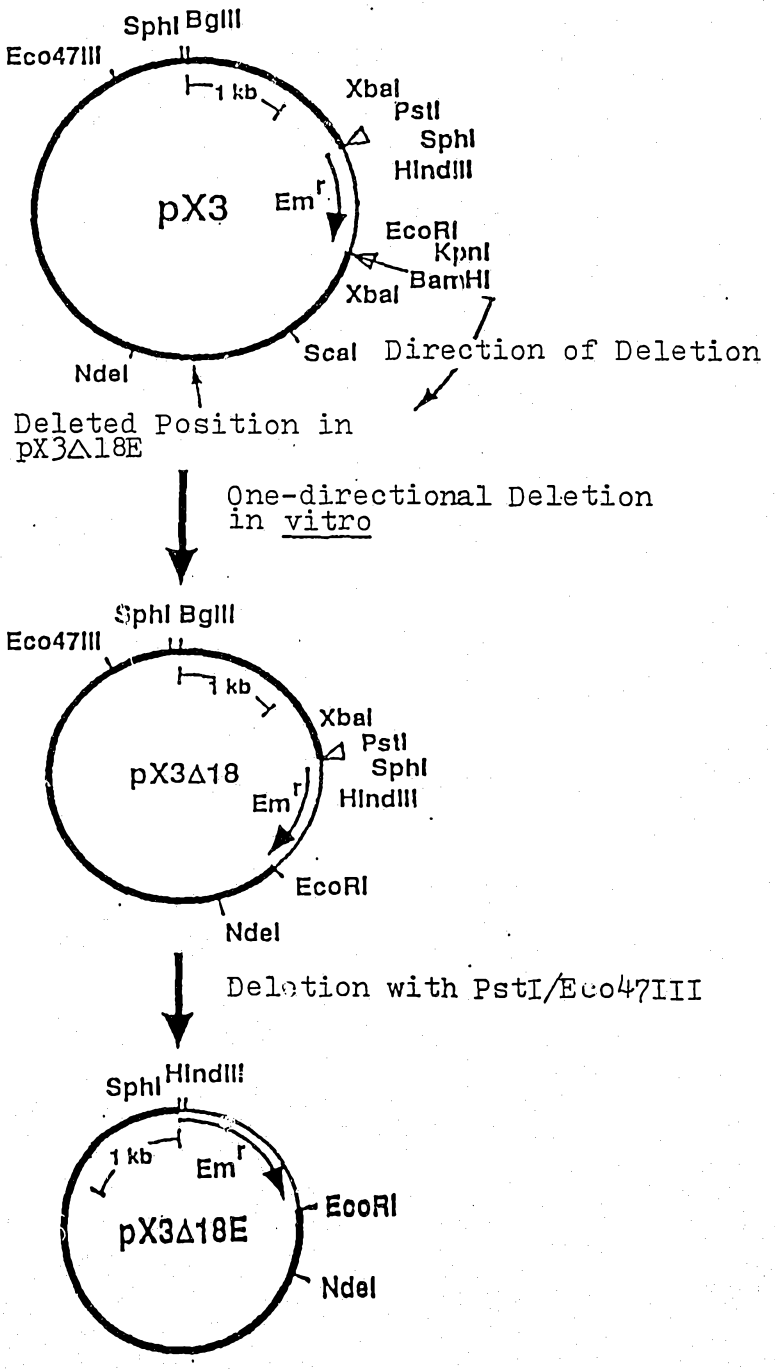

Em^r
ST-LDH
pBUL1
pXL38
BglII
XbaI
ScaI
KpnI
ScaI
XbaI
ScaI
BglII
BamHI
pXL39
pXL48
pXL49
1 kb

FIG. 6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XbaI
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HindIII
pX3
Em^r
EcoRI
KpnI
BamHI
XbaI
ScaI
NdeI
Direction of Deletion
Deleted Position in pX3Δ18E
One-directional Deletion in vitro
SphI
BglII
Eco47III
1 kb
XbaI
PstI
SphI
pX3Δ18
Em^r
HindIII
EcoRI
NdeI
Deletion with PstI/Eco47III
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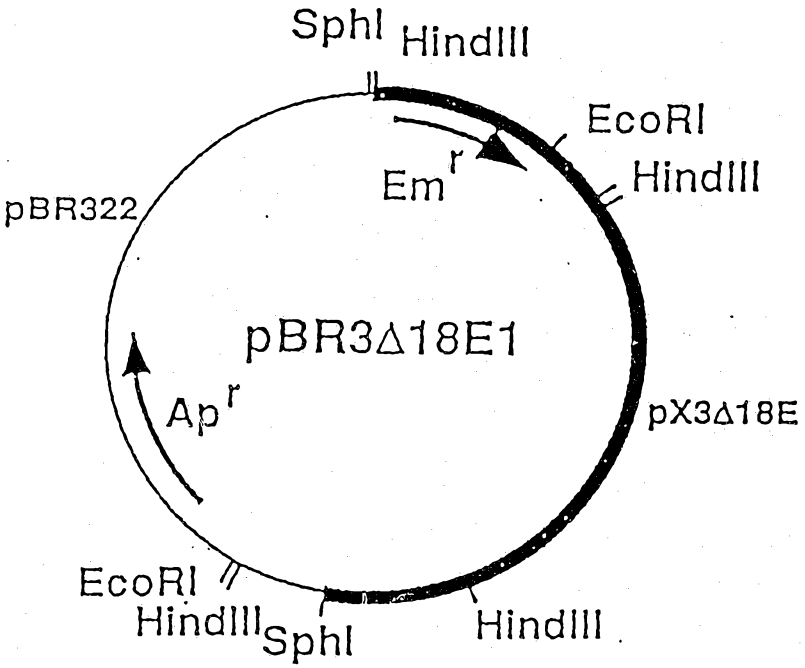
Em^r
EcoRI
pX3Δ18E
NdeI

FIG. 7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HindIII
EcoRI
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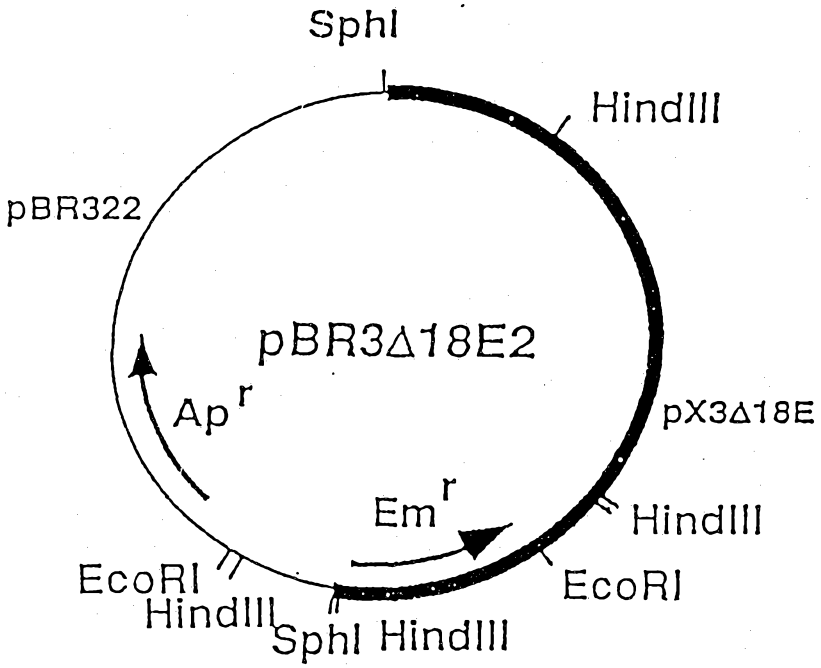
Ap r
pX3Δ18E
EcoRI
HindIII
SphI
HindIII

FIG. 8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HindIII
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Em r
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EcoRI
EcoRI
HindIII
SphI HindIII

FIG. 9

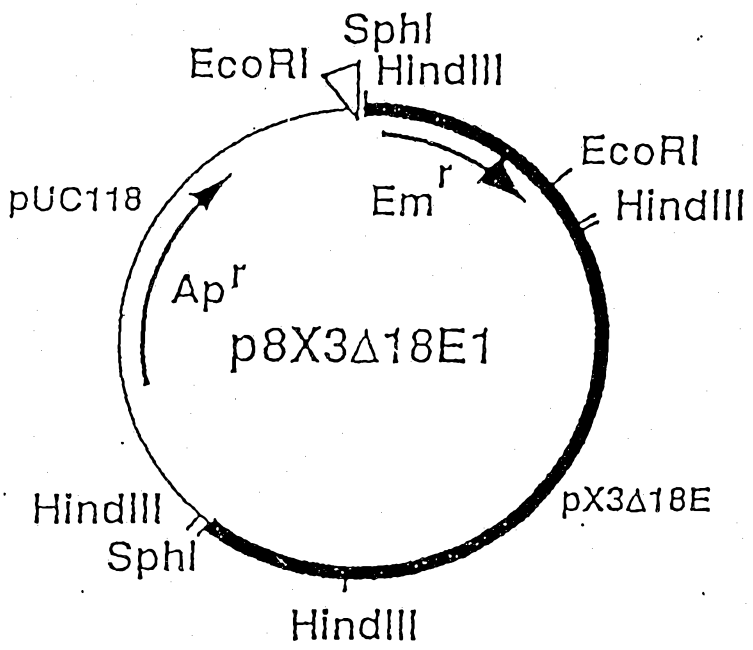
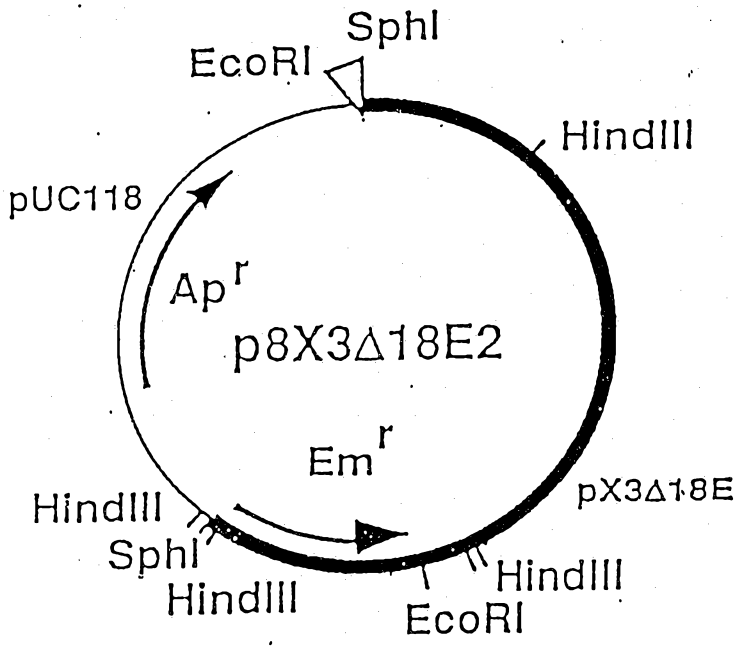

SphI
EcoRI
HindIII
EcoRI
HindIII
pUC118
Em r
Ap r
p8X3Δ18E1
HindIII
SphI
pX3Δ18E
HindIII

FIG. 10


SphI
EcoRI
HindIII
pUC118
Ap r
p8X3Δ18E2
Em r
pX3Δ18E
HindIII
SphI
HindIII
HindIII
EcoRI

国　際　調　査　報　告

国際出願番号PCT/JP92/00193

Ⅰ．発明の属する分野の分類

国際特許分類（IPC）　Int. Cl[5]
C12N15/74, 1/21//(C12N15/74, C12R1:225)

Ⅱ．国際調査を行った分野

調査を行った最小限資料

分類体系	分類記号
IPC	C12N15/00−15/90, C12N1:21

最小限資料以外の資料で調査を行ったもの

BIOSIS DATABASE

Ⅲ．関連する技術に関する文献

引用文献のカテゴリー※	引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示	請求の範囲の番号
A	Current Microbiology, 第10巻, 1984, Todd R. Klaenhammer「A General Method for Plasmid Isolation in Lactobacilli」P. 23−28	1−4
A	(BOLOGNA) 第4号, Microbiologica, 第4巻, 1981, Vescovo, M. et al.「Evidence of Plasmid Deoxyribonucleic Acid in Lactobacillus」p.413−420	1−4
A	Acta Microbiologica Bulgarica, 第27巻, 第0号, 1991, Miteva, V. et al.「Isolation and Characterization of Plasmids from Different Strains of Lactobacillus − bulgaricus Lactobacillus − helveticus and Streptococcus −thermophilus」P.3-8	1−4

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

Ⅳ．認　　証

国際調査を完了した日	国際調査報告の発送日
14. 05. 92	09.06.92
国際調査機関	権限のある職員
日本国特許庁（ISA/JP）	特許庁審査官　大久保　元　番　4B8828

様式PCT/ISA/210（第2ページ）（1981年10月）

INTERNATIONAL SEARCH REPORT

International Application No PCT/JP92/00193

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl^5 C12N15/74, 1/21//(C12N15/74, C12R1/225)

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	C12N15/00-15/90, C12N1/21

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

BIOSIS DATABASE

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	Current Microbiology, Vol. 10, 1984, Todd R. Klaenhammer "A General Method for Plasmid Isolation in Lactobacilli" P. 23-28	1-4
A	Microbiologica (BOLOGNA), Vol. 4, No. 4, 1981, Vescovo, M. et al. "Evidence of Plasmid Deoxyribonucleic Acid in Lactobacillus" P. 413-420	1-4
A	Acta Microbiologica Bulgarica, Vol. 27, No. 0, 1991, Miteva, V. et al. "Isolation and Characterization of Plasmids from Different Strains of Lactobacillus - bulgaricus Lactobacillus - helveticus and Streptococcus - thermophilus" P. 3-8	1-4

* Special categories of cited documents: [10]

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

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

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

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

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

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

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

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

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
May 14, 1992 (14. 05. 92)	June 9, 1992 (09. 06. 92)
International Searching Authority	**Signature of Authorized Officer**
Japanese Patent Office	