(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) **EP 2 949 753 A1**

(12) **EUROPEAN PATENT APPLICATION**

(43) Date of publication:
02.12.2015 Bulletin 2015/49

(51) Int Cl.:
C12N 15/70 *(2006.01)* ***C12N 15/61*** *(2006.01)*

(21) Application number: **14191756.7**

(22) Date of filing: **15.09.2004**

(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LI LU MC NL PL PT RO SE SI SK TR
Designated Extension States:
AL HR LT LV MK

(30) Priority: **15.09.2003 FI 20031319**

(62) Document number(s) of the earlier application(s) in accordance with Art. 76 EPC:
04767054.2 / 1 664 305

(71) Applicant: **FIT Biotech Oy**
33520 Tampere (FI)

(72) Inventors:
- **Tenson, Tanel**
  20103 Tartu (EE)
- **Laht, Silja**
  76901 Harku vald, Harjumaa (EE)
- **Adojaan, Maarja**
  61702 Tartu region (EE)
- **Männik, Andres**
  20409 Tartu (EE)
- **Toots, Urve**
  50415 Tartu (EE)
- **Ustav, Mart**
  50064 Tartu (EE)

(74) Representative: **Valea AB**
Box 7086
103 87 Stockholm (SE)

Remarks:
•Claims 16-27,31-36,40-46 are deemed to be abandoned due to non-payment of the claims fees (Rule 45(3) EPC).
•This application was filed on 04-11-2014 as a divisional application to the application mentioned under INID code 62.

(54) **Selection system containing non-antibiotic resistance selection marker**

(57) The present invention relates to a novel selection system, which is based on the use of an *ara*D gene, a mutated form of an *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof as a selection marker and to the use of a bacterial strain deficient of the *ara*D gene. The present invention further relates to novel vectors containing an *ara*D gene, a mutated form of an *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof and to novel bacterial strains deficient of the *ara*D gene. The present invention additionally relates to a method of selecting the cells transformed with a plasmid, which contains the gene of interest.

EP 2 949 753 A1

Description

Field of the invention

[0001] The present invention relates to a novel selection system, which is based on the use of an *ara*D gene, a mutated form of an *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof as a selection marker and to the use of a bacterial strain deficient of the *ara*D gene. The present invention further relates to novel vectors containing an *ara*D gene, a mutated form of an *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof and to novel bacterial strains deficient of an *ara*D gene. The present invention additionally relates to a method of selecting the cells transformed with a plasmid, which contains the gene of interest.

Background of the invention

[0002] An essential requirement for effective genetic engineering of bacteria and other cells propagated in cell cultures is the capacity to select the cells with a specific genotypic alteration. The most common selection strategy in recombinant DNA technology is to include a selection marker in the cloning vector or plasmid. A selection marker can be a cloned gene or a DNA sequence, which allows the separation of the host cells containing the selection marker from those not containing it. The selection marker together with a suitable selection medium maintains the cloning vector in the cells. Otherwise, since the replication of plasmids is an energetic burden for the bacterial host, in a growing culture the bacteria, which have lost the plasmid, would have a growth advantage over the cells with the plasmid.

[0003] For most purposes, an antibiotic resistance gene is a commonly used selection marker. However, for the production of recombinant therapeutics, where the goal is to generate a product, such as a DNA vaccine, in high yield for administration in patients, the use of antibiotic resistance genes presents problems: the spread of antibiotic resistant pathogens is a serious worldwide problem [Levy, S. B., J. Antimicrob. Chemother. 49 (2002) 25-30]. Therefore the antibiotic resistance genes cannot have extensive use in the pharmaceutical industry, and for instance, according to the regulations of the U.S. Food and Drug Administration, no antibiotic resistance genes are allowed in experimental DNA vaccines entering the third phase.

[0004] Alternatively, antibiotic-free selection systems have been suggested. Such antibiotic-free selection systems include bacterial toxin-antitoxin systems [Engelberg-Kulka, H. and Glaser, G., Annu Rev Microbiol 53 (1999) 43-70], genes responsible for resistance against heavy metals, such as tellurium [Silver, S. and Phung, L. T., Annu Rev Microbiol 50 (1996) 753-789], and systems, in which the plasmid encodes a gene complementing a host auxotrophy [Wang, M.D., et al., J. Bacteriol. 169 (1987) 5610-5614].

[0005] US Patent Application 2000/0014476 A1 generally discloses, inter alia, the use of a non-antibiotic selection marker, which may be a gene whose product is necessary for the metabolism of the cell under certain culturing conditions, such as a catabolism gene, which makes it possible for the cell to assimilate a certain substance present in the culture medium (specific carbon or nitrogen source) etc. No specific examples of such suitable genes are given. This approach is not necessarily applicable for commercial production, since the deletion an essential component, such as an amino acid or a carbon source, from the growth medium reduces the yield, which is not desirable. Additionally, the manipulation of the growth medium in terms of omitting an essential nutritient may considerably increase the cost of the growth medium, since commercially available nutritient mixtures must be replaced by individual nutritients.

[0006] For commercial therapeutic purposes it would be of advantage to use a gene, which is not essential for the growth of the host but whose manipulation still affects the growth in selected circumstances. Additionally, in view of the therapeutic use, it would be of advantage to use a gene, whose deletion leads to accumulation of compounds, which are toxic to the host cell but not toxic to mammalians, including humans. Also it would be of advantage to use smaller genes, which in turn would allow the construction of smaller plasmids for which the energy consumption for replication is smaller and thus the growth rate of bacterial culture and plasmid yield are improved.

Short description of the invention

[0007] The object of the present invention is to provide a novel antibiotic-free selection system, which avoids the problems of previously disclosed selection systems for use in the production of recombinant therapeutic products.

[0008] Another object of the invention is to provide a novel antibiotic-free selection system, which can be safely used in the production of recombinant therapeutic products in terms of the environment and the patient safety.

[0009] A further object of the invention is to provide a novel antibiotic-free selection system, which can be cost-effectively used in the production of recombinant therapeutic products using standard growth mediums.

[0010] A still further object of the invention is to provide a novel antibiotic-free selection system, which provides an increased growth rate and improved yield.

[0011] Yet another object of the present invention is to provide a novel vector containing a selection marker, which is

non-toxic to the environment and to humans and which is capable of a long-term maintenance in the host.

[0012] Yet another object of the present invention is to provide a novel host cell containing a gene defect, which is not hazardous to the environment.

[0013] Still another object of the present invention is to provide a method for selection of cells carrying a gene of interest for the production of recombinant therapeutic products.

[0014] It was surprisingly found that the objects of the present invention are met by the use of the *ara*D gene, a mutated form of an *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof as a selection marker and the use of a specific bacterial host deficient of the *ara*D gene.

[0015] Accordingly, the present invention provides a novel selection system comprising a bacterial cell deficient of an *ara*D gene into which a vector carrying an *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof has been added as a selection marker. One embodiment of the present invention relates to a selection system wherein the *ara*D gene is the *ara*D gene or the L-ribulose-5-phosphate 4-epimerase (EC 5.1.3.4.). Another embodiment of the present invention relates to a selection system wherein the *ara*D gene is mutated.

[0016] The present invention further provides novel vectors, which contain an *ara*D gene, a mutated form of an *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof as a selection marker.

[0017] The present invention further provides novel bacterial strains, which are deficient of the *ara*D gene.

[0018] The present invention further provides a method of selecting the cells transformed with a plasmid, which contains 1) the *ara*D gene, a mutated form of an *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof as a selection marker and 2) the gene of interest, the method comprising inserting said plasmid into the *ara*D deficient host cell and growing the cells in a growth medium containing arabinose.

Drawings

[0019]

Figure 1 shows the use of arabinose as a carbon source by the *E. coli* cells (Lin, 1987).

Figure 2 shows the map of S6wtd1 EGFP. The coding sequences for the d1EGFP, E2 and kanamycin resistance marker aminoglycoside-3'-O-phosphotransferase (kana) are indicated by arrows. Additional features are indicated by solid boxes: 10E2BS - ten BPV E2 binding sites with high affinity; CMV-tk - human cytomegalovirus immediately early promoter and HSV Th gene leader sequence; intron - rabbit beta-globin gene intron with optimized SD and SA sites; tkpa - HSV Tk gene polyadenylation signal; RSV LTR-Rous sarcoma virus long terminal repeat; bgh pA - bovine growth hormone gene polyadenylation signal; pUCori - bacterial origin of replication derived from the pUC18 plasmid.

Figure 3 shows the map of S6wtd1EGFP*kana*/*ara*D1. The coding sequences for the d1EGFP, E2, kanamycin resistance marker aminoglycoside-3'-O-phosphotransferase (kana) and L-ribulose-5-phosphate 4-epimerase (araD) are indicated by arrows. Additional features are indicated by solid boxes: 10E2BS - ten BPV E2 binding sites with high affinity; CMV-tk - human cytomegalovirus immediately early promoter and HSV Th gene leader sequence; intron - rabbit beta-globin gene intron with optimized SD and SA sites; tkpa - HSV Tk gene polyadenylation signal; RSV LTR - Rous sarcoma virus long terminal repeat; bgh pA - bovine growth hormone gene polyadenylation signal; pUCori - bacterial origin of replication derived from the pUC18 plasmid.

Figure 4 shows the map of S6wtd1EGFP*kana*/*ara*D2. The coding sequences for the d1EGFP, E2, kanamycin resistance marker aminoglycoside-3'-O-phosphotransferase (kana) and L-ribulose-5-phosphate 4-epimerase (araD) are indicated by arrows. Additional features are indicated by solid boxes: 10E2BS - ten BPV E2 binding sites with high affinity; CMV-tk - human cytomegalovirus immediately early promoter and HSV Th gene leader sequence; intron - rabbit beta-globin gene intron with optimized SD and SA sites; tkpa - HSV Tk gene polyadenylation signal; RSV LTR - Rous sarcoma virus long terminal repeat; bgh pA - bovine growth hormone gene polyadenylation signal; pUCori - bacterial origin of replication derived from the pUC18 plasmid.

Figure 5 shows the map of S6wtd1 EGFP/araD1. The coding sequences for the d1EGFP, E2 and L-ribulose-5-phosphate 4-epimerase (araD) are indicated by arrows. Additional features are indicated by solid boxes: 10E2BS - ten BPV E2 binding sites with high affinity; CMV-tk - human cytomegalovirus immediately early promoter and HSV Th gene leader sequence; intron - rabbit beta-globin gene intron with optimized SD and SA sites; tkpa-HSV Tk gene polyadenylation signal; RSV LTR - Rous sarcoma virus long terminal repeat; bgh pA - bovine growth hormone gene polyadenylation signal; pUCori - bacterial origin of replication derived from the pUC18 plasmid.

Figure 6 shows the map of S6wtd1 EGFP/araD2. The coding sequences for the d1EGFP, E2 and L-ribulose-5-phosphate 4-epimerase (*ara*D) are indicated by arrows. Additional features are indicated by solid boxes: 10E2BS - ten BPV E2 binding sites with high affinity; CMV-tk - human cytomegalovirus immediately early promoter and HSV Th gene leader sequence; intron - rabbit beta-globin gene intron with optimized SD and SA sites; tkpa-HSV Tk gene polyadenylation signal; RSV LTR - Rous sarcoma virus long terminal repeat; bgh pA - bovine growth hormone gene

polyadenylation signal; pUCori - bacterial origin of replication derived from the pUC18 plasmid.
Figure 7A and 7B shows the electrophoretic analysis of the plasmid DNA of the S6wtd1EGFP/*ara*D1 (7A) and S6wtd1 EGFP/araD2 (7B) extracted from the *E. coli* strain AG1delta *ara*D grown in different media.
Figure 8 shows the restriction pattern analysis of the plasmid DNA of the S6wtd1EGFP/*ara*D1 and S6wtd1EGFP/*ara*D2 extracted from the *E. coli* strain AG1delta*ara*D
Figure 9 shows the electrophoretic analysis of the S6wtd1 EGFP/araD2 in stability assay.
Figure 10A and 10B shows the restriction pattern analysis of the S6wtd1EGFP/*ara*D2 in stability assay.
Figure 11 shows the growth parameters of fed-batch fermentation of AG1Δ*ara*D S6wtd1EGFP/*ara*D2 measured and registered during fermentation. The abbreviations are as follows: sPump = feeding speed; pO2 = the oxygen concentration; Temp = growth temperature; mys = desired growth rate; OD = optical density at 600nm.
Figure 12 shows the scheme of lysis and purification of AG1Δ*ara*D S6wtd1EGFP/*ara*D2.
Figure 13 shows the *ara*D locus sequence of clone #13.
Figure 14 shows the map of plasmid p3hCG.
Figure 15 shows the map of plasmid paraDMgB.
Figure 16 shows the map of plasmid p3*ara*D1hCG.
Figure 17 shows the map of plasmid p3*ara*D2hCG.
Figure 18 shows the results of the analysis of L-arabinose sensitivity of *E. coli* strains with disrupted *ara*D.
Figure 19 shows the results of the analysis of the L-arabinose sensitivity in M9 and yeast extract medium with different glucose and arabinose concentrations.
Figure 20 shows the map of plasmid p2 MG C #11.
Figure 21 shows the map of plasmid p*ara*D MG C #145.
Figure 22 shows the *E. coli* genomic fragment containing the *sgb*E gene.
Figure 23 shows the *E. coli* genomic fragment containing *ula*F gene.

Detailed description of the invention

[0020] The present invention is based on an effort to find an alternative, antibiotic-free selection system, which could be used in the production of recombinant therapeutic products to be administered *in vivo,* especially in the production of DNA vaccines. Surprisingly it was found that the *ara*D gene involved in the pentose phosphate pathway of both prokaryotic and eukaryotic organisms, such as mammalians including humans, can be successfully used as a selection marker in an auxotrophic host cell for the plasmid. The use of the auxotrophy has the advantage of not involving a use or generation of toxic substances that could later contaminate the plasmid preparation.

[0021] An efficient selection system has been constructed on the basis of *ara*D/*ara*C genes [Ariza, R. R., et al., Carcinogenesis 14 (1993) 303-305]. However, this selection system has been used in the studies on the mechanisms of mutagenesis but not used before as a selection marker for plasmid maintenance. Ariza *et al.* used a strain where the *ara*C gene contains a termination codon and the *ara*D gene is inactivated. A product of the *supF* gene, which codes for a suppressor tRNA, was introduced on the plasmid. In the presence of active suppressor tRNA, enzymatically active product from *ara*C was produced causing cell growth arrest (because *ara*D was inactive). This system allows to study the suppression of mutations by supF tRNA: in case *sup*F is inactivated by mutation, the cells can grow on arabinose. Therefore, this selection system is based on *ara*C gene and not on *ara*D gene. *ara*D was not introduced into a plasmid, nor was the system designed or characterized for plasmid production purposes.

[0022] The *araD* gene codes for an enzyme which is responsible for epimerization of ribulose-5-phosphate to xylulose-5-phosphate (Fig. 1) and therefore allows the use arabinose in the pentose phosphate pathway [Engelsberg, E., et al., J. Bacteriol. 84: (1962) 137-146]. If *araD* is inactivated, ribulose-5-phosphate accumulates in the bacterial cell leading to growth arrest.

[0023] If the chromosomal copy of *araD* is inactivated in the host cell and an intact copy of the *araD* gene, a mutated form of the *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof is inserted into the plasmid, the growth advantage of the plasmid-containing cells in medium containing L-arabinose is achieved as a result from two effects. First, the plasmid-containing cells can use arabinose as a carbon source, and second, the toxic ribulose-5-phosphate does not accumulate. This allows the use of rich growth media supplemented with arabinose. In rich media the *E. coli* cells grow fast and the plasmid yield is high. Inexpensive standard components of the bacterial growth media, such as yeast extract, can be used as an amino acid source. The traces of ribulose-5-phosphate that theoretically could contaminate the plasmid preparation are not a problem, when the preparation is administered *in vivo,* as ribulose-5-phosphate can be efficiently metabolized by human cells and is not toxic.

[0024] The use of mutated form of the *ara*D gene offers particular advantages. Selection systems of the invention comprising a bacterial cell deficient of an *ara*D gene into which a vector carrying a mutated form of the *ara*D gene as a selection marker produce an optimal concentration of the *ara*D gene product L-ribulose-5-phosphate 4-epimerase to afford rapid uninhibited growth of the bacteria. Similar advantaged are obtained by the use selection systems containing

a vector carrying an intact *ara*D gene but comprising deletions or mutations elsewhere in the *ara*D gene locus.

[0025] The selection system of the invention comprises 1) a vector carrying an *ara*D gene, a mutated form of the *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof as a selection marker and 2) a specific bacterial strain deficient of the *ara*D gene into which the vector has been added. When the specific host deficient of the *ara*D gene is cultured in the presence of arabinose, the only surviving cells are those containing the vector, which contains an *ara*D gene, a mutated form of the *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof.

[0026] In the selection system of the invention any expression vector commonly used in the production of therapeutic products can be employed, whereby the *ara*D gene, a mutated form of the *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof is inserted into the vector using methods generally known in the art. In the present context, the *ara*D gene preferably comprises the sequence identified by SEQ ID NO. 1, by SEQ ID NO. 19, or a sequence hybridizable thereto. However, any applicable *ara*D genes are also contemplated. In the present context, the term "a catalytically active fragment of the *ara*D gene" is any gene fragment coding a polypeptide or a protein capable of epimerization of L-ribulose-5-phosphate to D-xylulose-5-phosphate. In a specific embodiment of the invention the *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof is inserted in the vector capable of a long-term maintenance and thereby capable of providing a stable expression of the desired antigen(s).

[0027] In another specific embodiment of the invention a mutated form of an *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof is inserted in the vector capable of a long-term maintenance and thereby capable of providing a stable expression of the desired antigen(s).

[0028] In a specifically preferred embodiment of the invention the vector used is an expression vector comprising:

(a) a DNA sequence encoding a nuclear-anchoring protein operatively linked to a heterologous promoter, said nuclear-anchoring protein comprising (i) a DNA binding domain which binds to a specific DNA sequence, and (ii) a functional domain that binds to a nuclear component, or a functional equivalent thereof; and
(b) a multimerized DNA sequence forming a binding site for the nuclear anchoring protein, wherein said vector lacks a papilloma virus origin of replication, and
(c) an *ara*D gene, a mutated form of an *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof.

[0029] Such vectors have been described in detail in the international patent application WO02/090558, which is incorporated herein by reference.

[0030] Most preferably the vector used in the selection method of the present invention is an expression vector comprising:

(a) the E2 protein of Bovine Papilloma Virus type 1 (BPV), and
(b) multiple binding sites of the BPV E2 protein incorporated into the vector as a cluster, where the sites can be as head-to-tail structures or can be included into the vector by spaced positioning, wherein said vector lacks a papilloma virus origin of replication, and
(c) the *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof.

[0031] In the selection system of the invention in principle any known host deficient of the *ara*D gene and suitable for use in the production of therapeutic products could be employed. In the present connection the term "deficient" denotes a host, in which the *ara*D gene is either totally deleted or inactivated by any known method.

[0032] In a preferred embodiment of the invention an *Escherichia coli* strain, preferably commercially available *E. coli* strains DH5alpha-T1, AG1 or JM109, from which the *ara*D gene has been deleted with generally known methods, such as those described below in the Examples, is used. In another preferred embodiment of the invention an *E. coli* strain, preferably *E. coli* strain DH5alpha-T1, AG1 or JM109, into which combined deletions have been made for depletion of other genes encoding proteins with L-ribulose-5-phosphate 4-epimerase activity. Alternatively, commercially available *E. coli* strains, preferably *E. coli* strains DH5alpha-T1, AG1 or JM109, in which the *ara*D gene and/or other genes encoding proteins with L-ribulose-5-phosphate 4-epimerase activity have been inactivated by any known method can be employed. In the method for selection of cells carrying a gene of interest for the production of recombinant therapeutic products, the gene of interest is inserted into host cells deficient of an *ara*D and/or other genes encoding proteins with L-ribulose-5-phosphate 4-epimerase activity using method well known in the art and the cells are cultured in a growth medium containing arabinose under culturing medium and conditions suitable the host in question.

[0033] Any growth medium suitable for culturing *E. coli* cells can be used. For commercial production the growth medium will naturally be optimized in terms of the yield. Examples of suitable growth media are commercially available growth media, such as M9 and LB (available from several manufacturers, such as Fermentas, Lithuania). The amount of arabinose added in the growth medium is not critical but naturally arabinose should be present in an amount that is

sufficient for the total culturing period. As low amount as 0.1 % has been found sufficient for the selection. Typically arabinose is added to the medium in an amount of about 0.1% to about 2.0%, preferably in an amount of about 0.2% to about 1,0%, most preferably 0.2% to about 0.5%. However the effect of L-arabinose is observed at concentrations as low as 0.01% and L-arabinose can be added up to 5% in the growth medium. In a special embodiment, where L-arabinose is used both as a selecting agent and as a limited carbon source, 0.2% of L-arabinose is a suitable amount to be added into the growth medium.

[0034] The selection system of the invention is suitable for use in any expression system. It is especially suitable for use in the expression of recombinant therapeutic products, such as DNA vaccines, intended for use *in vivo,* since the problems associated with the use of antibiotic resistance genes are avoided. Likewise the selection system of the invention is suitable for use in the production of recombinant proteins.

[0035] The possible contamination of arabinose in the final product resulting from the preparation process is inconsequential, since arabinose is editable sugar contained in foods naturally and as an additive and thus not toxic to mammalians including humans.

[0036] Additionally, the *araD* gene is smaller in size than the commonly used antibiotic resistance genes against, for instance, ampicillin and tetracyclin and of similar size to kanamycin and chloramphenicol resistance genes. This affords an additional advantage, since it allows the construction of small plasmids for which the energy consumption for replication is smaller than for large plasmids. Thereby both the growth rate of bacterial culture and plasmid yield are increased.

[0037] The present invention may be better understood by reference to the following non-limiting Examples, which are provided as exemplary of the invention. The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

Example 1

Cloning of *ara*D selection plasmids

[0038] For cloning *araD* selection constructs plasmid S6wtd1 EGFP (Figure 2) was used. It has pMB1 origin of replication and kanamycin resistance marker as functional elements of plasmid backbone. The kanamycin resistance in this plasmid is conferred by gene that is derived from *E. coli* transposon Tn903.

[0039] The *araD* gene was amplified using polymerase chain reaction (PCR) from *E. coli DH5α* chromosome according to standard procedure. The PCR product was cloned into selected plasmids in two different orientations with the primer pairs s6araDL1 + s6araDR1 or s6araDL1 + s6araDR1, generating products named *ara*D1 and *ara*D2, respectively:

s6*ara*DL1:

CGCCATGGTTCTCATGTTTGACAGCTTATCATCGATAAGCTTTA ATGCGGTAGTTTAGCACGAAGGAGTCAACATG (SEQ ID NO. 2);

s6araDR1:

CGCCATGGACTAGTAAAAAAAAGCCCGCTCATTAGGCGGGCT GTCATTACTGCCCGTAATATGC (SEQ ID NO. 3);

s6araDL2:

CGCCATGGACTAGTTCTCATGTTTGACAGCTTATCATCGATAAG CTTTAATGCGGTAGTTTAGCACGAAGGAGTCAACATG (SEQ ID NO. 4);

s6araDR2:

CGCCATGGAAAAAAAAGCCCGCTCATTAGGCGGGCTGTCAT-TACTGCCCGTAATATGC (SEQ ID NO. 5);

[0040] The primers were designed so that P2 promoter from plasmid pBR322 (used for driving the tetracycline resistance gene in pBR322) and termination sequence from *trp* operon of *E. coli* were added during PCR to the upstream and downstream of *ara*D coding sequence, respectively.

[0041] PCR products of 814 and 815 bp were cloned into pUC18 vector linearized with HincII (Fermentas, Lithuania) and correct sequences were verified by sequencing using universal sequencing primers

M13F22: GCCAGGGTTTTCCCAGTCACGA (SEQ ID NO. 6) and
M13R24: GAGCGGATAACAATTTCACACAGG (SEQ ID NO. 7) and *araD* specific primers
araD F311: CCAACTCACCGGCTGCTCTATC (SEQ ID NO. 8),
*ara*D F614: AATGCCGAAGATGCGGTGCATAAC (SEQ ID NO. 9),
*ara*D R700: TAACTGCGGCGCTAACTGAC (SEQ ID NO. 10), and
*ara*D R421: GGTTGCTGGAATCGACTGAC (SEQ ID NO. 11).

The mutations in amplified sequences were repaired by recombination of different clones.

[0042] For cloning *araD* into S6wtd1 EGFP, the vector was linearized by partial digestion with restriction enzyme PagI (position 4761) (Fermentas, Lithuania) and the DNA 5'-termini were dephosphorylated with Calf Intestine Alkaline Phosphatase (CIAP; Fermentas, Lithuania). *ara*D1 and *ara*D2 fragments were cut out from pUC18 with NcoI (Fermentas, Lithuania) and ligated to S6wtd1EGFP/PagI.

[0043] Both ligation mixtures were transformed into *E. coli DH5α* competent cells and plated onto dishes containing LB medium containing 50 μg/ml kanamycin and incubated at 37°C over night. Colonies were first analysed with colony PCR, after which the DNA was isolated and digested with different restriction enzymes.

[0044] The cloning resulted in plasmids S6wtd1EGFP*kana*/*ara*D1, S6wtd1EGFP*kana*/*ara*D2, which are shown in Figures 3 and 4.

[0045] To remove the kanamycin resistance marker gene from the plasmids, S6wtd1EGFP*kana*/*ara*D1 and S6wtd1EGFP*kana*/*ara*D2 were digested with restriction endonuclease BcuI (Fermentas, Lithuania) and a 6473 bp vector fragment was self-ligated.

[0046] The ligation mixtures were transformed into an *E. coli AG1 ΔaraD* strain (see Example 3) and plated onto dishes containing M9 media supplemented with 2% L-arabinose and incubated at 37°C for 36 hours. Colonies were first analyzed with colony PCR, after which the DNA was isolated and digested with different restriction enzymes. The cloning resulted in plasmids S6wtd1 EGFP/*ara*D1, S6wtd1 EGFP/*ara*D2, respectively, are shown in Figures 5 and 6.

[0047] The bacterial colonies containing S6wtd1EGFP/*ara*D1 and S6wtd1 EGFP/araD2 were grown in two different media: LB supplemented with 2.5% L-arabinose and M9 supplemented with 0.2% L-arabinose at 37°C with vigorous shaking. The cells were harvested and the plasmid DNA was extracted from the cell using QIAprep Spin Miniprep Kit (QIAGEN) and analysed by agarose gel electrophoresis (Figures 7A and 7B, respectively).

[0048] The plasmid DNA samples from cultures in LB and M9 media were analysed by agarose gel electrophoresis before and after digestion with restriction endonuclease PagI (Fermentas, Lithuania), (Figure 8). The predicted sizes of the fragments obtained in the PagI digestion were 3954 and 2519 bp for S6wtd1EGFP/*ara*D1 and 4315 and 2157 bp for S6wtd1EGFP/*ara*D2. Lambda DNA digested with Eco91I (M15 in Figure 8C) and lambda DNA digested with EcoRI/HindIII (Fermentas, Lithuania) (M3 in Figure 8C) were used as molecular weight markers. All analyzed bacterial clones contained the correct plasmid in the restriction enzyme analysis, but the DNA yield was very low when the plasmids were grown in LB media. Two of the analyzed bacterial clones from four S6wtd1 EGFP/araD2 clones (#13 and #14 in Figure 8B) had higher growth rate when grown in M9 media supplemented with 0,2% L-arabinose (Figures 7 and 8), which resulted in higher plasmid yield per culture.

[0049] Further analysis of these two clones with improved growth was performed. These two plasmids had the same structure as the other plasmids as judged by restriction analysis. The plasmids were extracted from the bacteria and further characterized by sequencing the *ara*D gene locus. The *ara*D locus sequence of clone #13 (SEQ ID NO. 18; SEQ ID NO. 19) indicated that *ara*D gene coding sequence carries a STOP codon instead of a codon for Glutamine in position 8 of L-ribulose-5-phosphate 4-epimerase. This mutation resulted from the replacement of Cytidine in codon 8 of L-ribulose-5-phosphate 4-epimerase (araD coding sequence) (5'-CAG-3') with the Thymidine, resulting in a STOP codon (5'-TAG-3'). The plasmid carrying such a mutation in *ara*D gene effectively provided the ability to grow in the selective medium in the presence of L-arabinose although the coding sequence contains the STOP codon. It has been demonstrated that the STOP codon UAG is effectively read through by the ribosomes of *Escherichia coli,* when such a STOP is in the beginning of the coding sequence [for reference, see review Murgola, E. J., Annu. Rev. Genet. 19 (1985) 57-80].

Without binding by the theory, we hypothesized that the high yield of the plasmid, which is an indication of rapid uninhibited growth of the bacteria, requires an optimal concentration of the *ara*D gene product L-ribulose-5-phosphate 4-epimerase.

[0050] The analysis of clone #14 *ara*D locus sequence indicated that the *ara*D coding sequence is perfect as predicted. However, the sequence rearrangements near the *ara*D promoter covering the E2 protein binding sites were observed (see Figure 13, SEQ ID NO. 18). These data suggested additionally that such rearrangements near the promoter might result in the down-regulation of the promoter activity, therefore the level of the *ara*D product.

Example 2

Cloning of mutated *ara*D selection plasmids

[0051] For cloning of mutated *araD* selection constructs plasmid p3hCG (Figure 14) carrying kanamycin resistance [transposon Tn5 derived kanamycin resistance marker (neo) gene] was cleaved with the restriction endonucleases BcuI and HindIII, the ends were filled in using Klenow Fragment (Fermentas, Lithuania) and the fragment with the size of 4647 bp was purified from the gel after agarose gel electrophoresis. The pMB1 origin of replication and the *ara*D sequence carrying the C to T mutation, which results in a STOP codon in position 8 of the *araD* gene coding sequence, was excised from the plasmid paraDMgB (Figure 15) with the restriction endonucleases BcuI and Eco52I, the ends were filled in using Klenow Fragment (Fermentas, Lithuania), and the DNA 5'-termini were dephosphorylated with Calf Intestine Alkaline Phosphatase (CIAP; Fermentas, Lithuania). The fragment with the size of 1532 bp was purified from the gel after agarose gel electrophoresis and ligated with the 4647 bp fragment obtained above. Escherichia coli AG1 *araD* deficient strain was transformed with this ligation mixture and plated onto agar plates containing selective M9 medium with 0.5% yeast extract, 2% L-arabinose and 25 μg/ml of kanamycin. The colonies were inspected 24 hours after the plating and showed that the size of the colonies was uniform. The plasmids were extracted from the bacteria and further characterized by sequencing of the *ara*D gene locus.

[0052] The cloning resulted in plasmids p3*ara*D1hCG and p3*ara*D2hCG, which are shown in Figures 16 and 17, respectively. According to the sequence analysis, the bacteria contained un-rearranged plasmids with the mutation C to T in codon 8 (p3araD1 hCG; Figure 16; p3*ara*D2hCG, Figure 17).

[0053] When this experiment was repeated with the wild type sequence and transformed plates were inspected 24 hours after the transformation, the result was different. Two types of colonies were observed: first, large size colonies, and small colonies, which had a retarded growth. The sequence analysis of these plasmids indicated that *araD* gene coding sequence carries a STOP codon instead of a codon for glutamine (plasmid #3A, *ara*D2) or the mutation had occurred in the Shine-Dalgarno sequence in the ribosomal binding site (AGGAG was replaced with AGTAG) (plasmid #2A, *ara*D2). Plasmid #7 (*ara*D1) had the correct sequence in all *araD* gene locus regions, however, the bacteria grew very slowly and resulted in a 10 times lower plasmid yield when were grown in liquid media.

Example 3

Construction of arabinose sensitive Δ*araD Escherichia coli* strains.

[0054] Three *E. coli* strains, DH5alpha T1, AG1 and JM109, were used to construct Δ*araD* mutants. The *araD* gene in *E. coli* genome was disrupted using the method described by Datsenko and Wanner [PNAS 97 (2000) 6640-6645]. This method exploits a phage λ Red recombination system. Briefly, the strategy of this system is to replace a chromosomal sequence with a selectable antibiotic resistance gene that is generated by PCR by using primers with homology extensions. This is accomplished by Red-mediated recombination in these flanking homologies.

[0055] For transformation of the pKD46 (Datsenko and Wanner, *supra),* which encodes the phage λ recombination system, *E. coli,* the cells were made chemically competent using RF1 and RF2 solutions:

RF1 100ml

RbCl 1	1.2 g
$MnCl_2 \cdot 4H_2O$	0.99 g
1 M KAc pH 7.5	3 ml
$CaCl_2 \cdot 2H_2O$	0.15 g
Glycerol	15 g
pH 5.8	(add CH_3COOH)

RF2 100ml

0.5 M MOPS	2 ml
RbCl	0.12 g
$CaCl_2 \cdot 2H_2O$	1.1 g
Glycerol	15 g
pH 6.8	(add NaOH)

[0056] The cells were grown in 2 ml of LB medium to OD_{600} 0.2-0.5. The culture was centrifuged and the pellet was resuspended in 1 ml of RF1. The mixture was kept on ice for 10 min and centrifuged. The pellet was suspended in 100 μl of RF2 and the suspension was kept on ice for 30-45 min. Approximately 50 ng of pKD43 was added and the cells were kept on ice for additional 30 min followed by heat shock of 5 min at 37°C. After incubation for 10 min on ice 900 μl of SOB medium was added to the transformed cells and the mixture was incubated at 37°C for one hour. Cells were plated on LB medium containing ampicillin (100 μg/ml). The colonies were picked from the transformation plates and grown in 2 ml of the same medium to OD_{600} of approximately 1 and glycerol stocks were made (2 ml culture + 0.6 ml 50% glycerol). The stocks were stored at -80°C.

[0057] For disruption of the *araD* gene a linear PCR product which contains kanamycin resistance gene was generated. Plasmid pKD13 (Datsenko and Wanner, PNAS vol. 97, no 12, June 2000) was used as the PCR template. Primers used were *ara*(pr1) and *ara*(pr4):

ara(pr1)

5'-CTCAAACGCCCAGGTATTAGAAGCCAACCTGGCGCTGCC-AAAACACGTGTAG GCTGGAGCTGCTTC 3' (SEQ ID NO. 12)

ara(pr4)

5'-GGTTTGATCACAAAGACGCCGCGCTCGCGATCAACGGCGC-ATTCCGGGGAT CCGTCGACC 3' (SEQ ID NO. 13)

[0058] These primers have the complement sequences with pKD13 for annealing in PCR and with the *ara*D gene for homologous recombination.

[0059] The PCR reaction mixture was as follows: PFU native buffer (5 μl), 10 mM dNTP (5 μl), primer *ara*(pr1) 10 μM (1 μl), primer *ara*(pr4) 10 μM (1 μl), pKD13 100 ng (2 μl), DMSO (4 μl), PFU 2.5 U (1 μl), and mQ water up to 50 μl.

[0060] The PCR procedure was as follows: denaturation 45 s, 96°C, annealing 45 s, 50°C, synthesis 2 min 30 s, 72°C, 25 cycles. The PCR product obtained was 1.4 kb.

[0061] Five reactions were performed simultaneously; the DNA was purified from 2% agarose gel using Ultrapure purification Kit (MoBio Labotratories Inc.) and eluted with 60 μl of water. The DNA was concentrated with ethanol precipitation and dissolved in 5 μl of water. The final concentration was 0.6 μg/μl. An aliquot of 1.5μl was used in one electroporation.

[0062] The PCR product was electroporated into DH5alpha T1 pKD46, AG1 pKD46 (Datsenko and Wanner, *supra),* and JM109 pKD46 *E. coli cells.* First, 200 ml of YENB medium containing 10 mM of L-arabinose for the induction of the recombination system and 100 μg/ml ampicillin was inoculated with an overnight culture of DH5alpha T1 pKD46, AG1 pKD46, and JM109 pKD46 *E. coli* cells. The cultures were grown at 30°C to OD_{600} 0.8 (DH5alpha T1 and JM109) and 0.6 (AG1). The bacteria was collected by centrifugation at 4,000 g for 10 min at 4°C, washed twice with 20 ml of sterile water and once with 20 ml of sterile water containing 10% glycerol. The cells were suspended in 300 μl water containing 10% glycerol. 40 μl of competent cells were used in one electroporation.

[0063] The electroporation was performed with BioRad *E. coli* Pulser using 0.2 cm cuvettes and 2.5 kV. The purified PCR product (1.5 μl) was added to the competent cells, kept on ice for 1 min, and immediately after the electroporation, 2 ml of warm SOB medium was added to the cells and the mixture was incubated at 37°C for 1 hour. The cells were plated on LB medium containg kanamycin (25 μg/ml). 100 pg of large kanamycin resistant plasmid (GTU-MultiHIV C-

clade) was used as a positive control, no plasmid was added to the negative control. The transformation efficiency was 10^6 for AG1 and 10^7 for JM109 for positive control. There were no colonies on the negative control plate, 215 colonies were obtained on JM109+PCR product plate, 70 colonies on AG1+PCR product plate and 50 colonies on DH5alpha T1+PCR product plate.

Example 4

Testing of the *E. coli* DH5alpha T1 *D*araD, AG1Δ*ara*D and JM109Δ*ara*D strains

[0064] The colonies obtained from the electroporation as described in Example 2 were tested for the presence of kanamycin resistance gene by colony PCR using primers araVlisF (5' CGGCACGAAGGAGTCAACAT 3'; SEQ ID NO. 14) and araVlisR (5' TGATAGAGCAGCCGGTGAGT 3'; SEQ ID NO. 15) which contain annealing sites on the *ara*D gene near the insertion site. A PCR product of 272 bp was expected from the *E. coli* DH5alpha T1, AG1 and JM109 strains without insertion in araD and a 1545 bp product, if the PCR product had been inserted in the *ara*D gene. Three colonies of DH5alpha T1 Δ*ara*D, nine colonies of AG1Δ*ara*D and 14 colonies of JM109Δ*ara*D out of 15 were checked and each gave the 1545 bp product. It was therefore concluded that these strains contained the kanamycin resistance gene insertion.

[0065] To confirm the insertion of kanamycin gene another colony PCR was performed using primers *kana*SF (5'TCAGATCCTTGGCGGCAAGA3'; SEQ ID NO. 16) and *ara*VR (5'TGTAATCGACGCCGGAAGGT3'; SEQ ID NO. 17). These primers produce a 435 bp product, if the kanamycin resistance gene has been inserted into the araD gene. Six colonies from AG1Δ*ara*D and JM109Δ*ara*D strains and three colonies of DH5alpha T1 Δ*ara*D strains were tested and all gave the correct product.

[0066] Six colonies of AG1Δ*ara*D and JM109Δ*ara*D, and three colonies of DH5alpha T1 Δ*ara*D were plated on LB medium containing 25 μg/ml of kanamycin and incubated at 37°C overnight to eliminate the pKD46 plasmid, which has a temperature sensitive replication origin. The cells were tested for ampicillin sensitivity by replica plating on LB medium and LB medium containing ampicillin. None grew on the medium containing ampicillin and it was concluded that the bacteria does not contain the pKD46 plasmid any more.

[0067] The arabinose sensitivity was tested on the produced AG1Δ*ara*D and JM109Δ*ara*D strains. One colony of AG1Δ*ara*D and one colony of JM109Δ*ara*D were each inoculated into 2 ml LB. The cultures were grown for 8 hours, diluted 1:100 into M9 medium containing 0.2% glycerol, 25 μg/ml kanamycin, 0.01% thiamine (0.05% proline for JM109Δ*ara*D) and different concentrations of L-arabinose were added in the growth medium. The cultures were grown overnight at 37°C in shaker incubator and OD_{600} was measured (Table 1).

Table 1. Testing of arabinose sensitivity.

L-arabinose %	AG1Δ*ara*D OD_{600}	JM109Δ*ara*D OD_{600}
0	3.2	1.9
0.1	0.03	0.03
0.2	0.030	0.026
0.5	0.030	0.020
1	0.024	0.025
2	0.017	0.021

[0068] As can be seen from Table 1, as low amount as 0.1% of L-arabinose is enough to inhibit the growth of the Δ*ara*D strains of the invention.

[0069] The arabinose sensitivity was further tested on AG1Δ*ara*D, DH5alphaT1 Δ*ara*D and JM109Δ*ara*D as above but using lower concentrations of L-arabinose. The results are given in Figure 18. As can be seen in Figure 18, as low an amount as 0.0005% of L-arabinose is enough to inhibit the growth of the Δ*ara*D strains of the invention.

[0070] Additionally the L-arabinose sensitivity was tested in M9 and yeast extract medium with different glucose and arabinose concentrations (0.2% glucose, 0.2% arabinose, 2% arabinose). The cultures were incubated at 37°C in a shaker incubator overnight. Then the OD_{600} was measured to quantitate the cell density. The results are given in Figure 19.

[0071] Both concentrations of arabinose (0,2% and 2%) inhibited the growth of the Δ*ara*D strains of the invention. However, the growth of strains with intact *araD* gene was not inhibited.

[0072] Additionally the plasmid DNA yield of the Δ*ara*D strains was tested.Plasmid S6wtd1EGFP*ara*D2 prepared in Example 1 was transformed into AG1Δ*ara*D and JM109Δ*ara*D strains. Competent cells were prepared with RF1 and RF2 solutions as described in Example 3.

[0073] The colonies from the transformation plates were inoculated into 2 ml of M9 medium containing 0.5% yeast

extract and 25 μg/ml kanamycin + 0.01% thiamine + L-arabinose (2% and 0.2%).

[0074] The cultures were incubated at 37°C for 17 hours. Then the OD_{600} was measured to quantitate the cell density and the plasmid DNA was extracted with Qiagen Miniprep Kit. Coefficient 2.8 (OD_{600}/ml) was used for miniprep isolation to get comparable results. The results are shown in Table 2.

[0075] DNA concentration was measured with spectrophotometer as OD at 260 nm. For microscopic analysis a drop of bacterial culture was applied on glass slide and covered with cover slip. The culture was visually inspected at a 100xmagnification with an objective in oil immersion.

Table 2. Plasmid DNA yield of Δ*araD* strains

Strain	L-arabinose (%)	OD_{600}	Plasmid DNA conc. (μg/μl)	Plasmid DNA yield (μg per ml of culture)	Appearance in microscope
AG1Δ*ara*D	2	7.6	0.039	5.3	no filaments
AG1Δ*ara*D	0.2	5.8	0.057	5.9	no filaments
JM109Δ*ara*D	2	4.9	0.043	3.8	very few filaments
JM109Δ*ara*D	0.2	4.3	0.038	2.9	very few filaments
DH5αT1Δ*ara*D	2	6.6	0.017	3.5	no filaments
DH5αT1Δ*ara*D	0.2	6.4	0.016	3.4	no filaments

[0076] According to these results 0.2% L-arabinose is sufficient for obtaining the plasmid copy number at the same level as with 2% arabinose.

[0077] For this plasmid AG1Δ*ara*D seems to be better, because the plasmid yield is somewhat higher and cell densities also.

Example 5.

Generation of an Escherichia coli strain with additional mutations within the genes potentially encoding L-ribulose-5-phosphate 4-epimerase.

[0078] *E. coli* chromosome contains two additional coding sequences for L-ribulose-5-phosphate 4-epimerases in different operons. The *ula*F and *sgb*E genes from L-ascorbate degradation pathway encode the genes with epimerase activity (Wen Shan Yew, Jhon A. Gerlt, J. Bacteriol. 184 (2002) 302-306. In order to increase the stringency of the selection and to avoid or knock out the possible adaptation mechanisms of *E. coli* strains due to other genes with epimerase activity, the coding sequences of the *Ula*F and SgbE genes in *E. coli* genome were interrupted. Such adaptation mechanisms could occur in long-term plasmid production under suitable conditions.

[0079] The *Ula*F and SgbE genes in *E. coli* strains DH5alphaT1Δ*ara*D and AG1Δ*ara*D were disrupted using the phage λ Red recombination system as described in Example 3.

[0080] First, the kanamycin-resistant gene in *E. coli* AG1Δ*ara*D and DH5αT1Δ*ara*D strains was eliminated. FLP recombinase expression plasmid pKD20 (Datsenko and Wanner, *supra)* is ampicillin resistant and temperature-sensitive. Kanamycin-resistant mutants were transformed with pCP20 (kanamycin-resistant gene is FRT-flanked), and ampicillin-resistant transformants were selected at 30°C (48 hours), after which the same colonies were purified non-selectively at 42°C (24 hours twice). Then they were tested for loss of kanamycin and ampicillin resistances.

[0081] The inactivation of the chromosomal *ula*F gene (SEQ ID NO. 20) by the phage λ Red recombination system was performed using the primers ulaFylem and ulaFalum:

ulaFylem

CAGCAGGTATTTGAAGCCAACATGGAGCTGCCGCGCTACG-GGCTGGTGTAGGCTGGAGCTGCTTC (SEQ ID NO. 21)

ulaFalum

AAACGGCTGCGGAATTAGACCAGTTATCTCCCGAGGAAGGAAA TTAATTCCGGGGATCCGTCGACC (SEQ ID NO. 22)

[0082] A lot of colonies were observed on both transformation plates. Fifteen colonies obtained from the electroporation were tested for the presence of the kanamycin resistance gene by colony PCR using primers ulaFvalisR and ulaFvalisF:

ulaFvalisR
AAACGGCTGCGGAATTAGACC (SEQ ID NO. 23)
ulaFvalisF
GCCGTACCTGATTGAGATGTGGAG (SEQ ID NO. 24)

[0083] These primers contain annealing sites on the *Ula*F gene near the insertion site. A PCR product of 864 bp was expected from the *E. coli* DH5alphaT1Δ*ara*D and AG1Δ*ara*D strains without insertion in *Ula*F and a 1527 bp product, if the PCR product had been inserted in the *Ula*F gene. To confirm the insertion of the kanamycin gene another colony PCR was performed using primers ulaFvalisR (SEQ ID NO 23) and kanaSF (SEQ ID NO 16).

[0084] These primers produce a 428 bp product, if the kanamycin resistance gene has been inserted into the *Ula*F gene. Four colonies from AG1ΔaraDΔ*ula*F and DH5alphaT1Δ*ara*DΔ*ula*F strains were tested and all gave the correct product. One colony from each strain was used further.

[0085] The elimination of the kanamycin-resistant gene in *E.coli* AG1Δ*ara*DΔ*ula*F and DH5alphaT1Δ*ara*DΔ*ula*F strains was performed as described above. The inactivation of the chromosomal *sgb*E gene (SEQ ID NO. 25) by the phage λ Red recombination system was performed as described in Example 3. The primers used were sgbEalum and sgbEylem:

sgbEalum

CGTTACAGCAAGGAACATATCAATTCGTAGTGCCGGGGCGATG AAGAATTCCGGGGATCCGTCGACC (SEQ ID NO. 26)

sgbEylem

GCAGGAGGCTGGATTTATATGTTAGAGCAACTGAAAGCCG-ACGTGGTGTAGGCTGGAGCTGCTTC (SEQ ID NO. 27)

[0086] A lot of colonies were observed on both transformation plates. Fifteen colonies obtained from the electroporation were tested for the presence of kanamycin resistance gene by the colony PCR using primers sgbEvalisR and sgbEvalisF:

sgbEvalisR
CGGCGTTACAGCAAGGAACATATC (SEQ ID NO. 28)
sgbEvalisF
ATTGAAGCGCGTATGCAGGAGG (SEQ ID NO. 29)

[0087] A PCR product of 792 bp was expected from the *E. coli* DH5alpha T1Δ*ara*DΔ*ula*FΔ*sgb*E and AG1Δ*ara*DΔ*ula*FΔ*sgb*E strains without insertion in *SgbE* and a 1413 bp product, if the PCR product had been inserted in the *Sgb*E gene. To confirm the insertion of kanamycin gene another colony PCR was performed using primers sgbEvalisR (SEQ ID NO. 28) and kanaSF (SEQ ID NO. 16):

Fifteen colonies from both strains were tested and four gave the correct product.

[0088] The arabinose sensitivity was tested on the *E. coli* DH5alphaT1 Δ*ara*DΔ*ula*FΔ*sgb*E and AG1Δ*ara*DΔ*ula*FΔ*sgb*E strains produced and compared to those of *E. coli* DH5alphaT1Δ*ara*D and AG1Δ*ara*D strains. One colony of each strain was inoculated into 2 ml of M9 medium containing 0.5% yeast extract, 25 μg/ml of kanamycin, 0.2% glucose only or 0.2% or 2% L-arabinose, respectively. The results are shown in Table 3.

Table 3. Testing of arabinose sensitivity

Strain	OD_{600} Glc	OD_{600} Glc+0,2% L-arabinose	OD_{600} Glc+2% L-arabinose
AG1 Δ*ara*D	7.3	0.82	0.26
DH5alphaT1 Δ*ara*D	7.7	0.95	0.35
AG1Δ*ara*D Δ*ula*FΔ*sgb*E	8.3	0.82	0.35
DH5alphaT1 Δ*ara*DΔ*ula*F Δ*sgb*E	7.5	0.75	0.28

[0089] As can be seen from Table 3, there were no essential differences in the arabinose sensitivity of the strains of the invention. Similarly, when the plasmid DNA yield of the Δ*ara*D and Δ*ara*DΔ*ula*FΔ*sgb*E strains was tested as described in Example 3 (the results are not shown), no differences were found between *E.coli* AG1Δ*ara*D and AG1Δ*ara*DΔ*ula*FΔ*sgb*E or DH5alphaT1Δ*ara*D and DH5alphaT1Δ*ara*DΔ*ula*FΔ*sgb*E strains.

Example 6

Stability of S6wtd1EGFP/*ara*D2

[0090] An important feature of the vaccination vector is the stability during propagation in bacterial cells. To test the stability of S6wtd1 EGFP/araD2 in bacteria the plasmid was transformed into the *E. coli* AG1Δ*ara*D and JM109Δ*ara*D strains prepared in Example 3 and the intactness of the vector was followed by the plasmid DNA analysis during four generations.

[0091] The plasmid S6wtd1 EGFP/araD2 was mixed with competent *E. coli* AG1Δ*ara*D and JM109Δ*ara*D cells and incubated on ice for 30 minutes. Subsequently, the cell suspension was subjected to a heat-shock for 3 minutes at 37°C followed by a rapid cooling on ice. One milliliter of LB medium was added to the sample and the mixture was incubated for 45 minutes at 37°C with vigorous shaking. Finally, a portion of the cells was plated onto M9 medium dishes containing 0.5% yeast extract, 2% L-arabinose and 25 μg/ml of kanamycin. On the next day, the cells from one colony were transferred onto the new dish containing the same medium. This procedure was repeated until four passages of bacteria had been grown. Two colonies from each passage of both bacterial strains were used to inoculate of 2 ml of M9 medium containing 0.5% yeast extract, 2% L-arabinose and 25 μg/ml of kanamycin incubated overnight at 37°C with vigorous shaking. The cells were harvested and the plasmid DNA was extracted from the bacteria using QIAprep Spin Miniprep Kit (QIAGEN). The plasmid DNA samples before (Figure 9) and after the digestion with restriction endonuclease HindIII (Figure 10) (Fermentas, Lithuania) were analyzed by agarose gel electrophoresis in comparison with the original S6wtd1EGFP/*ara*D2 DNA used for transformation (as control in Figures 9 and 10). Lambda DNA digested with EcoRI/HindIII (Fermentas, Lithuania) was used as a molecular weight marker (M3 in Figure 10).

[0092] Samples were digested with HindIII as shown in Figure 10A for *E. coli* AG1Δ*ara*D and in Figure 10B for JM109Δ*ara*D strain, patterns identical to the original S6wtd1EGFP/*ara*D2 plasmid DNA were observed. The predicted sizes of the fragments resulted by HindIII digestion are 3274, 1688 and 1510 bp. It can be concluded that the vaccination vector S6wtd1EGFP/*ara*D2 is stable when propagated in E. *coli* AG1Δ*ara*D and JM109Δ*ara*D strains.

Example 7

Comparison of an antibiotic selection system with the L-arabinose selection system of the invention

[0093] In the comparison of an antibiotic selection system with the L-arabinose selection system of the invention the following growth media were used.

For E. coli AG1 carrying plasmid p2 MG C #11:

Medium 1: M9 medium plus 0.5% yeast extract, 0.2% glucose and 25 μg/ml of kanamycin (selective medium);
Medium 2: M9 medium plus 0.5% yeast extract and 0.2% glucose (non-selective medium);
Medium 3:
M9 medium plus 0.5% yeast extract, 0.2% L-arabinose and 25 μg/ml of kanamycin; (selective medium); and
Medium 4: M9 medium plus 0.5% yeast extract and 0.2% L-arabinose (non-selective medium).

For *E. coli* AG1Δ*ara*D carrying p*ara*D MG C #145:

Medium 5:
M9 medium plus 0.5% yeast extract, 0.2% L-arabinose and 25 μg/ml of kanamycin (selective medium); and
Medium 6:M9 medium plus 0.5% yeast extract, 0.2% glucose and 25 μglml of kanamycin (non-selective medium).

[0094] The plasmids p2 MG C #11 (Figure 20) and pa*ra*D MG C #145 (Figure 21) were transformed into *E. coli* AG1 and into *E. coli* AG1Δ*ara*D carrying the mutation C to T in codon 8. The transformed bacterial colonies were grown at 37°C overnight in an incubator. Next morning the colonies were inoculated into the selective and non-selective liquid media as indicated above. The inoculated cultures were grown in a shaker in 2 ml of the respective medium until they reached the stationary phase, and the density of the cultures was measured at OD_{600}. The plasmid was extracted from the cultures and the plasmid DNA yield was determined by the measurement of the plasmid DNA at 260 nm. The plasmid yield was calculated on the basis that 50 μg yields to an optical density of 1 at 260 nm.

[0095] Then an aliquot of 20 μl from the stationary cultures was inoculated into fresh medium (dilution 100 times), and the cultures were grown until stationary phase (8-12 hours). The density of the cultures was measured at OD_{600}, the plasmid was extracted and the yield was determined, and again an aliquot was inoculated into 2 μl of the liquid medium. This procedure was repeated 7 times (preparations 1 to 7). The results of the experiment are provided in Table 5 below.

Table 5. Comparison of an antibiotic selection system with the L-arabinose selection system of the invention

Medium number/ preparation number	OD_{600}	Amount of plasmid DNA per 1 ml culture
1/1	6.215	6.35μg
1/7	3.278	2.3μg
2/1	6.652	6.15μg
2/7	5.133	0.65μg
3/1	7.317	10.9μg
3/7	3.046	1.6μg
4/1	6.874	6μg
4/7	4.634	0.75μg
5/1	7.271	6.45μg
5/7	7.014	5.15μg
6/1	6.131	5.3μg
6/7	6.031	4.4μg

[0096] It can be concluded from these data that a plasmid carrying the kanamycin resistance gene and conferring *E. coli* the resistance in the presence of kanamycin is lost in the consecutive dilution/growing steps of the culture under the non-selective as well as under selective conditions. The yield of the plasmid from 1 ml culture drops 3 times under the selective conditions and 10 times under the non-selective conditions at the seventh round of dilution (preparations 1/1 vs. 1/7 and 2/1 vs. 2/7, respectively, in Table 5). The same basic result is obtained, when the carbon source for *E. coli* carrying a plasmid with kanamycin resistance is L-arabinose instead of glucose (preparations 3/1 vs. 3/7 and 4/1 vs. 4/7, respectively, in Table 5). However, when the *ara*D selection system of the invention is used in the plasmid, the plasmid DNA yield is high under both selective (preparation 5/1 vs. 5/7 in Table 5) and non-selective (preparation 6/1 vs. 6/7 in Table 5) conditions. Both under selective and non-selective conditions the plasmid DNA yield dropped over 7 generations approximately 20%. This indicates clearly that the plasmids carrying *ara*D selection system of the invention are much more stable and grow efficiently under the selective as well as non-selective conditions.

Example 8

Fed-batch fermentation of AG1Δ*ara*D S6wtd1EGFP/*ara*D2

[0097] The *ara*D gene based selection system was also tested in fed-batch fermentation for the purpose of production of plasmid containing bacteria. A single colony was picked from AG1Δ*ara*D S6wtd1EGFP/*ara*D2 plate and inoculated into 250 ml M9 medium containing 0.5% yeast extract, 0.2% L-arabinose and 25 μg/ml of kanamycin and incubated overnight at 37°C with vigorous shaking. After 18 hours the OD_{600} of inoculum was 6.4. 160 ml of inoculum was added

to fermentor containing 5 l Fermenter Starting Medium (8 g/l KH_2PO_4; 10 g/l NaCl; 5 g/l NH_4Cl ; 5 g/l yeast extract; 2 g/l L-arabinose; 2 g/l $MgSO_4$, 25 mg/l kanamycin and 0.1 g/l thiamine; pH 6.7 with NH_4OH). After 5.5 hours of growth automatic feeding was started with given growth speed of 0.15 h^{-1} (allows carbon-source limited growth) with fermenter feeding medium (300 g/l L-arabinose; 150 g/l yeast extract; 50 mg/l kanamycin; 0.2 g/l thiamine). Feeding speed was controlled by computer according to formulae $F(t)=myS*S_{in}/S_f$ where myS is desired growth rate, S_{in} is the amount of carbon source added to the time point and S_f is carbon source concentration in feeding medium. The growth was followed by measuring OD_{600} and samples for plasmid DNA were taken. The data registered during fermentation is represented in Figure 11. Fermentation was terminated when 1 l of feeding medium was consumed. Final OD_{600} was 45. The bacterial mass was collected by centrifugation and washed once with 2 l STE buffer. Yield of bacterial biomass was 410 g wet weight. The data for plasmid DNA content is shown in Table 6.

Table 6. Plasmid DNA yield during AG1Δ*ara*D S6wtd1EGFP/*ara*D2 fermentation

Time	OD_{600}	Plasmid DNA conc. (μg/μl)	Plasmid DNA yield (μg per ml of culture)
Inoculum	6.4	0.04	4.6
4h	3.1	0.02	1.1
21 h	28	0.1	50
24 h	37	0.13	87
29 h	45	0.14	113

[0098] The data in Table 6 indicate that the L-arabinose selection system works very well at high cell densities. It is probably because more plasmid copies in bacterial cell gives an advantage in the conditions of L-arabinose limitation by enabling the bacterium to use sugar more rapidly.

Example 9

Purification of AG1Δ*ara*D S6wtd1EGFP/*ara*D2

[0099] The purification of AG1Δ*ara*D S6wtd1EGFP/*ara*D2 was performed as follows (Figure 12):

a) Feeding preparation

[0100] Clear lysate was prepared according to Qiagen's Plasmid Purification Handbook, exept RNase was not used.

[0101] 200g of *E. coli* cell paste was resuspended in 2000ml of Resuspension Buffer and later equal volumes of P2 and P3 for lysis and neutralization were used. The cell debris was removed by centrifugation at 6000g for 30 minutes at 4°C. Clear lysate was poured through the paper towel, 1/10 of 10% Triton X-114 (Sigma) was added and solution was left on ice for 1 hour. (Triton X-114 has been shown to effectively reduce the level of endotoxins in protein, Liu et al., Clinical Biochemistry, 1997) After one hour nucleic acids were precipitated with 0,6 volumes of cold isopropanol. Supernatant was decanted and precipitate was stored overnight at -20°C.

b) Plasmid DNA purification

[0102] Plasmid DNA purification was performed according to Amersham Pharmacia's three step supercoiled plasmid purification process, where few modifications were adopted.

Step 1. Precipitate was redissolved in 1500ml TE (10mM Tris-Cl, 1 mM EDTA; pH 8.0) and loaded for RNA removal and buffer exchange on Sepharose 6 FF (Amersham Pharmacia), previously equilibrated with Buffer A - 2M $(NH_4)_2SO_4$,100mM Tris Cl, 10mM EDTA, pH 7.5.

Step 2. Void volume was directed to the PlasmidSelect (Amersham Pharmacia) column (equilibrated with Buffer A) and after washing and elution with Buffer B2 (1,6M NaCl, 2M $(NH_4)_2SO_4$, 100mM Tris Cl, 10mM EDTA, pH 7.5), supercoiled plasmid DNA was captured.

Step 3. Eluted plasmid was diluted with five volumes of distilled, deionized water and loaded to SOURCE 30Q (Amersham Pharmacia) equilibrated with buffer C1 (0,4M NaCl, 100mM Tris Cl, 10mM EDTA, pH 7.5). After washing, purified plasmid was eluted with Buffer C2 (1 M NaCl, 100mM Tris Cl, 10mM EDTA, pH 7.5) and elution peak was collected. Fraction size was 150ml and it contained 100mg of endotoxins-free (<10 EU/mg) S6wtd1 EGFP/araD2 plasmid.

SEQUENCE LISTING

<110> FIT Biotech Oyj Plc.

<120> Novel selection system

<130> 2031002

<160> 29

<170> PatentIn version 3.1

<210> 1

<211> 780

<212> DNA

<213> Escherichia coli

<400> 1

gtttcgtttg attggctgtg gttttataca gtcattactg cccgtaatat gccttcgcgc 60

catgcttacg cagatagtgt ttatccagca gcgtttgctg catatccggt aactgcggcg 120

ctaactgacg gcagaatatc cccatataag cgacctcttc cagcacgatg gcgttatgca 180

ccgcatcttc ggcatttttg ccccatgcaa acgggccgtg ggaatggacc agaacgccgg 240

gcatttgcgc tgcatcgata ccctgttttt caaaggtttc tacgatgacg ttaccggttt 300

cccactcata ttcgccgttg atttctgcgt cggtcatttt gcgggtgcag ggaatggtgc 360

cgtagaaata gtcggcgtgg gtggtgccgg ttgctggaat cgactgaccc gcctgcgccc 420

agatggtggc gtggcgcgag tgcgtatgca caatgccgcc aatggagggg aatgcctgat 480

agagcagccg gtgagttggc gtgtcggagg agggcttttt cgtaccttca accacttcac 540

cggtttcgat gctaaccacg accatatcgt cagcggtcat gacgctgtaa tcgacgccgg 600

aaggtttgat cacaaagacg ccgcgctcgc gatcaacggc gctgacgttg ccccatgtga 660

gcgtgaccag gttgtgtttt ggcagcgcca ggttggcttc taatacctgg cgtttgagat 720

cttctaacat gttgactcct tcgtgccgga tgcgctttgc ttatccggcc tacaaaatcg 780

<210> 2

<211> 76

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer

<400> 2
cgccatggtt ctcatgtttg acagcttatc atcgataagc tttaatgcgg tagtttagca 60

cgaaggagtc aacatg 76

<210> 3

<211> 64

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer

<400> 3
cgccatggac tagtaaaaaa aagcccgctc attaggcggg ctgtcattac tgcccgtaat 60

atgc 64

<210> 4

<211> 81

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer

<400> 4
cgccatggac tagttctcat gtttgacagc ttatcatcga taagctttaa tgcggtagtt 60

tagcacgaag gagtcaacat g 81

<210> 5

<211> 58

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer

<400> 5
cgccatggaa aaaaaagccc gctcattagg cgggctgtca ttactgcccg taatatgc 58

<210> 6

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer

<400> 6
gccagggttt tcccagtcac ga 22

<210> 7

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer

<400> 7
gagcggataa caatttcaca cagg 24

<210> 8

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer

<400> 8
ccaactcacc ggctgctcta tc 22

<210> 9

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer

<400> 9
aatgccgaag atgcggtgca taac 24

<210> 10

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer

<400> 10
taactgcggc gctaactgac 20

<210> 11

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer

<400> 11
ggttgctgga atcgactgac 20

<210> 12

<211> 66

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer

<400> 12
ctcaaacgcc caggtattag aagccaacct ggcgctgcca aaacacgtgt aggctggagc 60

tgcttc 66

<210> 13

<211> 60

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer

<400> 13
ggtttgatca caaagacgcc gcgctcgcga tcaacggcgc attccgggga tccgtcgacc 60

<210> 14

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer

<400> 14
cggcacgaag gagtcaacat 20

<210> 15

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer

<400> 15
tgatagagca gccggtgagt 20

<210> 16

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer

<400> 16
tcagatcctt ggcggcaaga 20

<210> 17

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer

<400> 17
tgtaatcgac gccggaaggt 20

<210> 18

<211> 1030

<212> DNA

<213> Artificial

<400> 18
ggatccgacc ggcaacggta cagatccgac cggcaacggt acagatccga ccggcaacgg 60

tcagatccga ccggcaacgg tacagatccg accggcaacg gtacagatcc gaccggcaac 120

ggtacagatc cgaccggcaa cggtacagat ccgaccggca acggtacaga tccgaccggc 180

aacggtacag atccgaccgg caacggtaca gatcccccta gcgaattgac tagttctcat 240

gtttgacagc ttatcatcga taagctttaa tgcggtagtt tagcacgaag gagtcaacat 300

gttagaagat ctcaaacgcc aggtattaga agccaacctg gcgctgccaa aacacaacct 360

ggtcacgctc acatggggca acgtcagcgc cgttgatcgc gagcgcggcg tctttgtgat 420

caaaccttcc ggcgtcgatt acagcgtcat gaccgctgac gatatggtcg tggttagcat 480

cgaaaccggt gaagtggttg aaggtacgaa aaagccctcc tccgacacgc caactcaccg 540

gctgctctat caggcattcc cctccattgg cggcattgtg catacgcact cgcgccacgc 600

caccatctgg gcgcaggcgg gtcagtcgat tccagcaacc ggcaccaccc acgccgacta 660

tttctacggc accattccct gcacccgcaa aatgaccgac gcagaaatca acggcgaata 720

tgagtgggaa accggtaacg tcatcgtaga aacctttgaa aaacagggta tcgatgcagc 780

gcaaatgccc ggcgttctgg tccattccca cggcccgttt gcatggggca aaaatgccga 840

agatgcggtg cataacgcca tcgtgctgga agaggtcgct tatatgggga tattctgccg 900

tcagttagcg ccgcagttac cggatatgca gcaaacgctg ctggataaac actatctgcg 960

taagcatggc gcgaaggcat attacgggca gtaatgacag cccgcctaat gagcgggctt 1020

ttttttccat 1030

<210> 19

<211> 696

<212> DNA

<213> Escherichia coli

<400> 19

atgttagaag atctcaaacg ccaggtatta gaagccaacc tggcgctgcc aaaacacaac 60

ctggtcacgc tcacatgggg caacgtcagc gccgttgatc gcgagcgcgg cgtctttgtg 120

atcaaacctt ccggcgtcga ttacagcgtc atgaccgctg acgatatggt cgtggttagc 180

atcgaaaccg gtgaagtggt tgaaggtacg aaaaagccct cctccgacac gccaactcac 240

cggctgctct atcaggcatt cccctccatt ggcggcattg tgcatacgca ctcgcgccac 300

gccaccatct gggcgcaggc gggtcagtcg attccagcaa ccggcaccac ccacgccgac 360

tatttctacg gcaccattcc ctgcacccgc aaaatgaccg acgcagaaat caacggcgaa 420

tatgagtggg aaaccggtaa cgtcatcgta gaaacctttg aaaaacaggg tatcgatgca 480

gcgcaaatgc ccggcgttct ggtccattcc cacggcccgt ttgcatgggg caaaaatgcc 540

gaagatgcgg tgcataacgc catcgtgctg gaagaggtcg cttatatggg gatattctgc 600

cgtcagttag cgccgcagtt accggatatg cagcaaacgc tgctggataa acactatctg 660

cgtaagcatg gcgcgaaggc atattacggg cagtaa 696

<210> 20

<211> 687

<212> DNA

<213> Escherichia coli

<400> 20
atgcaaaagc taaaacagca ggtatttgaa gccaacatgg agctgccgcg ctacgggctg 60

gtgaccttta cctggggcaa cgtcagcgct atcgaccgcg aacgcgggct ggtggtgatc 120

aagcccagcg gcgttgccta cgaaaccatg aaagcggccg atatggtggt ggttgatatg 180

agcggcaagg tggtggaagg ggagtatcgc ccatcttccg acactgcgac gcatctcgaa 240

ctctaccgtc gttacccgtc gcttggtggc attgtccata cccactccac tcatgccacc 300

gcatgggcgc aggcggggct ggcgatcccg gcgttaggca ccacgcacgc cgactacttc 360

tttggcgaca ttccgtgtac gcgcgggtta agcgaagaag aggtgcaggg cgagtatgaa 420

ctgaacaccg gcaaagtgat tatcgaaacg ctgggcaacg ccgagccgct gcatacgccg 480

ggaattgtgg tgtatcagca cgggccgttc gcctggggga aagatgctca cgatgcggtg 540

cataacgcgg tggtgatgga agaagtggcg aaaatggcgt ggattgcccg cggcattaac 600

ccacaactca atcacatcga cagcttcctg atgaataaac acttcatgcg taaacacggt 660

cctaacgctt attacgggca gaagtag 687

<210> 21

<211> 65

<212> DNA

<213> Artificial

<400> 21
cagcaggtat ttgaagccaa catggagctg ccgcgctacg ggctggtgta ggctggagct 60

gcttc 65

<210> 22

<211> 66

<212> DNA

<213> Artificial

<400> 22
aaacggctgc ggaattagac cagttatctc ccgaggaagg aaattaattc cggggatccg 60

tcgacc 66

<210> 23

<211> 21

<212> DNA

<213> Artificial

<400> 23
aaacggctgc ggaattagac c 21

<210> 24

<211> 24

<212> DNA

<213> Artificial

<400> 24
gccgtacctg attgagatgt ggag 24

<210> 25

<211> 696

<212> DNA

<213> Escherichia coli

<400> 25
atgttagagc aactgaaagc cgacgtgctg gcggcgaatc tggcgcttcc cgctcaccat 60
ctggtgacgt tcacctgggg caatgtcagc gcggtagacg aaacgcggca atggatggta 120
atcaaacctt ccggcgtcga gtacgacgtg atgaccgccg acgatatggt ggtggttgag 180
atagccagcg gtaaggtggt ggaaggcagc aaaaaaccct cttccgatac accaacgcat 240
ctggcgctct accgtcgcta tgccgaaatt ggcggtattg tgcataccca ctcgcgccac 300
gccaccatct ggtcacaggc cgggctggat ctccccgcct ggggcaccac ccacgccgat 360
tatttttacg gtgccatccc ctgcacgcga cagatgaccg cagaggagat taacggcgaa 420
tatgaatatc agaccggcga agtgatcatt gaaaccttcg aagaacgtgg caggagtccg 480
gcacaaatcc cggcggtgct ggtgcattct cacggcccgt tcgcatgggg taaaaacgcc 540
gccgatgccg tgcataacgc cgtagtactc gaagaatgcg cctatatggg tctattctcg 600
cgccagcttg cgccgcagct ccctgcgatg caaaacgaac tgctggataa gcactacctg 660
cgtaagcatg gggccaatgc ctattacggg cagtaa 696

<210> 26

<211> 67

<212> DNA

<213> Artificial

<400> 26
cgttacagca aggaacatat caattcgtag tgccggggcg atgaagaatt ccggggatcc 60

gtcgacc 67

<210> 27

<211> 65

<212> DNA

<213> Artificial

<400> 27
gcaggaggct ggatttatat gttagagcaa ctgaaagccg acgtggtgta ggctggagct 60

gcttc 65

<210> 28

<211> 24

<212> DNA

<213> Artificial

<400> 28
cggcgttaca gcaaggaaca tatc 24

<210> 29

<211> 22

<212> DNA

<213> Artificial

<400> 29
attgaagcgc gtatgcagga gg 22

Claims

1. A selection system comprising a bacterial cell deficient of an *ara*D gene into which a vector carrying an *ara*D gene, or a catalytically active fragment thereof has been added as a selection marker.

2. A selection system according to claim 1, wherein the *ara*D gene is L-ribulose-5-phosphate 4-epimerase gene (EC 5.1.3.4.).

3. A selection system according to claim 1 or 2, wherein the *ara*D gene of the vector is mutated.

4. A selection system according to claim 3, wherein the mutation introduces a stop codon into position 8 of the *ara*D gene.

5. A selection system according to claim 1, wherein the bacterial cell is an *Escherichia coli* cell.

6. A selection system according to claim 5, wherein the *E. coli* is an *E. coli* strain JM109.

7. A selection system according to claim 5, wherein the *E. coli* is an *E. coli* strain DH5 alpha-T1.

8. A selection system according to claim 5, wherein the *E. coli* is an *E. coli* strain AG1.

9. A selection system according to any of claims 5-8, wherein said *E. coli* strain is deficient of the *araD* gene.

10. A selection system according to any of claims 5-8, wherein said *E. coli* strain is deficient of the *araD* gene and *ulaF* gene.

11. A selection system according to any of claims 5-8, wherein said *E. coli* strain is deficient of the *araD* gene and *sgbE* gene.

12. A selection system according to any of claims 5-8, wherein said *E. coli* strain is deficient of *ara*D gene, *ulaF* gene, and *sgb*E gene.

13. A vector comprising an mutated *ara*D gene with a stop codon at position 8, or a catalytically active fragment thereof as a selection marker.

14. A vector according to claim 13, wherein the vector is an expression vector comprising:

(a) a DNA sequence encoding a nuclear-anchoring protein operatively linked to a heterologous promoter, said nuclear-anchoring protein comprising (i) a DNA binding domain which binds to a specific DNA sequence, and (ii) a functional domain that binds to a nuclear component, or a functional equivalent thereof; and
(b) a multimerized DNA sequence forming a binding site for the nuclear anchoring protein, wherein said vector lacks a papilloma virus origin of replication, and
(c) the mutated *ara*D gene, or a catalytically active fragment thereof as a selection marker.

15. A vector according to claim 14, wherein the vector is an expression vector comprising:

(a) DNA sequence encoding a nuclear-anchoring protein operatively linked to a heterologous promoter, wherein the nuclear-anchoring protein is the E2 protein of Bovine Papilloma Virus type 1 (BPV), and
(b) a multimerized DNA sequence forming a binding site for the nuclear anchoring protein is of multiple binding sites the BPV E2 protein incorporated into the vector as a cluster, where the sites can be as head-to-tail structures or can be included into the vector by spaced positioning, wherein said vector lacks a papilloma virus origin of replication, and
(c) the mutated *araD* gene, or a catalytically active fragment thereof as a selection marker.

16. A vector according to claim 15 additionally comprising a deletion in the multimerized DNA sequence.

17. A vector according to claim 15 additionally comprising a mutation in Shine-Dalgarno sequence.

18. A selection system according to any of claims 1-12 comprising a vector according to any of claims 13-17.

19. Use of a vector comprising an *ara*D gene, a mutated form of an *ara*D gene, or a catalytically active fragment thereof as a selection marker, in a selection system.

20. Use of a vector according to claim 19 in a selection system, wherein the vector is an expression vector comprising:

(a) a DNA sequence encoding a nuclear-anchoring protein operatively linked to a heterologous promoter, said nuclear-anchoring protein comprising (i) a DNA binding domain which binds to a specific DNA sequence, and (ii) a functional domain that binds to a nuclear component, or a functional equivalent thereof; and
(b) a multimerized DNA sequence forming a binding site for the nuclear anchoring protein, wherein said vector lacks a papilloma virus origin of replication, and
(c) the *ara*D gene, a mutated form of an *ara*D gene, or a catalytically active fragment thereof as a selection marker.

21. Use of a vector according to claim 20 in a selection system, wherein the vector is an expression vector comprising:

(a) DNA sequence encoding a nuclear-anchoring protein operatively linked to a heterologous promoter, wherein the nuclear-anchoring protein is the E2 protein of Bovine Papilloma Virus type 1 (BPV), and
(b) a multimerized DNA sequence forming a binding site for the nuclear anchoring protein is of multiple binding sites the BPV E2 protein incorporated into the vector as a cluster, where the sites can be as head-to-tail structures or can be included into the vector by spaced positioning, wherein said vector lacks a papilloma virus origin of replication, and
(c) an *ara*D gene, a mutated form of an *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof as a selection marker.

22. Use of a vector according to claim 21 in a selection system, wherein the vector additionally comprises a deletion in the multimerized DNA sequence.

23. Use of a vector according to claim 21 in a selection system, wherein the vector additionally comprises a mutation in Shine-Dalgarno sequence.

24. Use of a vector according to any of claims 19-23, in a selection system according to any of claims 1-12.

25. *E. coli* strain DH5alpha-T1 deficient of the *araD* gene and *ulaF* gene.

26. *E. coli* strain DH5alpha-T1 deficient of the *araD* gene and *sgbE* gene.

27. *E. coli* strain DH5alpha-T1 deficient of the *araD* gene, *ulaF* gene, and *sgbE* gene.

28. *E. coli* strain AG1 deficient of the *araD* gene and *ulaF* gene.

29. *E. coli* strain AG1 deficient of the *araD* gene and *sgbE* gene.

30. *E. coli* strain AG1 deficient of the *araD* gene, *ulaF* gene, and *sgbE* gene.

31. *E. coli* strain JM109 deficient of the *araD* gene and *ulaF* gene.

32. *E. coli* strain JM109 deficient of the *araD* gene and *sgbE* gene.

33. *E. coli* strain JM109 deficient of the *araD* gene, *ulaF* gene, and *sgbE* gene.

34. Use of *E. coli* strain DH5alpha-T1 deficient of the *araD* gene and *ulaF* gene in a selection system.

35. Use of *E. coli* strain DH5alpha-T1 deficient of the *araD* gene and *sgbE* gene in a selection system.

36. Use of *E. coli* strain DH5alpha-T1 deficient of the *araD* gene, *ulaF* gene, and *sgbE* gene in a selection system.

37. Use of *E. coli* strain AG1 deficient of the *araD* gene and *ulaF* gene in a selection system.

38. Use of *E. coli* strain AG1 deficient of the *araD* gene and *sgbE* gene in a selection system.

39. Use of *E. coli* strain AG1 deficient of the *araD* gene, *ulaF* gene, and *sgbE* gene in a selection system.

40. Use of *E. coli* strain JM109 deficient of the *araD* gene and *ulaF* gene in a selection system.

41. Use of *E. coli* strain JM109 deficient of the *araD* gene and *sgbE* gene in a selection system.

42. Use of *E. coli* strain JM109 deficient of the *araD* gene, *ulaF* gene, and *sgbE* gene in a selection system.

43. A method of selecting the cells transformed with a plasmid containing an *ara*D gene, or a catalytically active fragment thereof as a selection marker and the gene of interest, the method comprising inserting the plasmid into the *ara*D deficient host cell and growing the cells in a growth medium containing arabinose.

44. A method according to claim 43 wherein the *ara*D gene is L-ribulose-5-phosphate 4-epimerase gene (EC 5.1.3.4.).

45. A method according to claim 43 or 44, wherein the *ara*D gene is mutated.

46. A method according to claim 45, wherein the mutation introduces a stop codon into position 8 of the *ara*D gene.

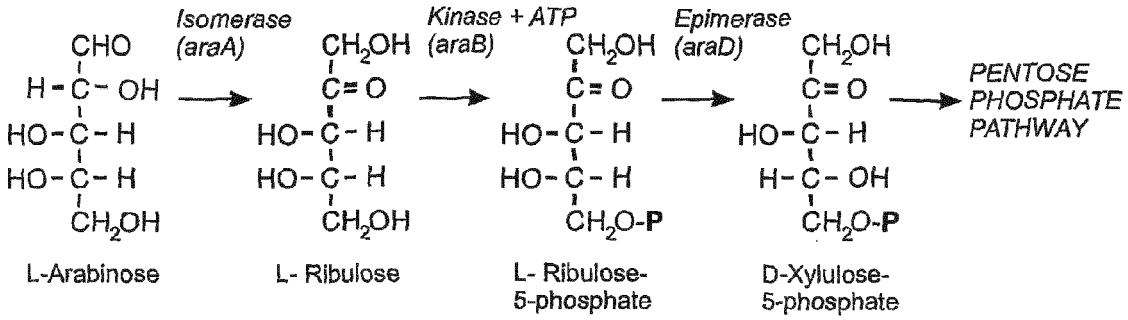

Isomerase
(araA)
Kinase + ATP
(araB)
Epimerase
(araD)
PENTOSE
PHOSPHATE
PATHWAY
L-Arabinose
L- Ribulose
L- Ribulose-
5-phosphate
D-Xylulose-
5-phosphate

Figure 1

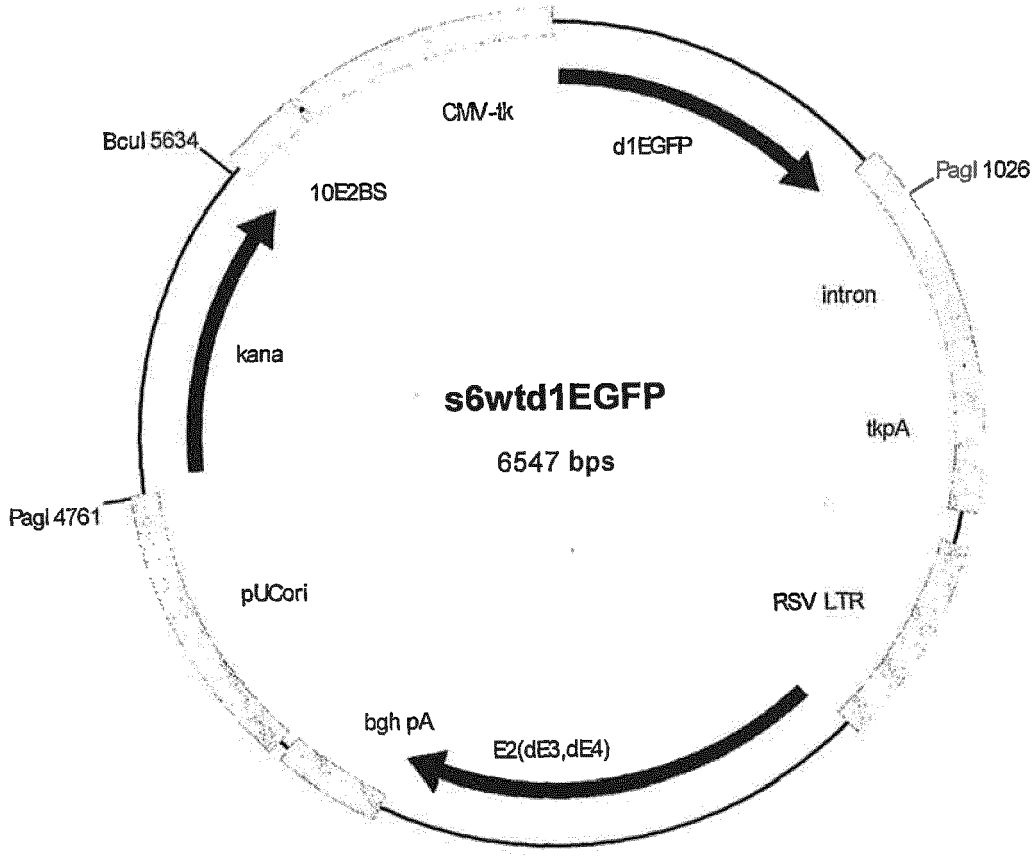

CMV-tk
d1EGFP
PagI 1026
intron
tkpA
RSV LTR
E2(dE3,dE4)
bgh pA
pUCori
PagI 4761
kana
10E2BS
BcuI 5634
s6wtd1EGFP
6547 bps

Figure 2

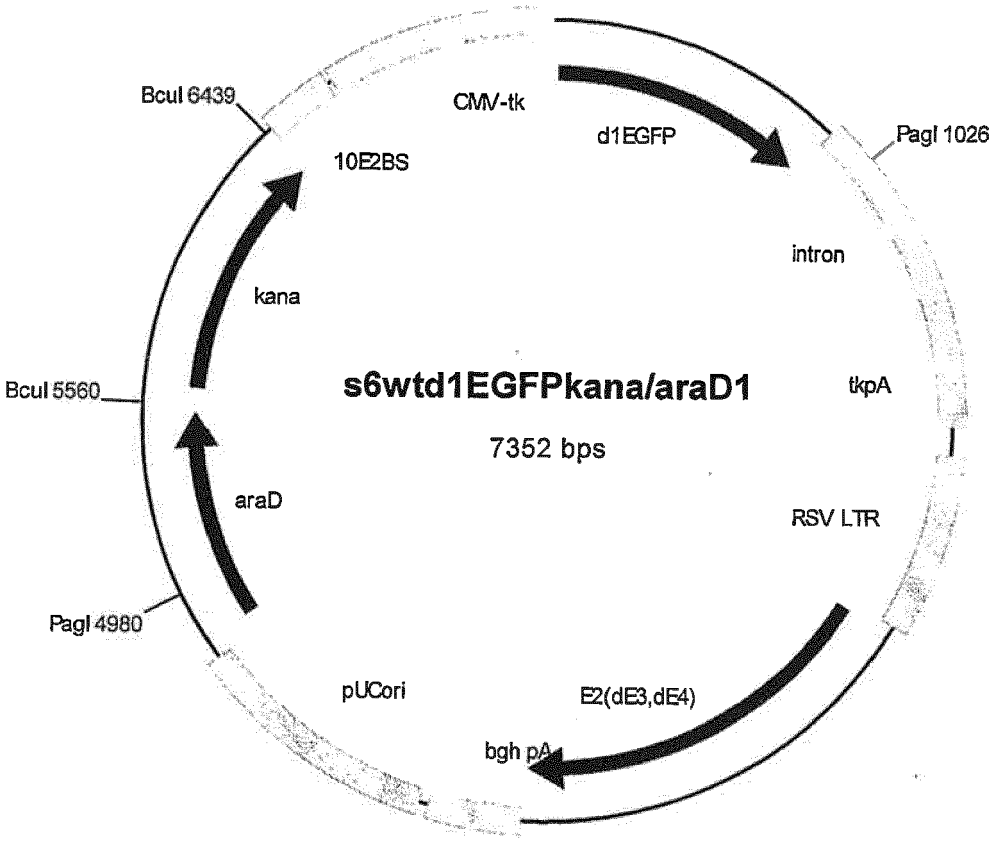

BcuI 6439
CMV-tk
d1EGFP
PagI 1026
10E2BS
intron
kana
s6wtd1EGFPkana/araD1
7352 bps
BcuI 5560
tkpA
araD
RSV LTR
PagI 4980
pUCori
E2(dE3,dE4)
bgh pA

Figure 3

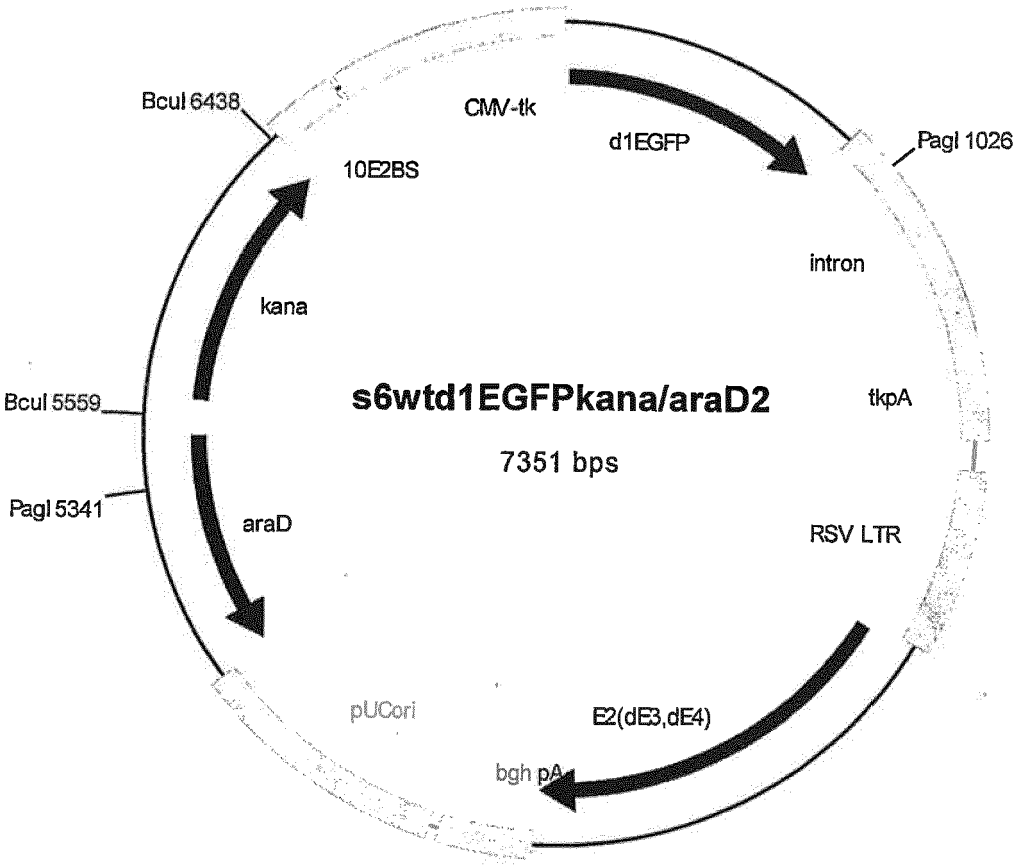

BculI 6438
CMV-tk
d1EGFP
PagI 1026
10E2BS
intron
kana
s6wtd1EGFPkana/araD2
7351 bps
BculI 5559
tkpA
PagI 5341
araD
RSV LTR
pUCori
E2(dE3,dE4)
bgh pA

Figure 4

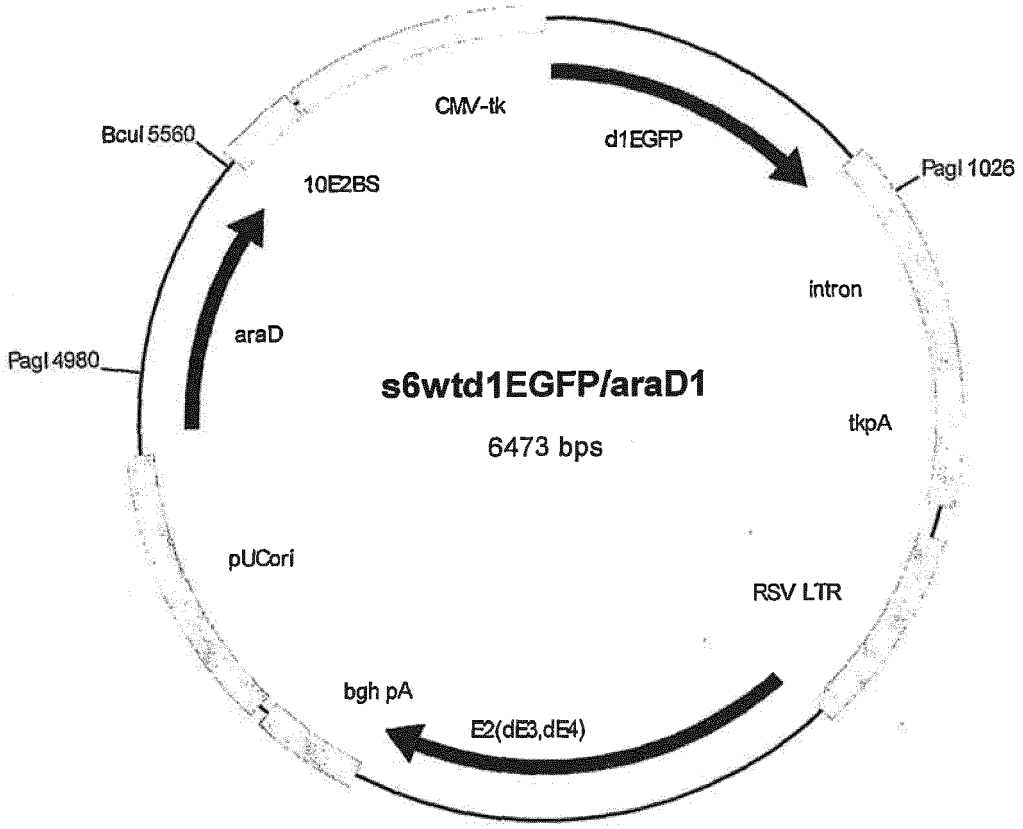

CMV-tk
d1EGFP
BcuI 5560
10E2BS
PagI 1026
intron
araD
PagI 4980
s6wtd1EGFP/araD1
6473 bps
tkpA
pUCori
RSV LTR
bgh pA
E2(dE3,dE4)

Figure 5

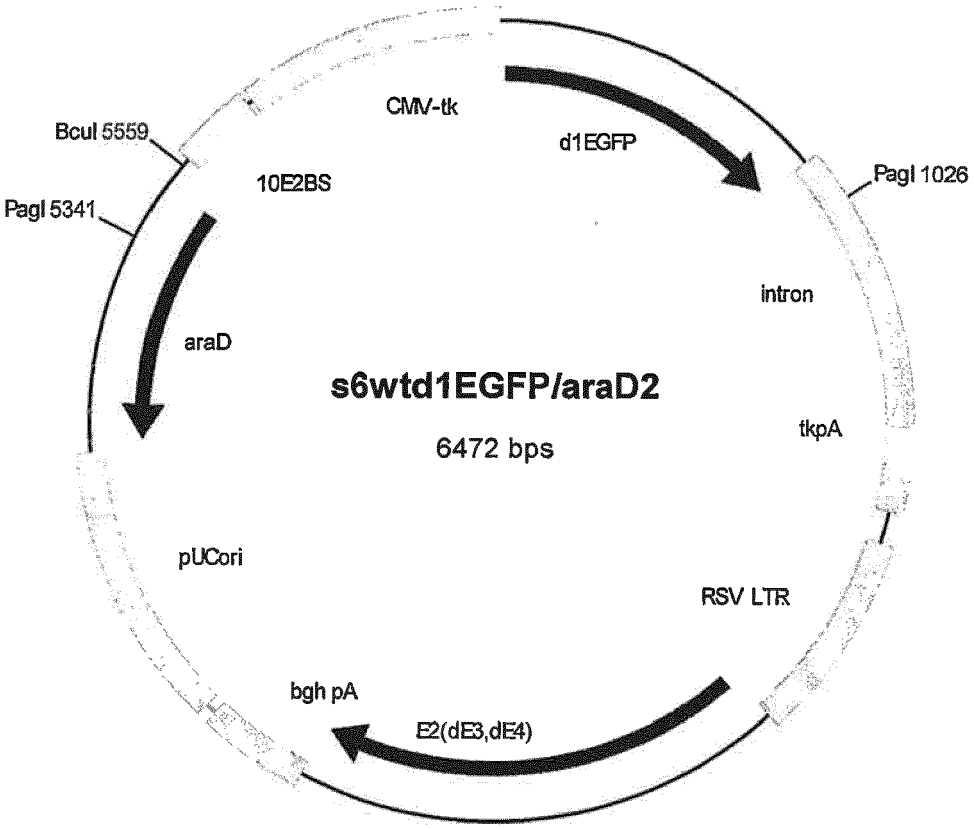

CMV-tk
d1EGFP
BcuI 5559
10E2BS
PagI 5341
PagI 1026
intron
araD
s6wtd1EGFP/araD2
6472 bps
tkpA
pUCori
RSV LTR
bgh pA
E2(dE3,dE4)

Figure 6

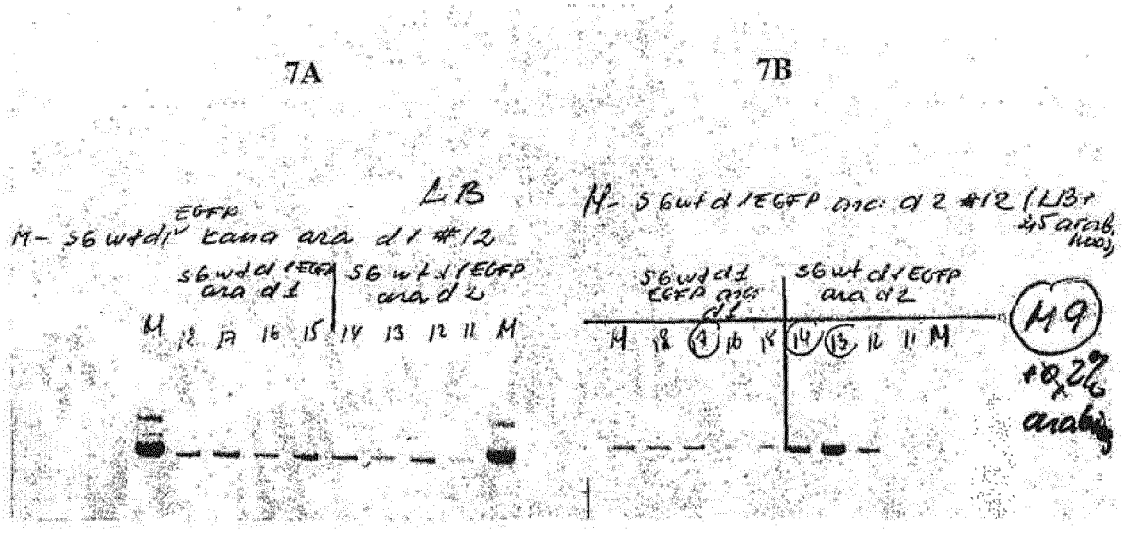


Figure 7

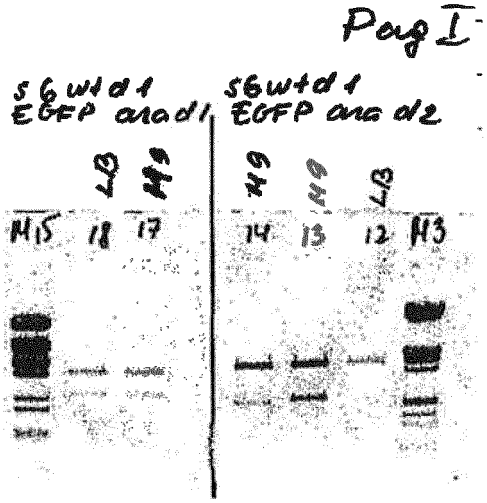

Pag I
S6wtd1 EGFP and d1
S6wtd1 EGFP and d2
LB
H9
H9
H9
LB
H15
18
17
14
13
12
H3

Figure 8

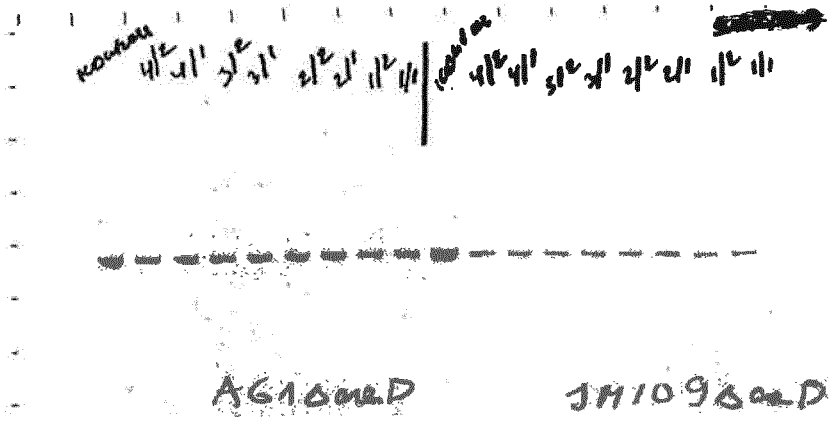


Figure 9

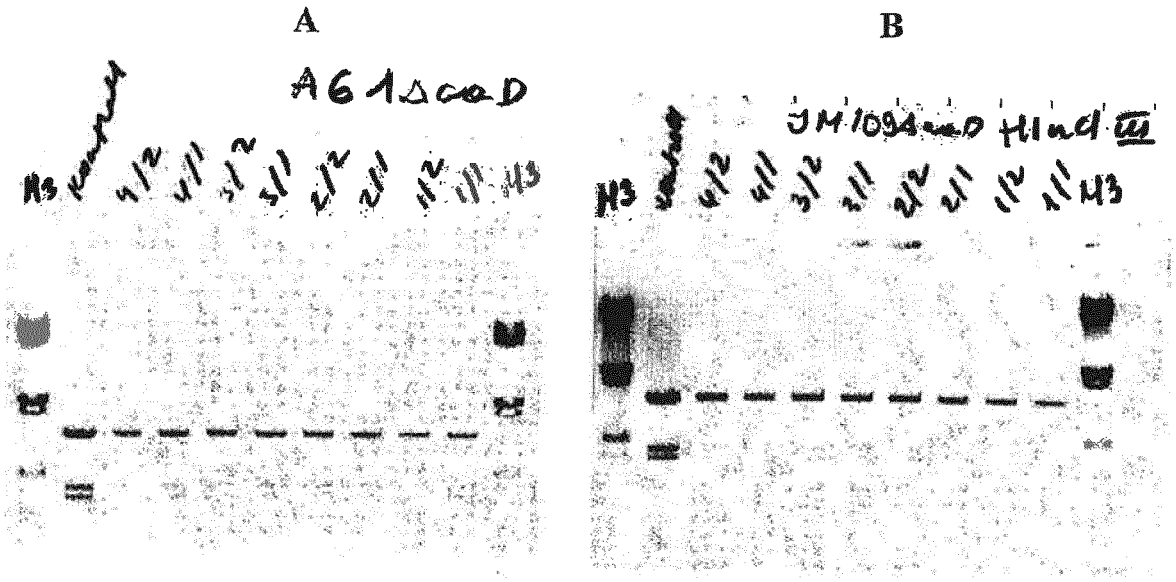


Figure 10

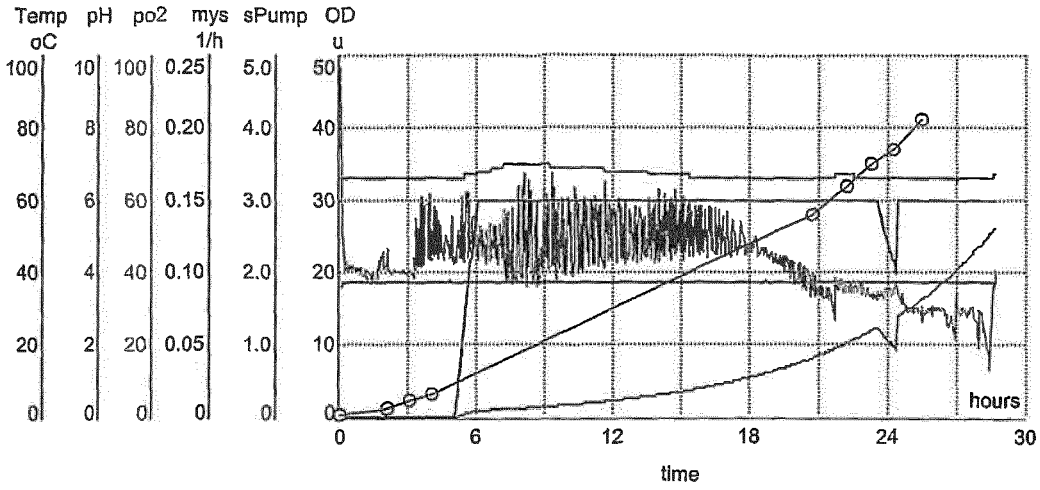

Temp
oC
pH
po2
mys
1/h
sPump
OD
u
100
80
60
40
20
0
10
8
6
4
2
0
100
80
60
40
20
0
0.25
0.20
0.15
0.10
0.05
0
5.0
4.0
3.0
2.0
1.0
0
50
40
30
20
10
0
hours
0
6
12
18
24
30
time

Figure 11

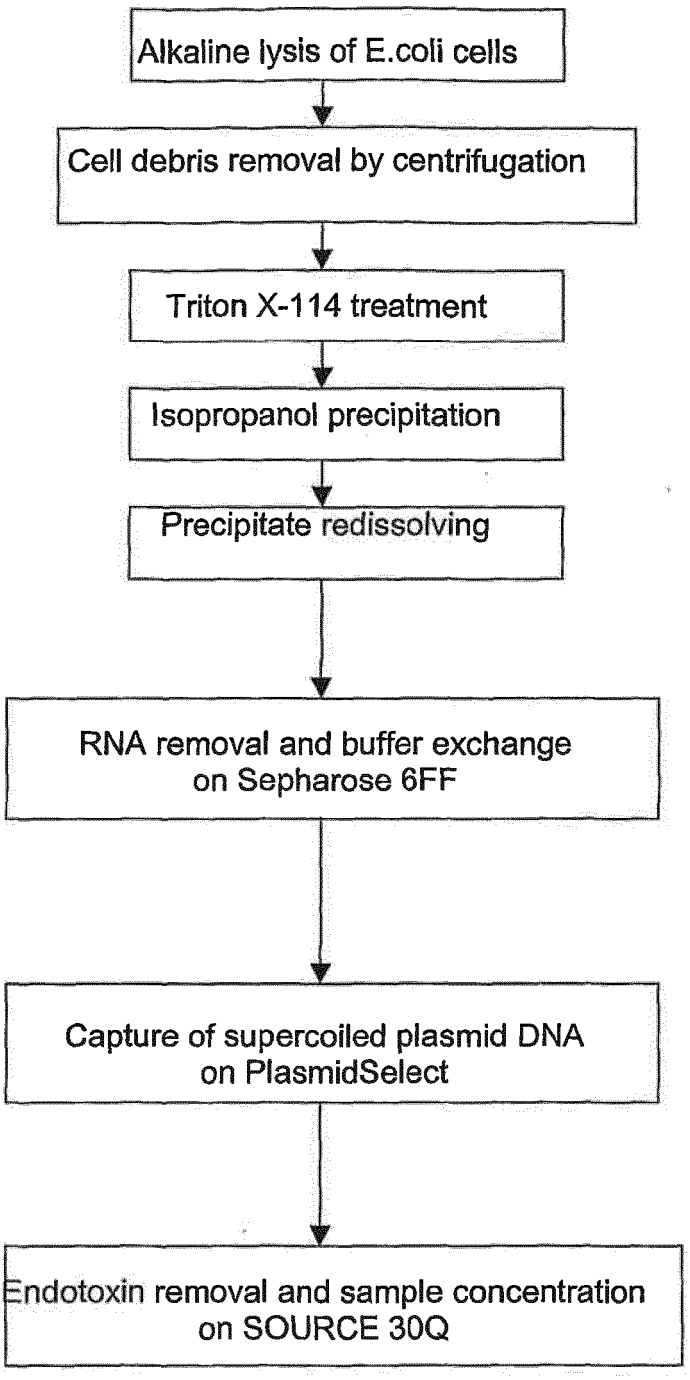

Alkaline lysis of E.coli cells
Cell debris removal by centrifugation
Triton X-114 treatment
Isopropanol precipitation
Precipitate redissolving
RNA removal and buffer exchange on Sepharose 6FF
Capture of supercoiled plasmid DNA on PlasmidSelect
Endotoxin removal and sample concentration on SOURCE 30Q

Figure 12


```
  1  GGATCCGACC GGCAACGGTA CAGATCCGAC CGGCAACGGT ACAGATCCGA

           <<.....................10E2BS.....................<

 51  CCGGCAACGG TCAGATCCGA CCGGCAACGG TACAGATCCG ACCGGCAACG

       <.....................10E2BS.....................<

101  GTACAGATCC GACCGGCAAC GGTACAGATC CGACCGGCAA CGGTACAGAT

       <.....................10E2BS.....................<
              >>deletion start in clone 14

151  CCGACCGGCA ACGGTACAGA TCCGACCGGC AACGGTACAG ATCCGACCGG

       <.....................10E2BS.....................<


201  CAACGGTACA GATCCCCCTA GCGAATTGAC TAGTTCTCAT GTTTGACAGC

       <..10E2BS.<<                          >>...promoter....>
deletion end in #14>>

                                                    T→mutation in #2A
                                                    ↑
251  TTATCATCGA TAAGCTTTAA TGCGGTAGTT TAGCACGAAG GAGTCAACAT

       >...........promoter...........>>    >>RBS.>>

                            T→STOP in clone 13          araD >>
                            ↑
301  GTTAGAAGAT CTCAAACGCC AGGTATTAGA AGCCAACCTG GCGCTGCCAA
       M  L  E  D   L  K  R   Q  V  L   E  A  N  L   A  L  P
       >.....................araD.....................>

351  AACACAACCT GGTCACGCTC ACATGGGGCA ACGTCAGCGC CGTTGATCGC
        K  H  N   L  V  T  L   T  W  G   N  V  S   A  V  D  R
       >.....................araD.....................>

401  GAGCGCGGCG TCTTTGTGAT CAAACCTTCC GGCGTCGATT ACAGCGTCAT
         E  R  G   V  F  V   I  K  P  S   G  V  D   Y  S  V
       >.....................araD.....................>

451  GACCGCTGAC GATATGGTCG TGGTTAGCAT CGAAACCGGT GAAGTGGTTG
       M  T  A  D   D  M  V   V  V  S   I  E  T  G   E  V  V
       >.....................araD.....................>
```

Figure 13

```
501  AAGGTACGAA AAAGCCCTCC TCCGACACGC CAACTCACCG GCTGCTCTAT
       E  G  T   K  K  P  S   S  D  T   P  T  H   R  L  L  Y
     >.........................araD.........................>

551  CAGGCATTCC CCTCCATTGG CGGCATTGTG CATACGCACT CGCGCCACGC
       Q  A  F   P  S  I   G  G  I  V   H  T  H   S  R  H
     >.........................araD.........................>

601  CACCATCTGG GCGCAGGCGG GTCAGTCGAT TCCAGCAACC GGCACCACCC
     A  T  I  W   A  Q  A   G  Q  S   I  P  A  T   G  T  T
     >.........................araD.........................>

651  ACGCCGACTA TTTCTACGGC ACCATTCCCT GCACCCGCAA AATGACCGAC
       H  A  D   Y  F  Y  G   T  I  P   C  T  R   K  M  T  D
     >.........................araD.........................>

701  GCAGAAATCA ACGGCGAATA TGAGTGGGAA ACCGGTAACG TCATCGTAGA
       A  E  I   N  G  E   Y  E  W  E   T  G  N   V  I  V
     >.........................araD.........................>

751  AACCTTTGAA AAACAGGGTA TCGATGCAGC GCAAATGCCC GGCGTTCTGG
     E  T  F  E   K  Q  G   I  D  A   A  Q  M  P   G  V  L
     >.........................araD.........................>

801  TCCATTCCCA CGGCCCGTTT GCATGGGGCA AAAATGCCGA AGATGCGGTG
       V  H  S   H  G  P  F   A  W  G   K  N  A   E  D  A  V
     >.........................araD.........................>

851  CATAACGCCA TCGTGCTGGA AGAGGTCGCT TATATGGGGA TATTCTGCCG
       H  N  A   I  V  L   E  E  V  A   Y  M  G   I  F  C
     >.........................araD.........................>

901  TCAGTTAGCG CCGCAGTTAC CGGATATGCA GCAAACGCTG CTGGATAAAC
     R  Q  L  A   P  Q  L   P  D  M   Q  Q  T  L   L  D  K
     >.........................araD.........................>

951  ACTATCTGCG TAAGCATGGC GCGAAGGCAT ATTACGGGCA GTAATGACAG
       H  Y  L   R  K  H  G   A  K  A   Y  Y  G   Q  -
     >.......................araD.....................>>
                                                terminator >>

1001 CCCGCCTAAT GAGCGGGCTT TTTTTTCCAT

     >........terminator.......>>
```

Figure 13 (cont.)

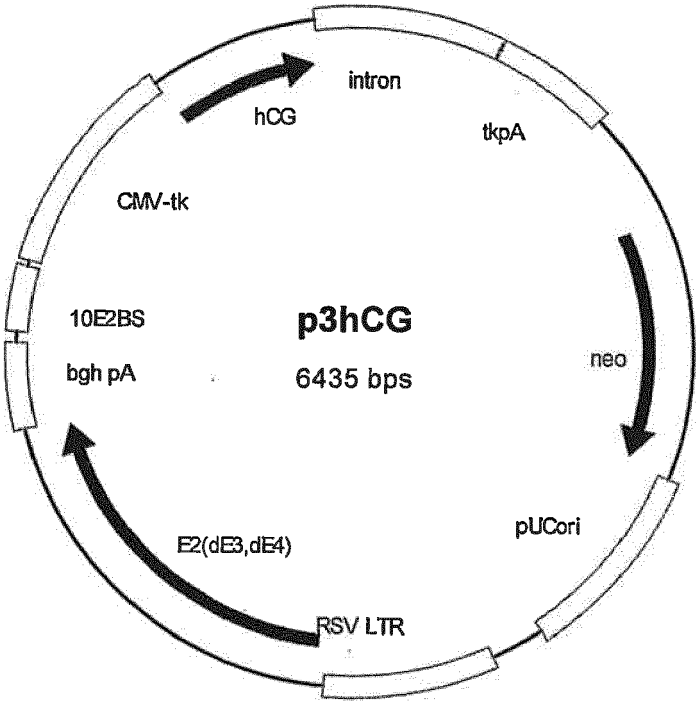

intron
hCG
tkpA
CMV-tk
10E2BS
p3hCG
6435 bps
neo
bgh pA
pUCori
E2(dE3,dE4)
RSV LTR

Figure 14

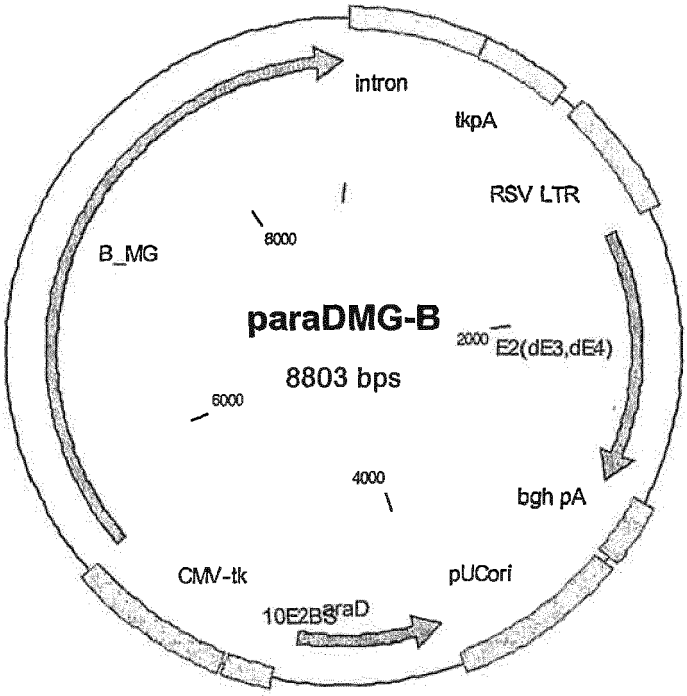

intron
tkpA
RSV LTR
8000
B_MG
paraDMG-B
2000
E2(dE3,dE4)
8803 bps
6000
4000
bgh pA
CMV-tk
pUCori
10E2B3
araD

Figure 15

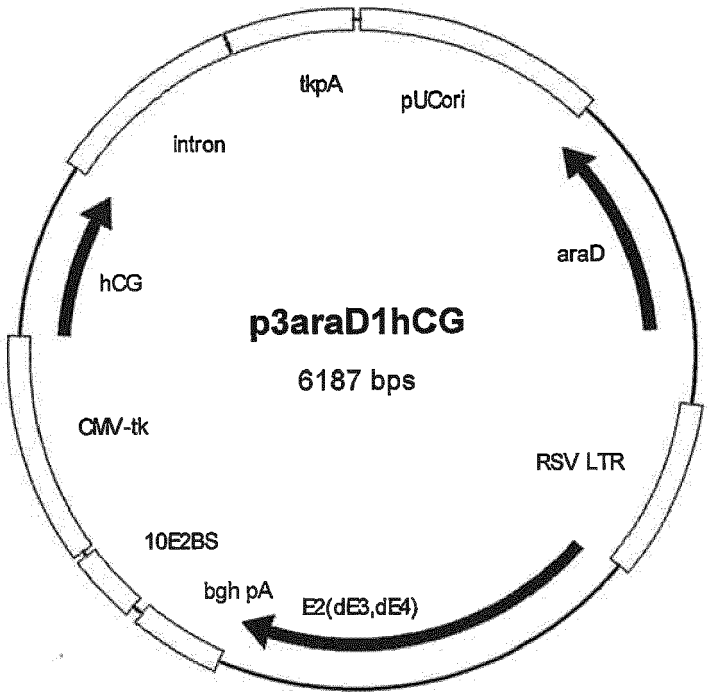

tkpA
pUCori
intron
araD
hCG
p3araD1hCG
6187 bps
CMV-tk
RSV LTR
10E2BS
bgh pA
E2(dE3,dE4)

Figure 16

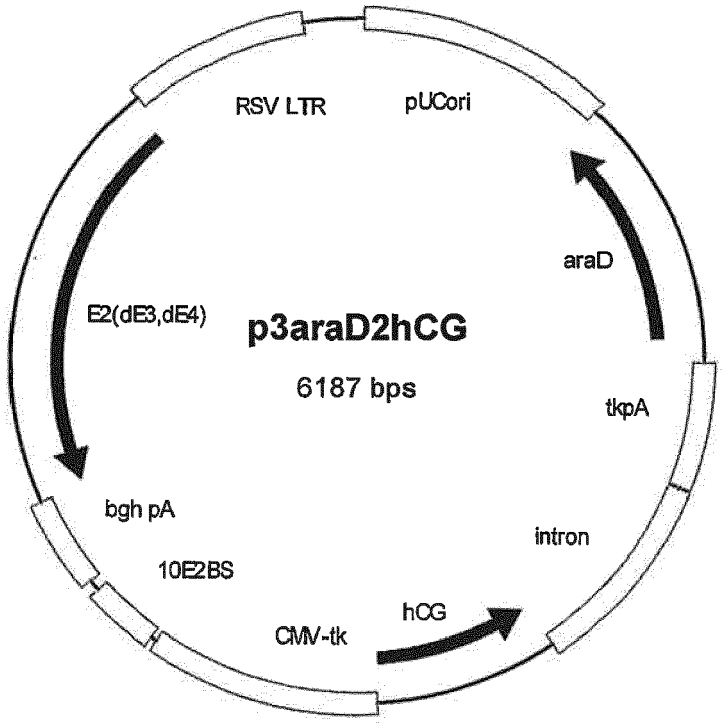

RSV LTR
pUCori
araD
E2(dE3,dE4)
p3araD2hCG
6187 bps
tkpA
bgh pA
intron
10E2BS
hCG
CMV-tk

Figure 17

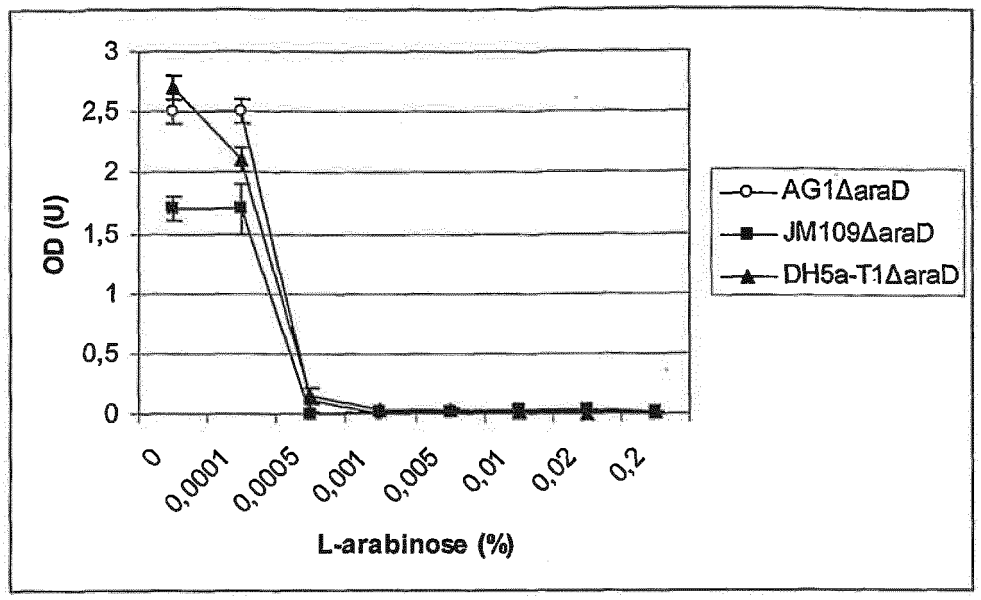

3
2,5
2
1,5
1
0,5
0
OD (U)
0
0,0001
0,0005
0,001
0,005
0,01
0,02
0,2
L-arabinose (%)
AG1ΔaraD
JM109ΔaraD
DH5a-T1ΔaraD

Figure 18

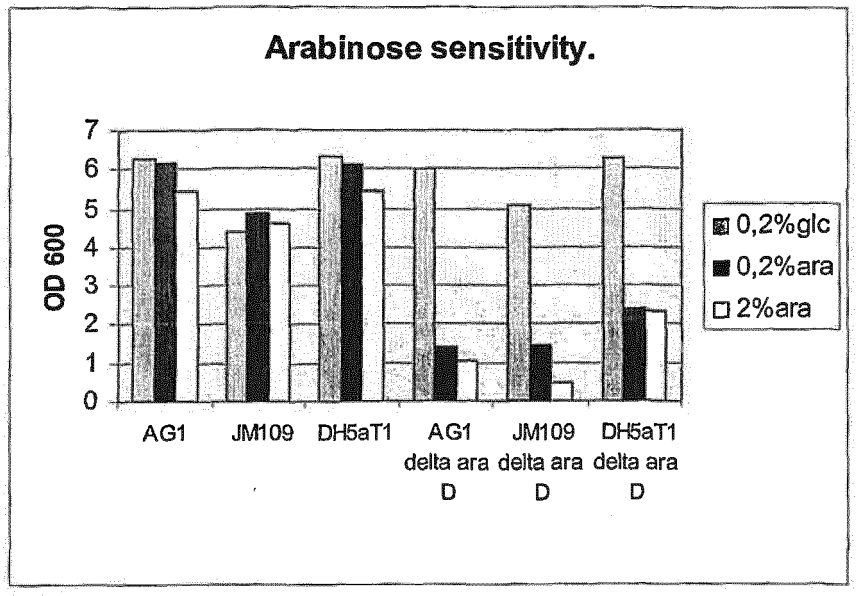

Arabinose sensitivity.
OD 600
7
6
5
4
3
2
1
0
AG1
JM109
DH5aT1
AG1 delta ara D
JM109 delta ara D
DH5aT1 delta ara D
0,2%glc
0,2%ara
2%ara

Figure 19

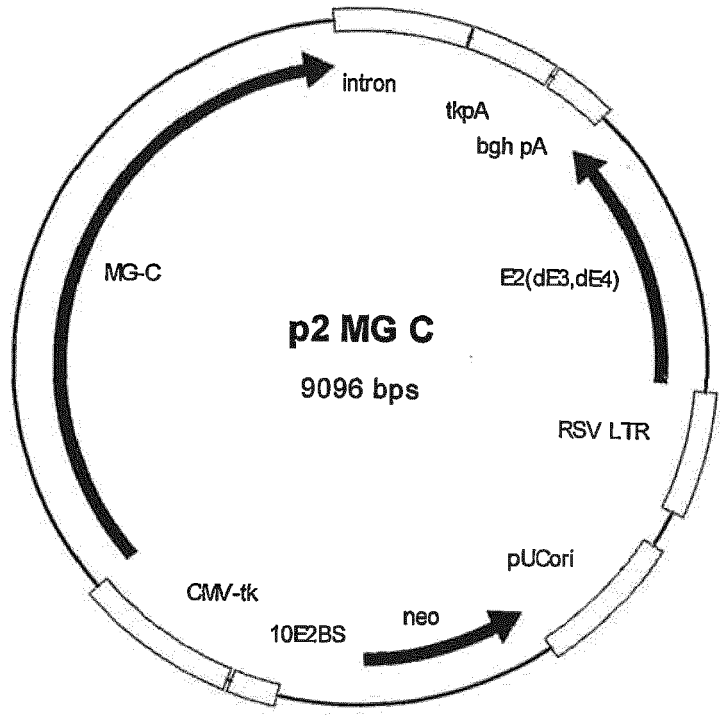

intron
tkpA
bgh pA
MG-C
E2(dE3,dE4)
p2 MG C
9096 bps
RSV LTR
pUCori
CMV-tk
10E2BS
neo

Figure 20

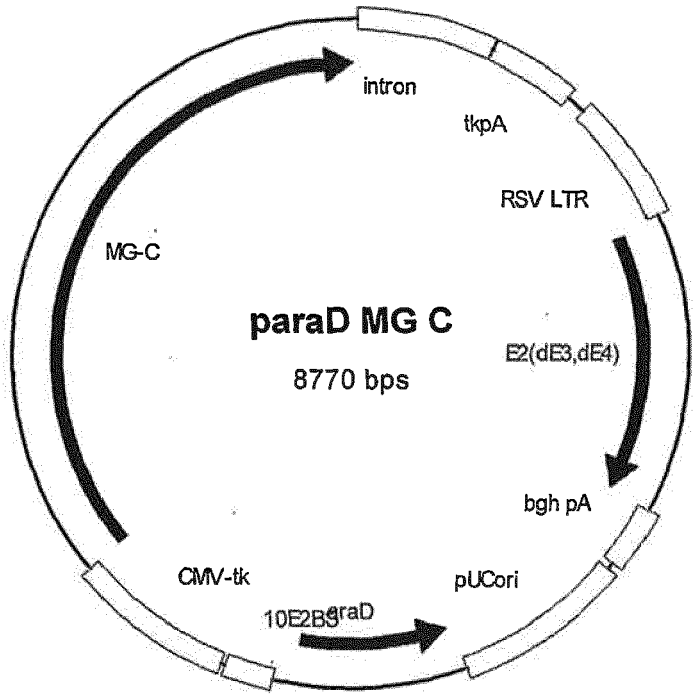
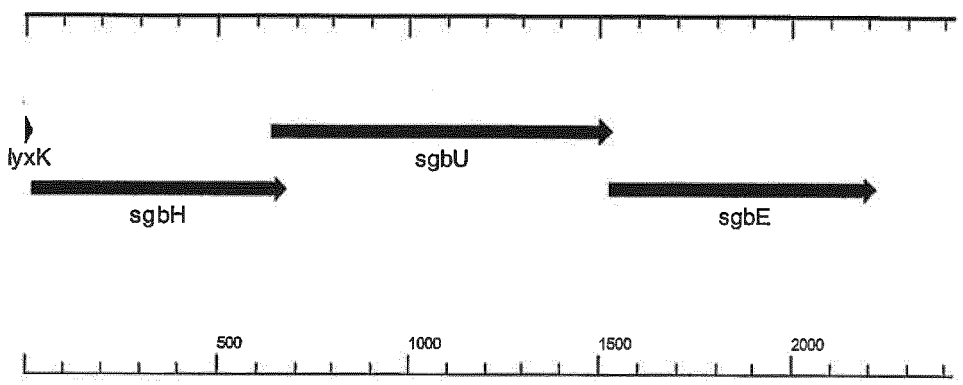

intron
tkpA
RSV LTR
MG-C
paraD MG C
8770 bps
E2(dE3,dE4)
bgh pA
CMV-tk
pUCori
10E2B5
paraD

Figure 21



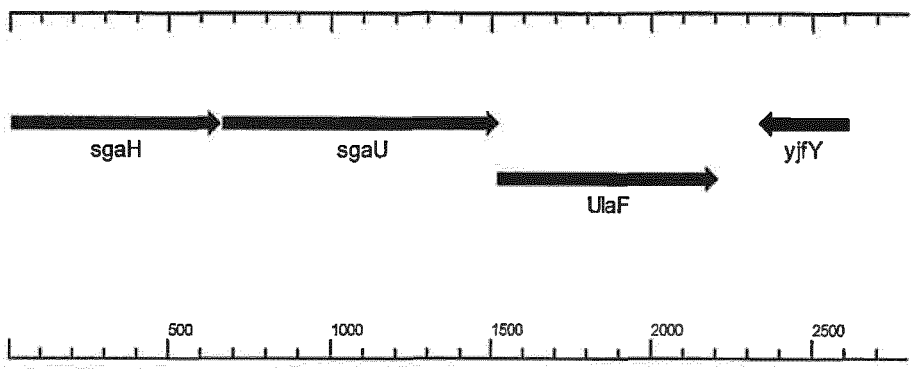
(2420 bps)

```
1400  ATAAACTGAA CTATCGCGGT TCTTTCCTGA TTGAGATGTG GACCGAAAAA
            >.......................sgbU.......................>

1450  GCCAAAGAGC CGGTGCTGGA GATTATTCAG GCGCGGCGTT GGATTGAAGC

      >.......................sgbU.......................>

1500  GCGTATGCAG GAGGCTGGAT TTATATGTTA GAGCAACTGA AAGCCGACGT
                                M  L   E  Q  L   K  A  D
      >.............sgbU.............>>
                                >>.........sgbE..........>

1550  GCTGGCGGCG AATCTGGCGC TTCCCGCTCA CCATCTGGTG ACGTTCACCT
      V  L  A  A   N  L  A   L  P  A   H  H  L  V   T  F  T
      >.......................sgbE.......................>
```

Figure 22

```
1600  GGGGCAATGT CAGCGCGGTA GACGAAACGC GGCAATGGAT GGTAATCAAA
        W  G  N   V  S  A  V   D  E  T   R  Q  W   M  V  I  K
      >.......................sgbE.......................>

1650  CCTTCCGGCG TCGAGTACGA CGTGATGACC GCCGACGATA TGGTGGTGGT
        P  S  G   V  E  Y   D  V  M  T   A  D  D   M  V  V
      >.......................sgbE.......................>

1700  TGAGATAGCC AGCGGTAAGG TGGTGGAAGG CAGCAAAAAA CCCTCTTCCG
      V  E  I  A   S  G  K   V  V  E   G  S  K  K   P  S  S
      >.......................sgbE.......................>

1750  ATACACCAAC GCATCTGGCG CTCTACCGTC GCTATGCCGA AATTGGCGGT
        D  T  P   T  H  L  A   L  Y  R   R  Y  A   E  I  G  G
      >.......................sgbE.......................>

1800  ATTGTGCATA CCCACTCGCG CCACGCCACC ATCTGGTCAC AGGCCGGGCT
        I  V  H   T  H  S   R  H  A  T   I  W  S   Q  A  G
      >.......................sgbE.......................>

1850  GGATCTCCCC GCCTGGGGCA CCACCCACGC CGATTATTTT TACGGTGCCA
      L  D  L  P   A  W  G   T  T  H   A  D  Y  F   Y  G  A
      >.......................sgbE.......................>

1900  TCCCCTGCAC GCGACAGATG ACCGCAGAGG AGATTAACGG CGAATATGAA
        I  P  C   T  R  Q  M   T  A  E   E  I  N   G  E  Y  E
      >.......................sgbE.......................>

1950  TATCAGACCG GCGAAGTGAT CATTGAAACC TTCGAAGAAC GTGGCAGGAG
        Y  Q  T   G  E  V   I  I  E  T   F  E  E   R  G  R
      >.......................sgbE.......................>

2000  TCCGGCACAA ATCCCGGCGG TGCTGGTGCA TTCTCACGGC CCGTTCGCAT
      S  P  A  Q   I  P  A   V  L  V   H  S  H  G   P  F  A
      >.......................sgbE.......................>

2050  GGGGTAAAAA CGCCGCCGAT GCCGTGCATA ACGCCGTAGT ACTCGAAGAA
        W  G  K   N  A  A  D   A  V  H   N  A  V   V  L  E  E
      >.......................sgbE.......................>

2100  TGCGCCTATA TGGGTCTATT CTCGCGCCAG CTTGCGCCGC AGCTCCCTGC
        C  A  Y   M  G  L   F  S  R  Q   L  A  P   Q  L  P
      >.......................sgbE.......................>

2150  GATGCAAAAC GAACTGCTGG ATAAGCACTA CCTGCGTAAG CATGGGGCCA
      A  M  Q  N   E  L  L   D  K  H   Y  L  R  K   H  G  A
      >.......................sgbE.......................>

2200  ATGCCTATTA CGGGCAGTAA TCCCTCACGC CGGGGCTTCA TCGCCCCGGC
        N  A  Y   Y  G  Q  -
      >.......sgbE.......>>

2250  ACTACGAATT GATATGTTCC TTGCTGTAAC GCCGCTTCCA CGCTGCTGGC

2300  G
```

Figure 22 (cont.)



(2792 bps)

```
1400  CGGGCCGTAC CTGATTGAGA TGTGGAGCGA AACGGCGGAA GACCCGGCGG

      >.......................sgaU.......................>

1450  CAGAAGTGGC GAAAGCGCGT GATTGGGTGA AAGCGCGCAT GGCGAAAGCG

      >.......................sgaU.......................>

1500  GGCATGGTGG AGGCGGCATA ATGCAAAAGC TAAAACAGCA GGTATTTGAA
                              M  Q  K   L  K  Q   Q  V  F  E
      >........sgaU.........>>
                           >>............UlaF.............>

1550  GCCAACATGG AGCTGCCGCG CTACGGGCTG GTGACCTTTA CCTGGGGCAA
        A  N  M   E  L  P   R  Y  G  L   V  T  F   T  W  G
      >.......................UlaF.......................>
```

Figure 23

```
1600  CGTCAGCGCT ATCGACCGCG AACGCGGGCT GGTGGTGATC AAGCCCAGCG
        N  V  S  A   I  D  R   E  R  G   L  V  V  I   K  P  S
      >.....................UlaF.....................>

1650  GCGTTGCCTA CGAAACCATG AAAGCGGCCG ATATGGTGGT GGTTGATATG
        G  V  A   Y  E  T  M   K  A  A   D  M  V   V  V  D  M
      >.....................UlaF.....................>

1700  AGCGGCAAGG TGGTGGAAGG GGAGTATCGC CCATCTTCCG ACACTGCGAC
        S  G  K   V  V  E   G  E  Y  R   P  S  S   D  T  A
      >.....................UlaF.....................>

1750  GCATCTCGAA CTCTACCGTC GTTACCCGTC GCTTGGTGGC ATTGTCCATA
        T  H  L  E   L  Y  R   R  Y  P   S  L  G  G   I  V  H
      >.....................UlaF.....................>

1800  CCCACTCCAC TCATGCCACC GCATGGGCGC AGGCGGGGCT GGCGATCCCG
        T  H  S   T  H  A  T   A  W  A   Q  A  G   L  A  I  P
      >.....................UlaF.....................>

1850  GCGTTAGGCA CCACGCACGC CGACTACTTC TTTGGCGACA TTCCGTGTAC
        A  L  G   T  T  H   A  D  Y  F   F  G  D   I  P  C
      >.....................UlaF.....................>

1900  GCGCGGGTTA AGCGAAGAAG AGGTGCAGGG CGAGTATGAA CTGAACACCG
        T  R  G  L   S  E  E   E  V  Q   G  E  Y  E   L  N  T
      >.....................UlaF.....................>

1950  GCAAAGTGAT TATCGAAACG CTGGGCAACG CCGAGCCGCT GCATACGCCG
        G  K  V   I  I  E  T   L  G  N   A  E  P   L  H  T  P
      >.....................UlaF.....................>

2000  GGAATTGTGG TGTATCAGCA CGGGCCGTTC GCCTGGGGGA AAGATGCTCA
        G  I  V   V  Y  Q   H  G  P  F   A  W  G   K  D  A
      >.....................UlaF.....................>

2050  CGATGCGGTG CATAACGCGG TGGTGATGGA AGAAGTGGCG AAAATGGCGT
      H  D  A  V   H  N  A   V  V  M   E  E  V  A   K  M  A
      >.....................UlaF.....................>

2100  GGATTGCCCG CGGCATTAAC CCACAACTCA ATCACATCGA CAGCTTCCTG
        W  I  A   R  G  I  N   P  Q  L   N  H  I   D  S  F  L
      >.....................UlaF.....................>

2150  ATGAATAAAC ACTTCATGCG TAAACACGGT CCTAACGCTT ATTACGGGCA
        M  N  K   H  F  M   R  K  H  G   P  N  A   Y  Y  G
      >.....................UlaF.....................>

2200  GAAGTAGAAC ACGCGCTGCG GAAATTTCCT TCCTCGGGAG ATAACTGGTC
      Q  K  -
      >....>> UlaF

2250  TAATTCCGCA GCCGTTTTTC AAAAAAAAGC CCCCTGCGAA GGGGGCAAAG

2300  C
```

Figure 23 (cont.)

Europäisches Patentamt
European Patent Office
Office européen des brevets

EUROPEAN SEARCH REPORT

Application Number
EP 14 19 1756

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	ARIZA R R ET AL: "A method for selection of forward mutations in supF gene carried by shuttle-vector plasmids", CARCINOGENESIS, XX, XX, vol. 14, no. 2, 1 January 1993 (1993-01-01), pages 303-305, XP002984154, * page 303 - page 305 * -----	28-30, 37-39	INV. C12N15/70 C12N15/61

TECHNICAL FIELDS SEARCHED (IPC)
C12N

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	3 September 2015	Scheffzyk, Irmgard

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- US 20000014476 A1 **[0005]**
- WO 02090558 A **[0029]**

Non-patent literature cited in the description

- **LEVY, S. B.** *J. Antimicrob. Chemother.,* 2002, vol. 49, 25-30 **[0003]**
- **ENGELBERG-KULKA, H. ; GLASER, G.** *Annu Rev Microbiol,* 1999, vol. 53, 43-70 **[0004]**
- **SILVER, S. ; PHUNG, L. T.** *Annu Rev Microbiol,* 1996, vol. 50, 753-789 **[0004]**
- **WANG, M.D. et al.** *J. Bacteriol.,* 1987, vol. 169, 5610-5614 **[0004]**
- **ARIZA, R. R. et al.** *Carcinogenesis,* 1993, vol. 14, 303-305 **[0021]**
- **ENGELSBERG, E. et al.** *J. Bacteriol.,* 1962, vol. 84, 137-146 **[0022]**
- **MURGOLA, E. J.** *Annu. Rev. Genet.,* 1985, vol. 19, 57-80 **[0049]**
- **DATSENKO ; WANNER.** *PNAS,* 2000, vol. 97, 6640-6645 **[0054]**
- **DATSENKO ; WANNER.** *PNAS,* June 2000, vol. 97 (12 **[0057]**
- **WEN SHAN YEW ; JHON A. GERLT.** *J. Bacteriol.,* 2002, vol. 184, 302-306 **[0078]**
- **LIU et al.** Triton X-114 has been shown to effectively reduce the level of endotoxins in protein. *Clinical Biochemistry,* 1997 **[0101]**