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WO-A1-2010/081110
US-A1- 2012 190 116
US-A1- 2013 045 539
US-A1- 2013 052 646
IBBA M ET AL: "RELAXING THE SUBSTRATE SPECIFICITY OF AN AMINOACYL-TRNA SYNTHETASE ALLOWS IN VITRO AND IN VIVO SYNTHESIS OF PROTEINS CONTAINING UNNATURAL AMINO ACIDS", FEBS LETTERS, ELSEVIER, AMSTERDAM, NL, vol. 364, 15 May 1994 (1994-05-15), pages 272-275, XP001021263, ISSN: 0014-5793, DOI: 10.1016/0014-5793(95)00408-2
DATTA D ET AL: "A designed phenylalanyl-tRNA synthetase variant allows efficient in vivo incorporation of aryl ketone functionality into proteins", JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, AMERICAN CHEMICAL SOCIETY, US, vol. 124, no. 20, 22 May 2002 (2002-05-22) , pages 5652-5653, XP002337184, ISSN: 0002-7863, DOI: 10.1021/JA0177096

Fortsættes ...

BARRETT ET AL.: 'Genetic tools for allelic replacement in Burkholderia species' APPLIED AND ENVIRONMENTAL MICROBIOLOGY vol. 74, no. 14, July 2008, pages 4498 - 4508, XP055324621
KRISTICH ET AL.: 'Development of a host-genotype-independent counterselectable marker and a high-frequency conjugative delivery system and their use in genetic analysis of Enterococcus faecalis' PLASMID vol. 57, 2007, pages 131 - 144, XP005907005

DESCRIPTION

CROSS REFERENCE TO A RELATED APPLICATION

FIELD

[0001] The present invention relates to selection markers of use in the preparation of recombinant *Clostridium* spp.

BACKGROUND

[0002] Processes for producing recombinant organisms are known. They typically involve transformation of an organism with an exogenous nucleic acid vector, which may integrate with the host genome or remain in a stable independent (for example, extra-chromosomal) state.

[0003] Integration of an exogenous nucleic acid into the host genome involves a double-crossover event between the vector and an endogenous nucleic acid. Double-crossover recombination happens at frequencies which are too low to reliably identify integrants by chance alone. Therefore, a means to select for one or both crossovers has a huge benefit on the frequency of identification in both time and labour.

[0004] Selection markers of use in screening for recombination events are known. Such markers are typically protein coding sequences that confer a selective advantage (positive-selection) or disadvantage (counter-selection) to a host organism. A number of positive-selection and counter-selection markers are known and can be of use in screening for organisms in which a desired recombination event has occurred. A positive-selection marker typically comprises a gene that when expressed allows an organism to survive in a particular growth environment. A counter-selection marker typically comprises a gene that when expressed produces a toxin which is lethal to an organism.

[0005] In bacteria other than Clostridia, there is a plethora of counter-selection markers available but unfortunately either due to physiological or genetic reasons, the vast majority do not work in Clostridia.

[0006] It is an object of the invention to overcome one or more of the disadvantages of the prior art, or to at least to provide the public with a useful choice.

SUMMARY OF INVENTION

[0007] In a first aspect, the invention provides the use of ThiK and/or PheS as a counter-selection marker in a method for producing a recombinant microorganism from a parental microorganism, wherein the parental microorganism is a *Clostridium* spp., and wherein the PheS includes at least one alteration compared to a wild-type PheS such that in use phenylalanine tRNA synthetase is able to aminoacylate tRNA using a phenylalanine analogue.

[0008] In a second aspect, the invention provides the use of a nucleic acid encoding ThiK and/or PheS in a plasmid of use in producing a recombinant microorganism from a parental microorganism, wherein the parental microorganism is a *Clostridium* spp., and wherein the PheS includes at least one alteration compared to a wild-type PheS such that in use phenylalanine tRNA synthetase is able to aminoacylate tRNA using a phenylalanine analogue.

[0009] In a third aspect, the invention provides the use of a plasmid comprising a nucleic acid encoding ThiK and/or PheS for producing a recombinant microorganism from a parental microorganism, wherein the parental microorganism is a *Clostridium* spp., and wherein the PheS includes at least one alteration compared to a wild-type PheS such that in use phenylalanine tRNA synthetase is able to aminoacylate tRNA using a phenylalanine analogue.

[0010] In a fourth aspect, the invention provides a method for the production of a recombinant microorganism from a parental microorganism, the method comprising at least the steps of:

1. a) transformation of a parental microorganism with a plasmid comprising
    1. 1. at least one nucleic acid sequence encoding at least one counter selection marker chosen from the group consisting of PheS and ThiK, wherein the PheS includes at least one alteration compared to a wild-type PheS such that in use phenylalanine tRNA synthetase is able to aminoacylate tRNA using a phenylalanine analogue;
    2. 2. at least one nucleic acid sequence encoding at least one positive selection marker; and,
    3. 3. two nucleic acid sequences homologous to selected regions around a target location within the genome of the parental microorganism, which allow for the recombination of the plasmid with the genome of the parental microorganism;
2. b) selecting one or more microorganisms that express the at least one positive selection marker; and,
3. c) selecting one or more microorganisms which do not express the at least one counter selection marker.

[0011] In one embodiment, the selection steps b) and c) are conducted simultaneously. In another embodiment, the selection steps b) anc c) are conducted sequentially.

[0012] In one embodiment, the plasmid further comprises at least one nucleic acid sequence of interest to be inserted into the parental genome.

[0013] In a fourth aspect, the invention provides a nucleic acid encoding a PheS, wherein the PheS is altered compared to a wild-type PheS such that in use phenylalanine tRNA synthetase is able to aminoacylate tRNA using a phenylalanine analogue, and wherein the wild-type PheS is derived from a *Clostridium* spp or is a functionally equivalent variant thereof.

[0014] In a fifth aspect, the invention provides a nucleic acid vector comprising a nucleic acid according to the fourth aspect of the invention. In one embodiment, the vector is a plasmid.

[0015] In one embodiment, the vector is a plasmid and also comprises one or more of:

1. a. at least one nucleic acid sequence encoding at least one positive selection marker; and,
2. b. two nucleic acid sequences homologous to selected regions around a target location within the genome of a parental microorganism, which allow for the recombination of the plasmid with the genome of the parental microorganism.

[0016] In one embodiment, the plasmid further comprises at least one nucleic acid sequence of interest which is desired to be inserted into the genome of a parental microorganism.

[0017] In a sixth aspect, the invention provides a PheS, wherein the PheS comprises one or more alteration compared to a wild-type PheS such that in use phenylalanine tRNA synthetase is able to aminoacylate tRNA using a phenylalanine analogue, and wherein the wild-type PheS is derived from a *Clostridium* spp or is a functionally equivalent variation thereof.

[0018] In a seventh aspect, the invention provides a cell comprising a nucleic acid according to the fourth aspect of the invention, a vector according to the fifth aspect of the invention and/or a PheS according to the sixth aspect of the invention.

[0019] In one embodiment of the above aspects of the invention, the wild-type PheS and/or wild-type nucleic acid encoding PheS is derived from a *Clostridium* spp or is a functionally equivalent variant thereof.

[0020] In one embodiment of the above aspects and embodiment of the invention, the at least one alteration in PheS compared to a wild-type includes one or more amino acid substitution, deletion and/or addition.

[0021] In one particular embodiment of the above aspects and embodiments of the invention, the at least one alteration in PheS is located within the substrate specificity site. In one embodiment, the substrate specificity site is located between amino acids 306 and 313 read relative to the amino acid position of wild-type PheS of *C. autoethanogenum* (SEQ ID 21). In one embodiment, the at least one alteration is an amino acid substitution at position 311. In one embodiment, the at least one alteration is substitution of Ala for Gly at amino acid 311.

[0022] In one embodiment of the above aspects and embodiments of the invention, the PheS is derived from *Clostridium autoethanogenum* or is a functionally equivalent variant thereof.

[0023] In one embodiment of the above aspects and embodiments of the invention, the alterned PheS comprises the amino acid sequence of SEQ ID No. 21.

[0024] In one embodiment of the above aspects and embodiments of the invention, the nucleic acid encoding PheS which includes at least one alteration compared to a wild-type PheS, comprises at least one alteration compared to a nucleic acid encoding a wild-type PheS. In one embodiment, the at least one alteration in a nucleic acid encoding PheS includes one or more nucleotide substitution, deletion and/or addition. In one embodiment, the one or more alteration in the nucleic acid sequence is located within a region of the nucleic acid which encodes the substrate specificity site of PheS. In one embodiment, the region of a nucleic acid encoding the substrate specificity site is located between bases 918 and 939, read relative to the nucleotide position of the gene encoding wild-type PheS of *C. autoethanogenum* (SEQ ID 12). In one embodiment, the at least one alteration is a nucleotide substitution at base 932.. In one embodiment, the at least one alteration is substitution of C for G at base 932.

[0025] In one embodiment of the above aspects and embodiments of the invention, the nucleic acid encoding PheS is derived from *Clostridium autoethanogenum* or is a functionally equivalent variant thereof.

[0026] In one embodiment of the above aspects and embodiments of the invention, the nucleic acid encoding altered PheS comprises the sequence of SEQ ID No. 14.

[0027] In one embodiment of the above aspects and embodiments of the invention, the phenylalanine analogue is chosen from chlorophenylalanine, fluorophenylalanine and bromophenylalanine. In one particular embodiment, the phenylalanine analogue is chosen from DL-4-chlorophenylalanine and *p*-chlorophenylalanine, p-fluoro-L-phenylalanine, p-fluoro-DL-phenylalanine, p-bromo-L-phenylalanine.

[0028] In one embodiment of the above aspects and embodiments of the invention, the ThiK and/or the nucleic acid encoding ThiK is from Herpes Simplex Virus 1 or Herpes Simplex Virus 2 (HSV-TK), VZV, CMV, HHV7, HHV7, HHV8, EBV or is a functionally equivalent variant of any one or more thereof.

BRIEF DESCRIPTION OF THE FIGURES

[0029] These and other aspects of the present invention, which should be considered in all its novel aspects, will become apparent from the following description, which is given by way of example only, with reference to the accompanying figures, in which:

Figure 1: Shows the map of plamid pMTL83155-HSV-tk

Figure 2: Shows PCR amplification of ~1.5 kb fragment spanning gram positive replicon and *catP* marker on pMTL83155 and pMTL83155-Hsv-tk (h1 and h2) in *C. autoethanogenum* transformants.

Unmodified *C. autoethanogenum* (C) was used as a control. 2 colonies each of LZ-pMTL83155 (P1 and P2) and LZ-pMTL83155-Hsv-tk (h1 and h2) were screened.

Figure 3: Shows pairwise translated nucleotide sequence alignment of pheS from *E. coli* MG1655 (Seq ID 13) and *C. autoethanogenum* with putative substrate specificity region bold and underlined.

Figure 4: Shows the map of plasmid pMTL85151-*pheS**

Figure 5: Shows a representative map of a plamid comprising HSV-tk.

Figure 6: Shows a representative map of a plamid comprising *pheS.*

Figure 7: Shows TAE agarose gel electrophoresis results from PCR screen with AM041 and AM042. Lane 1 contains GeneRuler 1kb Ladder (Thermo). Lane 2 contains PCR with no template added. Lane 3 PCR with wild type C. autoethanogenum genomic DNA as template showing expected wild type product size (3137 bp). Lanes 4-7 contain PCR with p-chlorophenylalanine and thiamphenicol resistant colonies as template. Lane 6 shows a PCR product of expected size (3570 bp) for successful double crossover replacement of native butanediol dehydrogenase gene with butanediol dehydrogenase gene from K. pneumonia.

Figure 8: Gene map showing the intron target sites (211s, 287a, 388a, 400s, 433s, and 552a). Primer binding sites are also shown (bottom, horizontal arrows).

Figure 9A -9C: Confirmation of the group II intron insertions. Fig. 9A: 433s and 388a (faint band), Fig. 9B: 211s, and 287a. Fig. 9C: 287a, and 433

BRIEF DESCRIPTION OF SEQUENCE INFORMATION

[0030] Prior to the figures shown herein after, the specification includes details of the sequences of nucleic acids and polypeptides relevant to the invention. The following sequences are provided:

Seq. ID.1: Nucleic acid sequence of pMK-RQ-Hsv-tk

Seq. ID.2: Nucleic acid sequence of pMTL83155-Hsv-tk

Seq. ID.3: Nucleic acid sequence of pMTL83155

Seq. ID.4: Nucleic acid sequence of primer **repHf**

Seq. ID.5: Nucleic acid sequence of primer **catr**

Seq. ID.6: Nucleic acid sequence of primer **fD1**

Seq. ID.7: Nucleic acid sequence of primer **rP2**

Seq. ID.8: 16s rRNA nucleic acid sequence of LZ-pMTL83155-1 obtained using primer rP2

Seq. ID.9: 16s rRNA nucleic acid sequence of LZ-pMTL83155-2 obtained using primer rP2

Seq. ID.10: 16s rRNA nucleic acid sequence of LZ-pMTL83155-hsv-tk-1 obtained using primer rP2

Seq. ID.11: 16s rRNA nucleic acid sequence of LZ-pMTL83155-hsv-tk-2 obtained using primer rP2

Seq. ID.12: Nucleic acid sequence encoding *pheS* of *C. autoethanogenum*

Seq. ID.13: Nucleic acid sequence encoding *pheS* of *E. coli* MG1655

Seq. ID.14: Nucleic acid sequence encoding altered *pheS** of *C. autoethanogenum*

Seq. ID.15: Forward primer sequence used for confirming the presence of PheS plasmid - M13F

Seq. ID.16: Reverse primer sequence used for confirming the presence of PheS plasmid - M13R

Seq. ID.17: Synthetic promoter *PpheS**

Seq. ID.18: Nucleotide sequence of pMTL85151*pheS**

Seq. ID.19: Nucleic acid sequence encoding HSV-TK of Human Herpesvirus 1 (Herpes simplex virus type 1)

Seq. ID. 20: Amino acid sequence of PheS of *E. coli* MG1655

Seq. ID. 21: Amino acid sequence of PheS of *C. autoethanogenum*

Seq. ID. 22: Nucleic acid sequence encoding ThiK of Human Herpesvirus 1 (Herpes simplex virus type 1)

Seq. ID. 23: Nucleic acid sequence encoding CatP of *Clostridium perfringens*

Seq. ID. 24: Nucleic acid sequence encoding ErmB of *Peptoclostridium difficile*

Seq. ID. 25: Nucleic acid sequence encoding TetA of *Escherichia coli*

Seq ID 26: Nucleic acid sequence of PheS* cassette and ColE1 with traJ PCR product for assembly of pPheS*-ErmB

Seq ID 27: Nucleic acid sequence of pPheS Fragment PCR product for assembly of pPheS-CaBDHXXKpBDH (Example 2)

Seq ID 30: Nucleic acid sequence of pCB102 origin of replication PCR product used for assembly of pPheS*-ErmB

Seq ID 33: Nucleic acid sequence of erythromycin resistance cassette PCR product used for assembly of pPheS*-ErmB

Seq ID 36: Nucleic acid sequence of pPhes-ErmB template for amplification of backbone plasmid for assembly of pPheS-CaBDHXXKpBDH

Seq ID 39: Nucleic acid sequence of upstream homology arm PCR product for assembly of pPheS-CaBDHXXKpBDH

Seq ID 42: Nucleic acid sequence of K. pneumoniae butanediol dehydrogenase gene PCR product for assembly of pPheS-CaBDHXXKpBDH

Seq ID 45: Nucleic acid sequence of chloramphenicol acetyltransferase cassette PCR product for assembly of pPheS-CaBDHXXKpBDH

Seq ID 48: Nucleic acid sequence of downstream homology arm PCR product for assembly of pPheS*-CaBDHXXKpBDH

Seq ID 53: Amino acid sequence of altered *pheS** of *C. autoethanogenum*

Seq ID 54: Amino acid sequence of Human Herpesvirus 1 (Herpes simplex virus type 1)

Seq ID 55: Nucleic acid sequence of primary:secondary alcohol dehydrogenase of *C. autoethanogenum*

Seq ID 56: Intron targeting region

Seq ID 57: Primer sequemce for 156F

Seq ID 58: Primer sequemce for 939R

[0031] Other sequences of relevance to the invention are described elsewhere herein. For example, see Table 3 in Example 2.

DETAILED DESCRIPTION OF THE INVENTION

[0032] The following is a description of the present invention, including preferred embodiments thereof, given in general terms. The invention is further elucidated from the disclosure given under the heading "Examples" herein below, which provides experimental data supporting the

invention, specific examples of various aspects of the invention, and means of performing the invention.

[0033] The production of recombinant microorganisms can involve introducing an exogenous nucleic acid into a parental microorganism, with a double-crossover recombination event occurring between the exogenous nucleic acid and the genome of the microorganism so that at least one desired genetic alteration can be introduced into the genome. Double-crossover recombination happens at frequencies that are typically too low to reliably identify integrants by chance alone. Therefore, the inventors believe that a means to select for one or both crossovers has a huge benefit on the frequency of identification in both time and labour. It has been noted by the inventors in their lab that the frequency of single-crossover recombination although low, can be found by screening an appropriate number of colonies, however, this has not been seen to be the case with the second crossover event. The present invention provides a means to select for the second event by counter selecting against a condition lethal gene product present in the exogenous nucleic acid introduced into the parental microorganism. This means that in any microorganisms in which only a single-crossover event has occurred in the presence of a counter selecting agent the expression of the condition legal gene product, will kill any cell which has not undergone the secondary crossover event and released the nucleic acid containing the gene encoding the counter selection marker.

[0034] Counter-selection markers are known. However, they are not necessarily transferable for use in different genera of bacteria. Unfortunately either due to physiological or genetic reasons, the vast majority do not work in Clostridia. The inventors have surprisingly identified that ThiK and an altered version of PheS can be used as counter-selection markers in *Clostridium* spp.

Definitions

[0035] "Exogenous nucleic acids" are nucleic acids which originate outside of the microorganism to which they are introduced. Exogenous nucleic acids may be derived from any appropriate source, including, but not limited to, the microorganism to which they are to be introduced, strains or species of organisms which differ from the organism to which they are to be introduced, or they may be artificially or recombinantly created.

[0036] A "genetic modification" should be taken broadly and is intended to include, for example, introducing a mutation to a genetic site, adding to or removing from the genome one or more nucleotides, substitution of one or more nucleotides with different nucleotides, substitution of a gene, removal of a gene, addition of a gene and the like.

[0037] Reference may be made herein to an "altered PheS", a "PheS which is altered" or a PheS including one or more or at least one "alteration" compared to a wild-type PheS such that in use phenylalanine tRNA synthetase is able to aminoacylate tRNA using a phenylalanine analogue. An "alteration" should be considered broadly and includes, for example, one or a

combination of substitution of one or more amino acid, deletion of one or more amino acid, and/or addition of one or more amino acid compared to a wild-type PheS. An "altered" PheS may also include one or more alterations in addition to those that allow phenylalanine tRNA synthetase to aminoacylate tRNA using a phenylalanine analogue, provided it is still able to substantially perform its desired function.

[0038] Reference may be made herein to a nucleic acid encoding a PheS comprising one or more alterations compared to a nucleic acid which encodings a wild-type PheS. An "alteration" should be considered broadly and includes, for example, one or a combination of substitution of one or more nucleotides, deletion of one or more nucleotide, and/or addition of one or more nucleotide compared to a nucleic acid encoding a wild-type PheS. The nucleic acid may also include one or more alterations in addition to those that allow phenylalanine tRNA synthetase to aminoacylate tRNA using a phenylalanine analogue, provided the PheS is still able to substantially perform its desired function.

[0039] One or more alteration of PheS or a nucleic acid encoding PheS may be described herein with reference to a specific region or amino acid or nucleotide position in a wild-type PheS (or nucleic acid encoding same) from a specific organism. It will be appreciated that the precise location of a particular region, amino acid or nucleotide may vary slightly from one PheS or nucleic acid encoding PheS to another, for example in different strains or species of organisms. To account for this variation, where the location of a specific region, nucleotide or amino acid is referred to herein, it is described as being "read in relation to" or "read relative to" the amino acid position of wild-type PheS of *C. autoethanogenum* (SEQ ID No. 21) (or to the wild-type Phe-S from *C. ljungdahlii* DSM13528 (GenBank: ADK16487.1)) or the nucleotide position of the nucleic acid encoding PheS of *C. autoethanogenum* (SEQ ID 12 herein) (or to the wild-type Phe-S from *C. ljungdahlii* DSM13528 (GenBank: ADK16487.1), wherein the first position amino acid in SEQ ID 21 (or ADK16487.1) and the first nucleotide in SEQ ID 12 (or ADK16487.1) are position 1. Such phrases should be taken broadly and are intended to encompass equivalent regions, amino acids or nucleotides in other PheS proteins (or nucleic acids encoding same) even though they may be at a different location. Persons of skill in the art to which the invention relates will be able to readily identify the location or position of a particular region, amino acid or nucleotide in a particular PheS or nucleic acid encoding same through routine sequence alignment and with the information contained herein.

[0040] Reference to a particular region of a PheS or nucleic acid "between" two particular amino acids or nucleotides should be taken to mean a region comprising said nucleotides or amino acids. In other words, the region includes the terminal nucleotides or amino acids referred to. For example, a substrate specific site between amino acids at position 306 and 313 includes the amino acids present at positions 306 and 313.

[0041] The term "phenylalanine analogue" should be taken broadly and includes an analogue or derivative of phenylalanine that can be incorporated into peptides and proteins in the place of phenylalanine resulting in toxicity to a microorganism. In one embodiment, the phenylalanine analogue is chosen from chlorophenylalanine, fluorophenylalanine and bromophenylalanine. In

one particular embodiment, the phenylalanine analogue is chosen from DL-4-chlorophenylalanine and *p*-chlorophenylalanine, p-fluoro-L-phenylalanine, p-fluoro-DL-phenylalanine, p-bromo-L-phenylalanine. Skilled persons may readily appreciate other phenylalanine analogues of use in the invention.

[0042] Reference may be made herein to a nucleic acid vector including a "nucleic acid sequence of interest" or like phrases. Such phrases should be taken broadly and include one or more nucleotide, gene, promoter, regulatory sequence, other genetic element and may be coding or non-coding. It may include a nucleotide sequence which is designed to introduce one or more genetic modification to one or more target location in the host genome, including one or a combination of a deletion, addition or subsitituion of one or more nucleotides. In some embodiments, the nucleic acid or nucleic acid sequence of interest may be designed to delete a gene present in the genome of the parental microorganism.

[0043] The expressions "target location" and "target nucleic acid sequence" as used herein should be taken broadly to include any site, region or nucleotide sequence in a parental or host genome where it is desired to introduce one or more genetic modification (including insertion, deletion and/or substitution of one or more nucleotides), and includes a gene, intergenic region, promoter and/or regulatory sequence of interest, for example.

[0044] Reference may be made herein to a vector of the invention including "two nucleic acid sequences homologous to selected regions around a target location" within the genome of a parental microorganism. Such nucleic acid sequences may also be referred to herein as "homology arms".

[0045] Reference may be made herein to proteins (PheS and/or ThiK) or nucleic acids encoding such proteins being "from" or "derived from" a particular organism. This should be taken broadly to mean that the protein or nucleic acid has the sequence of the relevant protein or nucleic acid encoding the relevant protein in that organism. It should not be taken to mean that the protein or nucleic acid has been physically taken from that organism. Such proteins and nucleic acids may be made using chemical synthesis and the like, for example.

[0046] A "parental microorganism" is a microorganism used to generate a recombinant microorganism according to the invention. In one embodiment, the parental microorganism may be one that occurs in nature (ie a wild type microorganism) or one which has been previously modified (a genetically modified or recombinant microorganism). According to the present invention, a "parental microorganism" is a *Clostridium* spp.

[0047] Skilled persons will be able to readily identify *Clostridium* ssp. microorganisms of use in the invention. However, by way of example, the group may include: *Clostridium autoethanogenum, Clostridium ljungdahlii, Clostridium ragsdalei, Clostridium carboxidivorans, Clostridium drakei, Clostridium scatologenes, Clostridium aceticum, Clostridium formicoaceticum, Clostridium magnum, Clostridium coskatii, Clostridium acetobutylicum, Clostridium beijerinckii, Clostridium sacharoperbutylacetonicum, Clostridium*

saccharobutylicum, Clostridium thermocellum, Clostridium cellulolyticum, Clostridium phytofermentans, Clostridium pasterianum, Clostridium kluyveri, Clostridium difficile, Clostridium botulinum, Clostridium sporogenes, Clostridium perfringens, Clostridium acetobutylicum, Clostridium acidisoli, Clostridium aciditolerans, Clostridium acidurici, Clostridium aerotolerans,Clostridium akagii, Clostridium aldenense, Clostridium algidicarnis, Clostridium algidixylanolyticum, Clostridium alkalicellulosi, Clostridium aminovalericum, Clostridium amygdalinum, Clostridium arcticum, Clostridium argentinense,Clostridium aurantibutyricum, Clostridium baratii, Clostridium botulinum, Clostridium bowmanii, Clostridium butyricum,Clostridium beijerinckii, Clostridium cadaveris, Clostridium caminithermale, Clostridium carboxidivorans, Clostridium carnis, Clostridium celatum, Clostridium celerecrescens, Clostridium cellulolyticum, Clostridium cellulosi, Clostridium chartatabidum, Clostridium clostridioforme, Clostridium coccoides, Clostridium cochlearium, Clostridium cocleatum, Clostridium colinum, Peptoclostridium difficile, Clostridium diolis, Clostridium disporicum, Clostridium drakei, Clostridium durum, Clostridium esterteticum, Clostridium fallax, Clostridium felsineum, Clostridium ervidum, Clostridium fimetarium,Clostridium formicaceticum, Clostridium ghonii, Clostridium glycolicum, Clostridium glycyrrhizinilyticum, Clostridium haemolyticum, Clostridium halophilum, Clostridium tetani, Clostridium perfringens, Clostridium phytofermentans, Clostridium piliforme, Clostridium polysaccharolyticum, Clostridium populeti, Clostridium propionicum, Clostridium proteoclasticum,Clostridium proteolyticum, Clostridium psychrophilum, Clostridium puniceum, Clostridium puri, Clostridium putrefaciens, Clostridium putrificum, Clostridium quercicolum, Clostridium quinii, Clostridium ramosum, Clostridium roseum, Clostridium saccharobutylicum, Clostridium saccharolyticum, Clostridium saccharoperbutylacetonicum, Clostridium sardiniense, Clostridium stercorarium, Clostridium sticklandii, Clostridium paradoxum, Clostridium paraperfringens, Clostridium paraputrificum, Clostridium pascui, Clostridium pasteurianum, Clostridium novyi, Clostridium septicum, Clostridium histolyticum, Clostridium hydroxybenzoicum, Clostridium hylemonae, Clostridium innocuum, Clostridium kluyveri, Clostridium lactatifermentans, Clostridium lacusfiyxellense, Clostridium laramiense, Clostridium lentocellum, Clostridium lentoputrescens, Clostridium methoxybenzovorans, Clostridium methylpentosum, Clostridium nitrophenolicum, Clostridium novyi, Clostridium oceanicum, Clostridium oroticum, Clostridium oxalicum, Clostridium tertium, Clostridium tetani, Clostridium tetanomorphum, Clostridium thermaceticum, Clostridium thermautotrophicum, Clostridium thermoalcaliphilum, Clostridium thermobutyricum, Clostridium thermocellum, Clostridium thermocopriae, Clostridium thermohydrosulfuricum, Clostridium thermolacticum, Clostridium thermopalmarium, Clostridium thermopapyrolyticum, Clostridium thermosaccharolyticum, Clostridium thermosulfiirigenes, Clostridium tyrobutyricum, Clostridium uliginosum, Clostridium ultunense, Clostridium villosum, Clostridium viride, Clostridium xylanolyticum, Clostridium xylanovorans, Clostridium bifermentans, and Clostridium sporogenes.

[0048] In one particular embodiment the parental organism is selected from a group of acetogenic *Clostridium* spp. In one particular embodiment, the parental microorganism is selected from the group of acetogenic carboxydotrophic organisms comprising the species *Clostridium autoethanogenum, Clostridium ljungdahlii, Clostridium ragsdalei, Clostridium carboxidivorans, Clostridium drakei, Clostridium scatologenes, Clostridium aceticum,*

Clostridium formicoaceticum, Clostridium magnum, and *Clostridium coskatii.*

[0049] In a one embodiment, the parental microorganism is selected from a cluster of carboxydotrophic Clostridia comprising the species *C. autoethanogenum, C. ljungdahlii,* and "*C. ragsdalei*" and related isolates. These include but are not limited to strains *C. autoethanogenum* JAI-1^T (DSM10061) (Abrini, Naveau, & Nyns, 1994), *C. autoethanogenum* LBS1560 (DSM19630) (WO/2009/064200), *C. autoethanogenum* LBS1561 (DSM23693), *C. ljungdahlii* PETC^T (DSM13528 = ATCC 55383) (Tanner, Miller, & Yang, 1993), *C. ljungdahlii* ERI-2 (ATCC 55380) (US patent 5,593,886), *C. ljungdahlii* C-01 (ATCC 55988) (US patent 6,368,819), *C. ljungdahlii* O-52 (ATCC 55989) (US patent 6,368,819), or "*C. ragsdalei* P11^T" (ATCC BAA-622) (WO 2008/028055), and related isolates such as "*C. coskatii*" (US patent 2011/0229947), and mutant strains thereof such as *C. ljungdahlii* OTA-1 (Tirado-Acevedo O. Production of Bioethanol from Synthesis Gas Using Clostridium ljungdahlii. PhD thesis, North Carolina State University, 2010).

[0050] These strains form a subcluster within the Clostridial rRNA cluster I (Collins *et al.,* 1994), having at least 99% identity on 16S rRNA gene level, although being distinct species as determined by DNA-DNA reassociation and DNA fingerprinting experiments (WO 2008/028055, US patent 2011/0229947).

[0051] The strains of this cluster are defined by common characteristics, having both a similar genotype and phenotype, and they all share the same mode of energy conservation and fermentative metabolism. The strains of this cluster lack cytochromes and conserve energy via an Rnf complex.

[0052] All strains of this cluster have a genome size of around 4.2 MBp (Köpke *et al.,* 2010) and a GC composition of around 32 %mol (Abrini *et al.,* 1994; Köpke *et al.,* 2010; Tanner *et al.,* 1993) (WO 2008/028055; US patent 2011/0229947), and conserved essential key gene operons encoding for enzymes of Wood-Ljungdahl pathway (Carbon monoxide dehydrogenase, Formyl-tetrahydrofolate synthetase, Methylene-tetrahydrofolate dehydrogenase, Formyl-tetrahydrofolate cyclohydrolase, Methylene-tetrahydrofolate reductase, and Carbon monoxide dehydrogenase/Acetyl-CoA synthase), hydrogenase, formate dehydrogenase, Rnf complex (*rnfCDGEAB*), pyruvate:ferredoxin oxidoreductase, aldehyde:ferredoxin oxidoreductase (Köpke *et al.,* 2010, 2011). The organization and number of Wood-Ljungdahl pathway genes, responsible for gas uptake, has been found to be the same in all species, despite differences in nucleic and amino acid sequences (Köpke *et al.,* 2011).

[0053] The strains of the cluster all have a similar morphology and size (logarithmic growing cells are between 0.5-0.7 x 3-5 µm), are mesophilic (optimal growth temperature between 30-37 °C) and strictly anaerobe (Abrini *et al.,* 1994; Tanner *et al.,* 1993)(WO 2008/028055). Moreover, they all share the same major phylogenetic traits, such as same pH range (pH 4-7.5, with an optimal initial pH of 5.5-6), strong autotrophic growth on CO containing gases with

similar growth rates, and a metabolic profile with ethanol and acetic acid as main fermentation end product, with small amounts of 2,3-butanediol and lactic acid formed under certain conditions (Abrini *et al.,* 1994; Köpke *et al.,* 2011; Tanner *et al.,* 1993)(WO 2008/028055). Indole production has been observed with all species. However, the species differentiate in substrate utilization of various sugars (e.g. rhamnose, arabinose), acids (e.g. gluconate, citrate), amino acids (e.g. arginine, histidine), or other substrates (e.g. betaine, butanol). Some of the species were found to be auxotrophic to certain vitamins (e.g. thiamine, biotin) while others were not. Reduction of carboxylic acids into their corresponding alcohols has been shown in a range of these organisms (Perez, Richter, Loftus, & Angenent, 2012).

[0054] The traits described are therefore not specific to one organism like *C. autoethanogenum* or *C. ljungdahlii,* but rather general traits for carboxydotrophic, ethanol-synthesizing Clostridia. The invention can be anticipated to work across not only these strains, but across all Clostridia species, although there may be differences in performance.

[0055] In particular embodiments, the parental microorganism is selected from the group comprising *Clostridium autoethanogenum, Clostridium ljungdahlii,* and *Clostridium ragsdalei.* In one embodiment, the group also comprises *Clostridium coskatii.* In one particular embodiment, the parental microorganism is *Clostridium autoethanogenum* DSM23693.

[0056] A parental microorganism may or may not contain nucleic acids encoding phenylalanine tRNA synthetase or express phenylalanine tRNA synthetase.

[0057] Throughout this specification exemplary sequence information is provided for PheS, altered PheS and ThiK proteins/peptides and nucleic acids encoding same. This information is provided to identify exemplary proteins/peptides and nucleic acids applicable to the invention and to allow a skilled person to practise specific embodiments of the invention without undue experimentation. It should be appreciated that nucleic acid and amino acid sequences may differ from one microorganism to another. Accordingly, the invention should not be construed as being limited to these specific embodiments but rather to extend to proteins/peptides and nucleic acids having different sequences but which are substantially capable of performing the same function. For PheS the desired function (as a subunit of phenylalanine tRNA synthetase) is aminoacylation of $tRNA^{Phe}$ with phenylalanine. For altered PheS the desired function (as a subunit of phenylalanine tRNA synthetase) is aminoacylation of tRNA with a phenylalanine analogue. For ThiK the desired function is to catalyse the reaction:

$$Thd + ATP \rightarrow TMP + ADP$$

where Thd is deoxythymidine, ATP is adenosine 5'-triphosphate, TMP is deoxythymidine 5'-phosphate and ADP is adenosine 5'-diphosphate.

[0058] Typically, such alternative or variant proteins/peptides will have at least approximately 75% amino acid sequence similarity to a PheS (including an altered PheS) or ThiK protein exemplified herein. In particular embodiments, such alternative proteins will have at least

approximately 80%, 85%, 90%, 95% or 99% sequence similarity to a PheS (including an altered PheS) or ThiK exemplified herein. In particular embodiments, such alternative proteins will have at least approximately 75%, 80%, 85%, 90%, 95% or 99% sequence identity to a PheS (including an altered PheS) or ThiK exemplified herein. At the nucleic acid level, genes encoding such alternative or variant proteins will typically have at least approximately 75% sequence homology to a nucleic acid encoding a PheS (including an altered PheS) or ThiK exemplified herein. In particular embodiments, such variant or alternative nucleic acids will have at least approximately 80%, 85%, 90%, 95% or 99% sequence homology to a nucleic acid encoding a PheS (including an altered PheS) or ThiK exemplified herein. In one particular embodiment, such nucleic acids will have at least approximately 75%, 80%, 85%, 90%, 95% or 99% sequence identity to a nucleic acid encoding a PheS (including an altered PheS) or ThiK exemplified herein. Alternative or variant nucleic acids or proteins/peptides as described may be referred to herein as "functionally equivalent variants".

[0059] It should also be appreciated that the functionally equivalent variant of PheS, altered PheS, or ThiK need not have the same level of activity as a protein/peptide of which it is a variant. All that is required is that some level of the desired activity is retained. Assays of use in assessing the activity of PheS, an altered PheS or ThiK will be known by skilled persons. However, by way of example: The function or activity of Phe S can be tested using methods which measure aminoacylation. The authors used velocities of aminoacylation and kinetic parameters of pheS to test activity variations of pheS in utilising phenylalanine (Kast et al., 1991 (J. Mol. Biol 222: 99-124)). The function or activity of an altered PheS can be conducted by observing growth in the presence of a toxic analogue of phenylanine using methods known for culturing or growing microorganisms (Kast et al., 1991 (J. Mol. Biol 222: 99-124)). The function or activity of ThiK can be tested using an activity assay as described by Brockenbrough et al (Nucl Med Biol. 2007, 34(6):619-23) and Jonsson & McIvor (Anal Biochem. 1991, 199(2):232-7) or with commercially available ELISA kits as for example from BioVendor (Cat. No. 901/902).

[0060] Reference to "transforming" a parental microorganism should be taken broadly to include any means of transferring or introducing an exogenous nucleic acid into a microorganism which are known in the art. By way of example, "transforming" includes, but is not limited to transfection, transduction, conjugation, and electroporation.

Aspects and Embodiments of Invention

[0061] The invention provides the use of ThiK and/or PheS as a counter-selection marker in a method for producing a recombinant microorganism from a parental microorganism. It also provides nucleic acid(s) encoding Thik and/or PheS, nucleic acid vectors comprising said nucleic acid(s), and the use of said nucleic acid(s) and/or plasmids for producing a recombinant microorganism from a parental microorganism. In addition, the invention provides a method of producing a recombinant microorganism from a parental microorganism. In accordance with the invention, the parental microorganism is a *Clostridium* spp., as described

herein before, and the PheS includes at least one alteration compared to a wild-type PheS such that in use phenylalanine tRNA synthetase is able to aminoacylate tRNA using a phenylalanine analogue.

PheS

[0062] PheS is the alpha subunit of the two subunit protein phenylalanine tRNA synthetase. Phenylalanine tRNA synthetase is responsible for aminoacylation of $tRNA^{Phe}$ with phenylalanine which is critical for protein production in a cell. The enzyme catalyses the acelation of phenylalanine to its cognate tRNA. The resultant $tRNA^{Phe}$ is delivered to the ribosome by elongation factors then subsequently bound to its cognate anti-codon present upon the mRNA. Once bound, the amino acid is covalently attached to its preceding amino-acid thereby increasing the peptide chain.

[0063] A PheS of the invention is one which includes at least one alteration compared to a wild type PheS such that in use phenylalanine tRNA synthetase is able to aminoacylate tRNA using phenylalanine analogues. Incorporation of phenylananie analogues into cellular proteins results in unstable or non-functional proteins. Thus, any cell including the altered PheS will typically not be able to survive.

[0064] The wild-type PheS on which the altered PheS is based may be from any appropriate source including any number of different self replicating organisms, such as plants, animals, fungi and microorganisms. In one particular embodiment, the PheS is from a microorganism. In one embodiment, the PheS is from a *Clostridium* spp or is a functionally equivalent variant thereof. By way of example only, the *Clostridium* spp. may include:

[0065] *Clostridium autoethanogenum, Clostridium ljungdahlii, Clostridium ragsdalei, Clostridium carboxidivorans, Clostridium drakei, Clostridium scatologenes, Clostridium aceticum, Clostridium formicoaceticum, Clostridium magnum, Clostridium coskatii, Clostridium acetobutylicum, Clostridium beijerinckii, Clostridium sacharoperbutylacetonicum, Clostridium saccharobutylicum, Clostridium thermocellum, Clostridium cellulolyticum, Clostridium phytofermentans, Clostridium pasterianum, Clostridium kluyveri, Clostridium difficile, Clostridium botulinum, Clostridium sporogenes, Clostridium perfringens, Clostridium acetobutylicum, Clostridium acidisoli, Clostridium aciditolerans, Clostridium acidurici, Clostridium aerotolerans, Clostridium akagii, Clostridium aldenense, Clostridium algidicarnis, Clostridium algidixylanolyticum, Clostridium alkalicellulosi, Clostridium aminovalericum, Clostridium amygdalinum, Clostridium arcticum, Clostridium argentinense, Clostridium aurantibutyricum, Clostridium baratii, Clostridium botulinum, Clostridium bowmanii, Clostridium butyricum, Clostridium beijerinckii, Clostridium cadaveris, Clostridium caminithermale, Clostridium carboxidivorans, Clostridium carnis, Clostridium celatum, Clostridium celerecrescens, Clostridium cellulolyticum, Clostridium cellulosi, Clostridium chartatabidum, Clostridium clostridioforme, Clostridium coccoides, Clostridium cochlearium, Clostridium*

cocleatum, Clostridium colinum, Clostridium difficile, Clostridium diolis, Clostridium disporicum, Clostridium drakei, Clostridium durum, Clostridium esterteticum, Clostridium fallax, Clostridium felsineum, Clostridium ervidum, Clostridium fimetarium, Clostridium formicaceticum, Clostridium ghonii, Clostridium glycolicum, Clostridium glycyrrhizinilyticum, Clostridium haemolyticum, Clostridium halophilum, Clostridium tetani, Clostridium perfringens, Clostridium phytofermentans, Clostridium piliforme, Clostridium polysaccharolyticum, Clostridium populeti, Clostridium propionicum, Clostridium proteoclasticum, Clostridium proteolyticum, Clostridium psychrophilum, Clostridium puniceum, Clostridium puri, Clostridium putrefaciens, Clostridium putrificum, Clostridium quercicolum, Clostridium quinii, Clostridium ramosum, Clostridium roseum, Clostridium saccharobutylicum, Clostridium saccharolyticum, Clostridium saccharoperbutylacetonicum, Clostridium sardiniense, Clostridium stercorarium, Clostridium sticklandii, Clostridium paradoxum, Clostridium paraperfringens, Clostridium paraputrificum, Clostridium pascui, Clostridium pasteurianum, Clostridium novyi, Clostridium septicum, Clostridium histolyticum, Clostridium hydroxybenzoicum, Clostridium hylemonae, Clostridium innocuum, Clostridium kluyveri, Clostridium lactatifermentans, Clostridium lacusfiyxellense, Clostridium laramiense, Clostridium lentocellum, Clostridium lentoputrescens, Clostridium methoxybenzovorans, Clostridium methylpentosum, Clostridium nitrophenolicum, Clostridium novyi,Clostridium oceanicum, Clostridium oroticum, Clostridium oxalicum, Clostridium tertium, Clostridium tetani, Clostridium tetanomorphum, Clostridium thermaceticum, Clostridium thermautotrophicum, Clostridium thermoalcaliphilum, Clostridium thermobutyricum, Clostridium thermocellum, Clostridium thermocopriae, Clostridium thermohydrosulfuricum, Clostridium thermolacticum, Clostridium thermopalmarium, Clostridium thermopapyrolyticum, Clostridium thermosaccharolyticum, Clostridium thermosulfiirigenes, Clostridium tyrobutyricum, Clostridium uliginosum, Clostridium ultunense, Clostridium villosum, Clostridium viride, Clostridium xylanolyticum, Clostridium xylanovorans, Clostridium bifermentans, and Clostridium sporogenes.

[0066] In certain embodiments, the PheS is from a microorganism selected from the group of *Clostridium* spp or is a functionally equivalent variant thereof. In one embodiment, the PheS is from a microorganism selected from a group of acetogenic *Clostridium* spp or is a functionally equivalent variant thereof. In one particular embodiment, PheS is from a microorganisms selected from the group of acetogenic carboxydotrophic organisms comprising the species *Clostridium autoethanogenum, Clostridium ljungdahlii, Clostridium ragsdalei, Clostridium carboxidivorans, Clostridium drakei, Clostridium scatologenes, Clostridium aceticum, Clostridium formicoaceticum, Clostridium magnum,* and *Clostridium coskatii,* or is a functionally equivalent variant of any one or more thereof.

[0067] In a one embodiment, PheS is selected from a cluster of carboxydotrophic Clostridia comprising the species *C. autoethanogenum, C. ljungdahlii,* and "*C. ragsdalei*" and related isolates, which have been described herein before.
By way of example only, appropriate wild-type PheS proteins (and corresponding nucleic acid sequences) include those described in public databases, such as GenBank, as follows: PheS from *Clostridium ljungdahlii* DSM13528 (GenBank ADK16487.1); *Clostridium carboxidivorans* P7 (GenBank: EET86555.1); phenylalanyl-tRNA synthetase subunit alpha [*Clostridium kluyveri*]

WP_012620882.1; phenylalanyl-tRNA synthetase subunit alpha [*Clostridium perfringens* str. 13] GenBank: BAB81592.1; phenylalanyl-tRNA synthetase subunit alpha [*Clostridium botulinum* A str. ATCC 3502] GenBank: YP_001255621.1; phenylalanyl-tRNA synthetase subunit alpha [*Clostridium sporogenes*] GenBank: WP_003495653.1; phenylalanyl-tRNA synthetase subunit alpha [*Clostridium beijerinckii* NCIMB 8052] GenBank: YP_001308703.1; phenylalanyl-tRNA synthetase subunit alpha [*Clostridium acetobutylicum* ATCC 824] GenBank: NP_348973.1; phenylalanyl-tRNA synthetase subunit alpha [*Clostridium thermocellum* ATCC 27405] GenBank: YP_001036648.1. In one embodiment, the PheS is from *C. autoethanogenum* and has the amino acid sequence of SEQ ID No.21. Functionally equivalent variants of these exemplary proteins may also be of use.

[0068] In one embodiment, the at least one alteration in PheS compared to a wild-type PheS is located within a region which comprises the substrate specificity site. In one embodiment, the substrate specificity site is located between amino acids 306 and 313, read in relation to the wild-type PheS from *C. autoethanogenum* SEQ ID No. 21 (or to the wild-type Phe-S from *C. ljungdahlii* DSM13528 (GenBank: ADK16487.1)).

[0069] It should be appreciated that the precise location of the substrate specificity site may vary from one particular PheS protein to another. Accordingly, the invention should be taken to include PheS proteins which have been altered outside of the site defined by amino acids 306 to 313 above, and confer on phenylalanine tRNA synthetase the ability to aminoacylate tRNA using phenylalanine analogues. Persons of general skill in the art to which the invention relates will readily be able to identify the appropriate site based on alignment of the amino acid sequence with that of *C. autoethanogenum* SEQ ID 21 (or to the wild-type Phe-S from *C. ljungdahlii* DSM13528 (GenBank ADK16487.1), described above. However, by way of example, in PheS in *E. coli* the substrate specificity site is defined by amino acids at positions 289 to 296.

[0070] In one embodiment, the at least one alteration is one or more amino acid substitution, addition and/or deletion. In one embodiment, the at least one alteration is an amino acid substitution at position 311, read relative to the amino acid sequence of *Clostridium autoethanogenum* PheS (SEQ ID 21). In one embodiment, the at least one alteration is substitution of Ala for Gly at amino acid 311. In other embodiments, the alternation is one or a combination of amino acid substitutions at positions 310 and 312, read relative to the amino acid sequence of *Clostridium autoethanogenum* PheS (SEQ ID 21).

[0071] In one embodiment, the altered PheS comprises or consists of the amino acid sequence of SEQ ID No. 53.

[0072] The invention also relates to nucleic acids encoding an altered PheS of the invention. In one embodiment, the at least one alteration is located within a region of the nucleic acid which encodes the substrate specificity site, as referred to herein before.

[0073] In one embodiment, the region encoding the substrate specificity site is located

between bases 918 and 939, read in relation to the nucleic acid encoding PheS in *C. autoethanogenum* (SEQ ID 12 herein) (or to the wild-type Phe-S from *C. ljungdahlii* DSM13528 (GenBank: ADK16487.1). However, the site may differ from one nucleic acid to another (for example nucleic acids from different species or organisms), to reflect the precise location of the substrate specificity site on a wild-type PheS protein, as described hereinbefore. Skilled persons will readily be able to identify the appropriate region in alternative nucleic acids through standard sequence alignments.

[0074] In one embodiment, the at least one alteration is one or more nucleotide substitution, addition and/or deletion.

[0075] In one embodiment, the at least one alteration is a nucleotide substitution at base 932 read relative to the *Clostridium autoethanogenum* gene encoding PheS (SEQ ID 12). In one embodiment, the at least one alteration is substitution of C for G at base 932. In one embodiment, the nucleic acid comprises or consists of the sequence of SEQ ID No. 14.

[0076] Nucleic acids encoding an altered PheS in accordance with the invention can be generated using any number of known methods in the art, based on the information herein, the amino acid sequence (and/or the nucleic acid sequence encoding the amino acid sequence) of exemplary wild-type PheS proteins, and the genetic code, for example. However, by way of example, they may be produced by chemical synthesis or via standard recombinant techinques.

[0077] By way of example, exemplary nucleic acids encoding wild-type PheS are provided herein and in publicly available databases such as GenBank as follows: *E. coli* K12 (NC_000913.2), Gene ID: 946223, EcoGene:EG10709; *Clostridium ljungdahlii* DSM 13528, GenBank: CP001666.1, GI:300433347.

[0078] It should be appreciated that a nucleic acid encoding an altered PheS can be codon optimised for the particular *Clostridium* spp. in which it is to be expressed. This can be achieved using standard codon optimisation techniques.

ThiK

[0079] ThiK is a protein which functions to catalyse the reaction:

$$\text{Thd} + \text{ATP} \rightarrow \text{TMP} + \text{ADP}$$

where Thd is deoxythymidine, ATP is adenosine 5'-triphosphate, TMP is deoxythymidine 5'-phosphate and ADP is adenosine 5'-diphosphate. HSV-TK, for example, catalyses the phosphorylation of deoxythymidine.

[0080] ThiK of use in the invention may be derived from any appropriate organism. However,

by way of example, ThiK may be from Herpes Simplex Virus 1 or Herpes Simplex Virus 2 (HSV-TK), VZV, CMV, HHV7, HHV7, HHV8, EBV Alternatively, functionally equivalent variants of ThiK from HSV-TK could be used.

[0081] By way of example only, ThiK proteins include those described in public databases such as GenBank as follows: AB009254.2. Functionally equivalent variants of this exemplary protein may also be of use.

[0082] In one embodiment, the ThiK comprises the amino acid sequence of SEQ ID No.54.

[0083] The invention also relates to nucleic acids encoding a ThiK. Skilled persons will readily appreciate the appropriate nucleotide sequence of nucleic acids encoding ThiK, having regard to the amino acid sequence of the exemplary ThiK proteins provided herein, and the genetic code. However, by way of example, exemplary nucleic acids encoding ThiK are provided in public databases such as GenBank AB009254.2, JQ895546.1, AY575235.1, AF243479.1, AY575236.2, HQ123159.1

[0084] However, by way of example, the nucleic acids may have a nucleotide sequence of SEQ ID 19 or SEQ ID 22 as described herein.

[0085] In one embodiment, the nucleic acid encoding ThiK has the nucleotide sequence of SEQ ID No. 19.

[0086] Nucleic acids encoding a ThiK in accordance with the invention can be generated using any number of known methods in the art, based on the information herein, the amino acid sequence (and/or the sequence of nucleic acids encoding the amino acid sequence) of exemplary ThiK proteins, and the genetic code, for example. However, by way of example, they may be produced by chemical synthesis or via standard recombinant techinques.

[0087] It should be appreciated that a nucleic acid encoding ThiK can be codon optimised for the particular *Clostridium* spp in which it is to be expressed. This can be achieved using standard codon optimisation techniques.

Nucleic Acid Vectors

[0088] The invention also provides a nucleic acid vector comprising a nucleic acid which encodes ThiK and/or an altered PheS in accordance with the invention. The vector may be of any original or nature, as will be understood by persons skilled in the art to which the invention relates, including for example those suitable for cloning and expression and transformation.

[0089] In one embodiment, the nucleic acid vector is one suitable for generating a recombinant microorganism of the invention. In this embodiment, the nucleic acid vector is a plasmid which comprises at least a nucleic acid encoding an altered PheS and/or a ThiK as described herein

before. In one particular embodiment, the vector further comprises at least:

1. (a) at least one nucleic acid sequence encoding at least one positive selection marker;
2. (b) two nucleic acid sequences homologous to selected regions around a target location or nucleic acid sequence within the genome of a parental microorganism, which allow for the recombination of the plasmid with the genome of the parental microorganism.

[0090] In one embodiment, the vector further comprises at least one nucleic acid of interest which is desired to be inserted or integrated into the genome of a parental microorganism.

[0091] In one embodiment, the nucleic acid encoding the positive selection marker is positioned on the plasmid vector outside of the homology arms. In another embodiment, the nucleic acid encoding the positive selection marker is located between the homology arms.

[0092] Where the vector is to be used for producing a recombinant microorganism in accordance with the invention it will be adapted in use to allow for the expression of the nucleic acids encoding the one or more selection marker. Accordingly, it will also include at least one promoter able to drive expression of the selection markers contained in the plasmid. The at least one promoter may comprise a part of the at least one nucleic acid sequence encoding at least one counter-selection marker or a part of the at least one nucleic acid sequence encoding at least one positive selection marker, or it may be a separate nucleic acid contained within the plasmid, which is separated from the nucleic acid(s) encoding the one or more selection markers by intervening nucleotides. In one embodiment, the promoter may be inducible. In another embodiment the promoter is constitutive.

[0093] Skilled persons will readily appreciate promoters of use in a plasmid of the invention. However, by way of example, these may include the Ppta-ack promoter (described herein in the Examples section), the lac promoter, ara, tet, or T7 system.

[0094] In one particular embodiment, the plasmid includes a strong promoter which is able to drive expression of the selection marker(s). In one embodiment, the plasmid includes a strong promoter to drive expression of at least a nucleic acid encoding a counter-selection marker. This is particularly the case where an altered PheS is used for counter-selection and the host genome includes a nucleic acid encoding PheS. Ideally the strong promoter will be sufficient to drive expression of the altered PheS at at least the same level, and preferably at an increased level, compared to expression of a nucleic acid encoding PheS which is present in the host genome. Alternatively, one or more other regulatory element, such as an operator and/or enhancer, could be included on the plasmid in addition to a promoter, to increase expression of one or more selection marker. Examples of strong promoters of use in the invention include, for example, T3 promoter, T7 promoter, PrRNA promoter, Ptrc promoter, or those exemplified in the Examples section hereinafter.

[0095] The at least one positive selection marker may be chosen from any number of known

positive selection markers which will be readily appreciated by persons skilled in the area of technology to which the invention relates. However, by way of example, CatP (chloramphenicol acetyltransferase), ErmB or TetA could be used [Heap et al., 2009 (J Microbial Methods; Jul; 78(1); 78-85). Skilled persons will readily appreciate the nucleotide sequence for nucleic acids encoding these positive selection markers, based on published information, and the genetic code. However, by way of example, GenBank: WP_002570989 (CatP), YP_007078965 (ErmB), NP_957551.1 (TetA); CatP (SEQ ID 23); ErmB (SEQ ID 24); TetA (SEQ ID 25).

[0096] The homology arms allow for homologus recombination of the vector with the host genome. While it may be preferred that the arms have 100% complementarity to the region in the genome which they are targeted to, this is not necessary, provided that the sequence is sufficiently complementary to allow for targeted recombination with the genetic region of interest. Typically, the arms will have a level of homology which would allow for hybridisation to a target region under stringent conditions, as defined in Sambrook et al (Molecular Cloning: A laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989). As will be appreciated by persons of skill in the art, the homology arms may be designed to hydridise to nucleic acid sequences within the genome which are adjacent to each other or separated from each other by one or more nucleotides.

[0097] Skilled persons will be able to readily design homology arms sufficient to allow for targeted homologous recombination having regard to publicly available sequence information for a given parental microorganism. Exemplary information is provided in the Examples section herein after.

[0098] A plasmid may also comprise one or more additional elements including one or more regulatory elements, one or more origin of replication, one or more multicloning site, among other elements. In one particular embodiment, the plasmids are adapted to allow for the disruption of a gene native to (or at least already present in) a parental microorganism, for example. In another embodiment, the plasmids are adapted to allow for integration and expression of one or more genes encoded by the plasmid. The plasmids may be in the form of naked nucleic acids as well as nucleic acids formulated with one or more agents to facilitate delivery to a cell (for example, liposome-conjugated nucleic acid, an organism in which the nucleic acid is contained).

[0099] As described herein before, phenylalanine tRNA synthetase is made up of two subunits, of which one is PheS. The second subunit may be present in the genome of a parental microorganism to be transformed. Accordingly, in the presence of a vector of the invention, a microorganism is able to produce phenylalanine tRNA synthetase, albeit one which is altered and able to aminoacylate tRNA using phenylalanine analogues. In addition, while the altered PheS may be based on PheS from any organism, as described previously herein, in a preferred embodiment it should be one which is compatible with the other subunit expressed by the parental microorganism. If the subunits are not compatible they will not form a functional enzyme. Skilled persons will readily be able to identify whether or not phenylalanine tRNA synthetase subunits are compatible using standard assays for testing the activity and function

of the enzyme - as are described herein before. However, the inventors contemplate that a PheS from any *Clostridium* spp. will be compatible with a PheT subunit from any other *Clostridium* spp. In one particular embodiment, the PheS subunit is from the same species of Clostridia as the parental microorganism. In one particular embodiment, the altered PheS is based on the PheS of the phenylalanine tRNA synthetase expressed by the parental microorganism to be transformed. In another embodiment, the plasmid of the invention may also include a nucleic acid encoding a PheT subunit, which together with the altered PheS, forms an active phenylalanine tRNA synthetase, albeit an altered one able to aminoacylate tRNA using phenylalanine analogues. This may be useful, for example, where the parental microorganism does not contain a nucleic acid encoding the PheT subunit.

[0100] A plasmid may be replicating or non-replicating.

[0101] Nucleic acid vectors of use in the invention may be constructed using any number of techniques standard in the art. For example, chemical synthesis or recombinant techniques may be used. Such techniques are described, for example, in Sambrook et al (Molecular Cloning: A laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989). Further exemplary techniques are described in the Examples section herein after. Essentially, the individual nucleic acids, including a nucleic acid encoding a counter-selection marker, nucleic acid encoding a positive selection marker, homology arms, nucleic acid of interest, and optionally other nucleic acids will be operably linked to one another so that they can perform their desired function.

[0102] Any one of a number of plasmid vectors known in the art may be suitable for use in the invention. However, by way of example, vectors from PMTL80000 series would be suitable. Specific examples are provided in the Examples section herein after.

[0103] In certain embodiments of the invention, a nucleic acid vector is one suitable for generating or cloning a nucleic acid encoding a ThiK or an altered PheS of the invention. In this case, the vector need not be adapted to express the ThiK or an altered PheS. Any number of known nucleic acid vectors may be used, including plasmids and viral vectors. Such vectors may include one or more regulatory elements, an origin of replication, a multicloning site and/or a selectable marker, among other elements, sites and markers, as will be known to persons skilled in the art.

Cells

[0104] The invention also provides a cell comprising a nucleic acid of the invention, a vector, a PheS and/orThiK according to the the invention. The cell may be of any origin and may include those of use in cloning or preparing a vector in accordance with the invention. In one embodiment, the cell is *E.coli* or a *Clostridium* spp.

Methods

[0105] As described herein before, the invention provides a method for the production of a recombinant microorganism from a parental microorganism. The method generally comprises at least the steps of: transforming a parental microorganism with a plasmid as described herein before, selecting one or more microorganisms that express at least the one positive selection marker and selecting one or more microorganisms which do not express the at least one counter-selection marker.

[0106] A parental microorganism may be transformed with a plasmid of the invention using any number of techniques known in the art for producing recombinant microorganisms. By way of example only, transformation (including transduction or transfection) may be achieved by electroporation, electrofusion, ultrasonication, polyethylene glycol-mediated transformation, chemical or natural competence, protoplast transformation, prophage induction or conjugation. Suitable transformation techniques are described for example in, Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A laboratory Manual, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, 1989.

[0107] By way of example only, electroporation has been described for several carboxydotrophic acetogens such as *C. ljungdahlii* (Köpke et al. 2010, Poc. Nat. Acad. Sci. U.S.A. 107: 13087-92; Leang et al., 2012, Appl. Environ. Microbiol.; PCT/NZ2011/000203; WO2012/053905), and *C. autoethanogenum* (PCT/NZ2011/000203; WO2012/053905) and is a standard method used in many Clostridia such as *C. acetobutylicum* (Mermelstein et al., 1992, Biotechnology, 10, 190-195), *C. cellulolyticum* (Jennert et al., 2000, Microbiology, 146: 3071-3080) or *C. thermocellum* (Tyurin et al., 2004, Appl. Environ. Microbiol. 70: 883-890).

[0108] By way of further example, electrofusion has been described for acetogenic *Clostridium* sp. MT351 (Tyurin and Kiriukhin, 2012, J Biotech: 1-12).

[0109] A further exemplary technique includes the prophage induction described for carboxydotrophic acetogen such as *C. scatologenes* (Prasanna Tamarapu Parthasarathy, 2010, Development of a Genetic Modification System in Clostridium scatologenes ATCC 25775 for Generation of Mutants, Masters Project Western Kentucky University).

[0110] By way of further example, the conjugation methods of Herbert et al., 2003, (FEMS Microbiol. Lett. 229: 103-110) and Williams et al., 1990 (J. Gen. Microbiol. 136: 819-826) may be used.

[0111] It should be appreciated that the plasmid may be delivered to a parental microorganism as naked nucleic acid or may be formulated with one or more agents to facilitate the tranformation process (for example, liposome-conjugated nucleic acid, an organism in which the nucleic acid is contained).

[0112] In certain embodiments, due to the restriction systems which are active in the

microorganism to be transformed, it is necessary to methylate any nucleic acid (for example a plasmid of the invention) to be introduced into the microorganism. This can be done using a variety of techniques, including those described below.

[0113] By way of example, in one embodiment, a recombinant microorganism of the invention is produced by a method comprises the following steps:

- introduction into a shuttle microorganism of (i) at least one plasmid to be introduced to the parental microorganism as described herein and (ii) a methylation construct/vector comprising a methyltransferase gene;
- expression of the methyltransferase gene;
- isolation of the at least one plasmid from the shuttle microorganism; and,
- introduction of the at least one plasmid into a destination microorganism.

[0114] In one embodiment, the methyltransferase gene is expressed consitutively. In another embodiment, expression of the methyltransferase gene is induced.

[0115] The shuttle microorganism is a microorganism, preferably a restriction negative microorganism, that facilitates the methylation of the nucleic acid sequences that make up a plasmid of the invention. In a particular embodiment, the shuttle microorganism is a restriction negative E. *coli, Bacillus subtillis, or Lactococcus lactis.*

[0116] The methylation construct/vector comprises a nucleic acid sequence encoding a methyltrans feras e.

[0117] Once the one or more plasmid and the methylation construct/vector are introduced into the shuttle microorganism, the methyltransferase gene present on the methylation construct/vector is induced. Induction may be by any suitable promoter system although in one particular embodiment of the invention, the methylation construct/vector comprises an inducible lac promoter and is induced by addition of lactose or an analogue thereof, more preferably isopropyl-β-D-thio-galactoside (IPTG). Other suitable promoters include the ara, tet, or T7 system. In a further embodiment of the invention, the methylation construct/vector promoter is a constitutive promoter.

[0118] In a particular embodiment, the methylation construct/vector has an origin of replication specific to the identity of the shuttle microorganism so that any genes present on the methylation construct/vector are expressed in the shuttle microorganism.

[0119] Expression of the methyltransferase enzyme results in methylation of the genes present on the one or more plasmid to be introduced to a parental microorganism. The plasmid may then be isolated from the shuttle microorganism according to any one of a number of known methods. For example, commercially available kits such as Qiagen or Zymo may be used according to the manufacturer's instructions.

[0120] In one particular embodiment, both the methylation construct/vector and the one or more plasmid of the invention are concurrently isolated.

[0121] The one or more plasmid destined for the parental microorganism may be introduced into the microorganism using any number of known methods. However, by way of example, the methodology described hereinbefore, or in the Examples section hereinafter may be used.

[0122] It is envisaged that a methyltransferase gene may be introduced into a shuttle microorganism and over-expressed. Thus, in one embodiment, the resulting methyltransferase enzyme may be collected using known methods and used in vitro to methylate one or more plasmid to be introduced into the parental microorganism. The one or more plasmid may then be introduced into the destination (parental) microorganism. In another embodiment, the methyltransferase gene is introduced into the genome of the shuttle microorganism followed by introduction of the one or more plasmid destined for the parental microorganism into the shuttle microorganism, isolation of the one or more plasid from the shuttle microorganism and then introduction of the one or more plasmid into the destination (parental) microorganism.

[0123] It is envisaged that the one or more plasmid destined for the parental microorganism and the methylation construct/vector as defined above may be combined to provide a composition of matter. Such a composition has particular utility in circumventing restriction barrier mechanisms to produce the recombinant microorganisms of the invention.

[0124] In one particular embodiment, the methylation construct/vector is a plasmid.

[0125] Skilled persons will appreciate a number of suitable methyltransferases of use in producing microorganisms in accordance with the invention. However, by way of example the Bacillus subtilis phage ΦT1 methyltransferase and the methyltransferase described in WO2012053905 may be used. Nucleic acids encoding suitable methyltransferases will be readily appreciated having regard to the sequence of the desired methyltransferase and the genetic code.

[0126] Any number of constructs/vectors adapted to allow expression of a methyltransferase gene may be used to generate the methylation construct/vector. However, by way of example those mentioned in WO2012053905 may be used.

[0127] Once a plasmid has been introduced into a desired parental microorganism, a first selection occurs. This involves selecting one or more microorganisms that express at least the one positive selection marker.

[0128] Such microorganisms may be identified and selected using any number of known techniques, having regard to the positive selection marker being used. However, by way of general example, the microorganisms may be cultured in or on a media which contains a toxin which would kill any microorganisms which do not express the positive selection marker. By

way of specific example, the microorganisms may be grown in the presence of a toxic antibiotic, with the plasmid of the invention including a nucleic acid encoding a product conferring antibiotic resistance to the microorganism. Those microorganisms in which the plasmid is present will survive and those that do not will die.

[0129] Further examples of methodology and conditions of use in selecting microriganisms expressing a positive selection marker are described, for example, in Sambrook *et al*, 1989 (as previously described herein). Additional examples are provided in the Examples section herein after.

[0130] The methods of the invention also include a second selection. This involves selecting one or more microorganism that does not express at least one counter selection marker.

[0131] In the case of use of ThiK as a counter-selection marker, selection of one or more microorganisms which do not express this counter-selection marker involves culturing the microorganisms in or on a media containing a guanosine analgoue. In one particular embodiment, the guanosine analogue is ganciclovir. Those microorganisms which contain and express a nucleic acid encoding the ThiK counter-selection marker will not survive in the presence of the guanosine analogue. Accordingly, those microorganisms which survive are selected as having undergone the desired double-crossover recombination event.

[0132] In the case of use of an altered PheS as a counter-selection marker, selection of one or more microorganisms which do not express this counter-selection marker involves culturing the microorganisms in or on a media containing a phenylalanine analogue. In one particular embodiment, the phenylalanine analogue is as herein before exemplified. Those microorganisms which contain and express a nucleic acid encoding the altered PheS counter-selection marker will not survive in the presence of the phenylalanine analogue. Accordingly, those microorganisms which survive are selected as having undergone the desired double-crossover recombination event.

[0133] When using an altered PheS as a counter selection marker, it may be necessary to also include phenylalanine in the media.

[0134] The methods of the invention include both simultaneous and consecutive selection steps. For example, one could select microorganisms for single crossover events using the positive selection maker and subsequently select microorganisms for double crossover events using the counter-selection marker. Alternatively, the positive and counter-selection can occur simultaneously. By way of example, where the nucleic acid encoding the positive selection marker is positioned on the plasmid vector outside of the homology arms one may consecutively select for single-crossover events, then counter-select, selecting for the double-crossover events. By way of further example, where the nucleic acid encoding the positive selection marker is located between the homology arms (and is therefor integrated into the gemone of the parental microorganism), positive selection and counter-selection may occur simultaneously; any cell which has the positive selection marker integrated into the genome

and is resistant to the counter-selection marker, will have had a double crossover event occur.

[0135] Any media suitable for the culturing of one or more microorganisms may be used in a method of the invention. Skilled persons will readily appreciate appropriate media based on published information and having regard to the nature of the invention and the parental microorganisms described herein. Preferably, the media will be a media in which little to no phenylalanine is present, or at least a level of phenylalanine which does not out-compete the phenylalanine analgoues during counter-selection. By way of example, any appropriate minimal medium would be suitable, such as: Clostiridia Minimal Medium, Minimal defined medium (MDM), supplemented defined medium (SDM) and complete defined medium (CDM). Specific examples are provided herein after in the Examples section.

[0136] Once one or more microorganism is selected in accordance with the invention, it may be cultured and optionally stored for future use using known methodology.

[0137] The invention will now be described, by way of example only, with reference to the following Examples.

EXAMPLES

[0138] The following examples describe construction of plasmids for counterselectable markers HSV-Tk and PheS*, functionality HSV-Tk and PheS* for of counterselection in *Clostridium autoethanogenum,* and use of HSV-Tk and PheS* to facilitate homologous recombination gene replacement on the genome of *Clostridium autoethanogenum.* The same principle can also be applied to other memebrs of the *Clostridium* family, as the no homologue of the HSV-Tk gene exists in any sequenced Clostridium and the pheS genes or Clostridia species are highly conserved.

[0139] Standard Recombinant DNA and molecular cloning techniques were used in this invention and are described by Sambrook *et al*, 1989 and Ausubel *et al*, 1987. *E. coli* strain TOP10 (Life Technologies) and Clostridium autoethanogenum DSM10061 and DSM23693 (a derivate of DSM10061) were used. E. coli were grown in LB and SOB medium as described by Sambrook *et al*, 1989 and Ausubel *et al*, 1987, while *Clostridium autoethanogenum* was grown in anaerobic PETC medium (Table 1).

Table 1: PETC media (ATCC media 1754; atcc.org/Attachments/2940.pdf)

Media component	**Concentration per 1.0L of media**
NH_4Cl	1 g
KCl	0.1 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.8 g
KH_2PO_4	0.1 g

Media component	**Concentration per 1.0L of media**
$CaCl_2$	0.02 g
Trace metal solution	10 ml
Wolfe's vitamin solution	10 ml
Yeast Extract	1 g
Resazurin (2 g/L stock)	0.5 ml
MES	2g
Reducing agent	0.006-0.008 % (v/v)
Distilled water	Up to 1 L, pH 5.5 (adjusted with HCl)
Wolfe's vitamin solution	**per L of Stock**
Biotin	2 mg
Folic acid	2 mg
Pyridoxine hydrochloride	10 mg
Thiamine.HCl	5 mg
Riboflavin	5 mg
Nicotinic acid	5 mg
Calcium D-(+)-pantothenate	5 mg
Vitamin B_{12}	0.1 mg
p-Aminobenzoic acid	5 mg
Thioctic acid	5 mg
Distilled water	To 1 L
Trace metal solution	**per L of stock**
Nitrilotriacetic Acid	2 g
$MnSO_4.H_2O$	1 g
$Fe\ (SO_4)_2(NH_4)_2.6H_2O$	0.8 g
$COCl_2.6H_2O$	0.2 g
$ZnSO_4.7H_2O$	0.2 mg
$CuCl_2.2H_2O$	0.02 g
$NaMoO_4.2H_2O$	0.02 g
Na_2SeO_3	0.02 g
$NiCl_2.6H_2O$	0.02 g
$Na_2WO_4.2H_2O$	0.02 g
Distilled water	To 1 L
Reducing agent stock	per 100 mL of stock

Trace metal solution	per L of stock
NaOH	0.9 g
Cystein.HCl	4g
Na2S	4g
Distilled water	To 100 mL

Example 1: Functionality of HSV-Tk and PheS* for of counterselection in *Clostridium autoethanogenum*

Construction of plasmids:

[0140] *Construction of plasmids containing Hsv-tk:* DNA sequence of Human Herpes Virus thymidine kinase (*Hsv-tk*) was obtained from NCBI (Nucleic acid and amino acid). The codons in *Hsv-tk* gene were optimized to suit *C. autoethanogenum* and synthesized by GeneArt and delivered in their standard vector pMK-RQ (Seq. ID. 1 - pMK-RQ-HSV-tk)).

[0141] *HSV-tk* was released from pMK-RQ vector by digesting with Nde1 and Nhe1 restriction enzymes (New England Biolabs) and cloned into a modified version of the *E. coli-Clostridium* shuttle vector pMTL83151 (FJ797651.1; Nigel Minton, University of Nottingham; Heap *et al.,* 2009) which is referred to as pMTL83155 (SEQ ID 3), between the same sites in *E. coli* strain TOP10 (Life Technologies) to create pMTL83155-Hsv-tk (Seq. ID. 2, Figure 1). The pMTL83155 plasmid contains the promoter sequence of *C. autoethanogenum* phosphate acetyl transferase gene between Not1 and Nde1 sites (Seq. ID. 3).

[0142] *Construction of mutated pheS*:* The native *pheS* was identified in the genome from the sequence of *C. autoethanogenum* DSM 10061 (Seq.ID.12). By comparing the sequence of *E. coli* MG1655 (Seq. ID. 13) and *C. autoethanogenum*'s *pheS* by sequence alignment, the putative substrate specificity site was identified based upon homology of the amino acid sequence of amino acids between G284 and G298 of *E. coli* (amino acid sequence of PheS from E. coli MG1655 is SEQ ID 20) and amino acids G301 and G315 of *C. autoethanogenum* (Figure 3). A single point mutation was introduced at base 932, substituting C for G, resulting in the codon encoding glycine instead of alanine (Seq. ID. 14).

[0143] *Construction of plasmids containing pheS*:* The modified *pheS** was transcriptionally coupled to a synthetic promoter and RBS site (PpheS*; Seq.ID. 17) upstream of the start codon to allow high constitutive expression. The construct was also flanked by *Pme*I restriction sites to enable cloning of the gene into alternative vectors. Synthesis and subcloning into vector pMTL85151 utilizing restriction enzyme *Pme*I was carried out by GeneArt resulting in the final vector pMTL85151-pheS* (Seq. ID. 18; Figure 4).

Sensitivity testing:

[0144] *Toxicity testing of DL-4-chlorophenylanine:* *E. coli* Top10 and *C. autoethanogenum* DSM23693 harbouring the empty vector pMTL85151 were grown initially in the presence of DL-4-chlorophenylanine on plates and in liquid media to ascertain if the counter-selection marker has any effect on growth of the organisms. It was noted that the chemical did not impede growth of either organism in liquid media or on plates and colonies grew to the same size after 24/48 hours for *E. coli* and *C. autoethanogenum,* respectively.

[0145] *Testing pheS* in E. coli:* To test the ability of the counter selection marker to work in *E. coli,* the plasmid pMTL85151-pheS* was transformed into TOP10 and grown under chloramphenicol selection only. Once the culture had reached an OD of 0.5, representing an exponential growth phase, 100 ul was plated onto LB plates containing chloramphenicol and DL-4-chlorophenylalanine as well as chloramphenicol alone as a control, in triplicate, and incubated for 24 hours at 37°C. After 24 hours the plates were inspected and noted that on the chloramphenicol plates alone, there was a lawn of large colonies as expected, however, on the plates containing both chloramphenicol and DL-4-chlorophenylalanine, there was a light shading of small colonies suggesting that DL-4-chlorophenylanine was affecting the growth of the *E. coli* harbouring pMTL85151-pheS*. After 36 hours, the double selection plates had outgrown to the same level as the chloramphenicol alone plates.

Transformation of C. autoethanogenum and confirmation of plasmids:

[0146] *Transformation of C.autoethanogenum:* The pMTL83155, pMTL83155-Hsv-tk and pMTL85155-PheS plasmids were introduced into *C. autoethanogenum* DSM23693 (a derivate of DSM10061) as described in WO2012053905. Outgrowth was performed in PETC broth and spread on PETC- agar media supplemented with 15 (µg/ml thiamphenicol (Sigma) and 10 (µg/ml trimethoprim (Sigma). Colonies were observed after 3 days of incubation at 37° C in pressurized gas jars with 20 psi of a gas mix of 48%CO, 2% H2, 20% CO2, 30% N2. Streaks of single colonies were made on PETC-agar media containing 15 (µg/ml thiamphenicol.

[0147] *Screening of transformants for the presence of plasmids:* 2 colonies from LZ-pMTL83155 and LZ-pMTL83155-hsv-tk transconjugantswere randomly screened for the presence of pMTL83155 and pMTL83155-Hsv-tk plasmidsby PCR using primers repHf (Seq. ID. 4) and catr (Seq. ID. 5) spanning the Gram-positive replicon and *catP* positive selection marker in pMTL83155 and pMTL83155-Hsv-tk. Unmodified *C. autoethanogenum* was used as a control in these PCRs. The Maxime PCR PreMix Kit was used for PCR. 16s rDNA was also PCR amplified from these transformants using primers fD1 (Seq. ID. 6) and rP2 (Seq. ID.7) and Maxime PCR PreMix Kit.

[0148] PCR with repHF and catR primers amplified ~1.5 kb bands from LZ-pMTL83155-1 and

- 2 and LZ-pMTL83155-hsv-tk-1 and -2 (Figure 2). No amplification was detected from unmodified *C. autoethanogenum* sample, confirming the presence of plasmids only in the transformants. The sequencing of 16s rRNA from LZ-pMTL83155-1 (Seq. ID. 8) and -2 (Seq. ID. 9) and LZ-pMTL83155-hsv-tk-1 (Seq. ID. 10) and -2 (Seq. ID. 11) further confirmed the clones to be *C. autoethanogenum*.

[0149] Confirmation of *C. autoethanogenum* transformants harbouring plasmid pMTL85151-pheS* was carried out on three independent colonies using PCR primers specific to the plasmid (Seq. ID. 15 and 16), and primers fD1 (Seq. ID. 6) and rP2 (Seq. ID.7) to sequence the 16s rRNA.

Functionality of HSV-Tk and PheS* as counterselectable markers in Clostridium autoethanogenum:

[0150] *Sensitivity of C. autoethanogenum transformants to ganciclovir:* The sensitivity of LZ-pMTL83155 and LZ-pMTL83155-hsv-tk to ganciclovir was tested by plating them on PETC agar media containing 20nM ganciclovir only and PETC agar media containing 20nM ganciclovir and 15µg/ml thiamphenicol. Colonies on ganciclovir plates were observed only with LZ-pMTL83155 transformants and not with LZ-pMTL83155-hvs-tk (Table 2). The presence of *Hvs-tk* gene confers toxicity to ganciclovir.

[0151] *Sensitivity of C. autoethanogenum harbouring plasmid pMTL85151-pheS* to DL-4-chlorophenylanine:* The three independent transformants of *C. atoethanogenum* DSM23693 harbouring pMTL85151-pheS*, as well as *C. autoethanogenum* DSM23693 harbouring pMTL85151, were grown in liquid PECT media supplemented with thiamphenicol and grown at 37°C under CO only conditions. After 24 hours, 100 ul of each of the three independent, as well as the control pMTL85151, were plated into PETC-MES agar supplemented with either thiamphenicol alone, or thiamphenicol and DL-4-chlorophenylalanine and incubated for 48 hours. After 48 hours, the plates were inspected and the plates containing only thiamphenicol had a lawn of colonies for all 4 strains, whereas, the plates containing double selection only had 3, 4, and 7, colonies for each of the independent transformants containing pheS*, in contrast, the *C. autoethanogenum* transformants harbouring pMTL85151 showed the same results as the thiamphenicol only plate, suggesting DL-4-chlorophenyalanine has no effect upon this strain.

Table 2: Sensitivity of *C. autoethanogenum* transconjugants to different prodrugs in the presence of corresponding CSM

CSM	Prodrug	Concentration	LZ-pMTL83155	LZ-pMTL83155-hsv-tk
Hsv-tk	Ganciclovir	20 nM	Lawn	0-5
PheS	DL-4-Chlorophenlanine	0.20%	Lawn	7-Mar

This demonstrateds that both HSV-T and PheS* in combination with prodrugs Ganciclovir and

DL - 4-Chlorophenlanine are effective for counterselection in *C. autoethanogenum*

Example 2 - Use of PheS* to facilitate homologous recombination gene replacement on the genome of *Clostridium autoethanogenum*

[0152] This example describes replacing a native Clostridium autoethanogenum R-specific 2,3-butanediol dehydrogenase gene with S-specific 2,3-butanediol dehydrogenase gene from Klebsiella pneumoniae through homologous recombination facilitated by PheS* as a counter selectable marker in Clostridium autoethanogenum DSM23693 that has an inactivated secondary alcohol dehydrogenase.

Construction of C. autoethanogenum DSM23693 strain that has an inactivated secondary alcohol dehydrogenase

[0153] A strain of *Clostridium autoethanogenum* DSM23693 was constructed that has an inactivated secondary alcohol dehydrogenase (SEQ ID NO: 55) using the ClosTron System. (Heap et al 2007). The intron design tool hosted on the ClosTron.com website was used to design a 344 bp targeting region (SEQ ID NO: 56), as well as identify six target sites (Fig. 8) on the sense and antisense strands. The targeting region was chemically synthesised in the vector pMTL007C-E2 containing a Retro-transposition Activated ermB marker (RAM).

[0154] The vectors were introduced into *C.autoethanogenum* DSM23693 as described in WO2012/053905. Single colonies grown on PETC MES with 15 µg/ml thiamphenicol were streaked on PETC MES with 5 µg/ml clarothromycin. Colonies from each target were randomly picked, and screened for the insertion using flanking primers 155F (SEQ ID NO: 57), and 939R (SEQ ID NO: 58). Amplification was performed using the iNtron Maxime PCR premix. A PCR product of 783 bp indicated a wild-type genotype, while a product size of approximately 2.6 kb suggests the insertion of the group II intron in the target site (Fig. 9). The loss plasmid was checked by amplification of the resistance marker (*catP*), and the gram positive origin of replication (pCB102). This strain was used as base strain replacing a native Clostridium autoethanogenum R-specific 2,3-butanediol dehydrogenase gene with S-specific 2,3-butanediol dehydrogenase gene from Klebsiella pneumoniae through homologous recombination facilitated by PheS*.

[0155] *Construction of plasmid pPheS-ErmB.* The fragment containing PheS* cassette (Seq. ID 27) and ColE1 with traJ (Seq ID No. 26) was amplified from pMTL85151-PheS* (Seq ID No. 18) with primers PheS-repH-F (SEQ ID No. 28) and traJ-ermB-R (SEQ ID No. 29). The fragment containing pCB102 origin of replication (Seq ID No. 30) was amplified from the pMTL80000 series (Heap et al., 2009) using primers RepH-ermB-F (SEQ ID No. 31) and RepH-pheS-R (SEQ ID No. 32). The erythromycin resistance cassette (SEQ ID No. 33) was amplified from the pMTL80000 series (Heap et al., 2009) using primers ermB-traJ-F (SEQ ID

No. 34) and ermB-repH-R ((SEQ ID No. 35). The described PCR products contained overlaps to facilitate seamless assembly. They were assembled using the GENEART Seamless Cloning and Assembly kit from Life Technologies. The resulting plasmid, pPheS*-ErmB was verified by restriction digestion and fragment analysis.

[0156] *PCR amplification of plasmid parts for homologous recombination.* The vector backbone containing PheS* (SEQ ID No. 27) was amplified from pPheS*-ErmB (SEQ ID No. 36) using primers AM015 (SEQ ID No. 37) and AM035 (SEQ ID No. 38). The upstream homology arm (SEQ ID No. 39) was amplified from *C. autoethanogenum* genomic DNA using primers AM016 (SEQ ID No. 40) and AM017 (SEQ ID No. 41). The *K. pneumoniae* butanediol dehydrogenase gene (SEQ ID No. 42) was amplified, using primers AM018 (SEQ ID No. 43) and AM019 (SEQ ID No. 44), from pMTL85141-P-alsS-budA-budC (Kopke et. al. 2014). The chloramphenicol acetyltransferase expression cassette (SEQ ID No. 45) was amplified from the pMTL80000 series (Heap et al., 2009) using primers AM020 (SEQ ID No. 46) and AM021 (SEQ ID No. 47). The downstream homology arm (SEQ ID No. 48) was amplified from *C. autoethanogenum* genomic DNA using primers AM022 (SEQ ID No. 49) and AM036 (SEQ ID No. 50).

[0157] *Construction of pheS* containing plasmid for homologous recombination.* The PCR products above contained overlaps to facilitate seamless assembly. They were assembled using the GENEART Seamless Cloning and Assembly kit from Life Technologies. The resulting plasmid, pPheS*-CaBDHXXKpBDH was verified by restriction digestion and fragment analysis.

[0158] *Introduction of plasmid, counter selection, and screening for integration.* The pPheS*-CaBDHXXKpBDH was introduced as described in WO2012053905 into a strain of *C. autoethenogenum* DSM23693 that has a ClosTron-inactivated secondary alcohol dehydrogenase at position 287 as described above. Transformants were selected by their ability to grow on PETC-agar medium supplemented with 15 µg/ml thiamphenicol (Sigma) and 10 µg/ml trimethoprim (Sigma). To select for successful homologous recombination double cross, colonies were restreaked on PETC-agar medium supplemented with 15 µg/ml thiamphenicol and 2 mg/ml p-chlorophenylalanine. Colonies which grew were screened for integration by PCR with primers AM041 (SEQ ID No. 51) and AM042 (SEQ ID No. 52) which flank the integration site outside the homology arms. Successful integration was identified as yielding a PCR product 3570 base pairs in length compared to 3137 base pairs for the wild type (Figure 7). The PCR product was sequenced for fidelity by Sanger sequencing and found to be exactly the expected insertion sequence, confirming successful integration of the fragment facilitated by the pheS* counterselectable marker.

Table 3 - Primers used in Example 2

Primer name	**Sequence**	**SEQ ID**
AM015	CTTGCCTTGCTCGTCGGT	37
AM016	GACGAGCAAGGCAAGCAATTATAGTGAAAGATGTGAAGG	40
AM017	ACCTTTTTCATAATTATCTCTCCTTTTTTATAATAGTATGG	41

Primer name	Sequence	SEQ ID
AM018	AGAGATAATTATGAAAAAGGTTGCATTAGTTAC	43
AM019	CCTTACAATTTAATTAAATACCATACCACCGTC	44
AM020	GTATTTAATTAAATTGTAAGGATCCTAGTCAG	46
AM021	GTACTTTTTATGAGCTCTTAACTATTTATCAATTC	47
AM022	AGAGCTCATAAAAAGTACTCATAGAATTGATTAAAAAATG	49
AM035	AAGTGATAGTCAAAAGGCATAACAGTG	38
AM036	ATGCCTTTTGACTATCACTTATACATCTCCTTTAAATCCATTTG	50
AM041	CTGGAAAAGAACTCTTAGC	51
AM042	TGCGGTGGAATACAATGG	52
PheS-repH-F	GCAAGTTGAAAAATTCACGAAAGTTACACGTTACTAAAGG	28
traJ-ermB-R	CACTATCAACACACTCTTAAGCTTGCCTTGCTCGTCGGTG	29
RepH-ermB-F	GCTTTTGT AAA TTTGCA T AAAAA T AAGAAGCCTGCA TTTG	31
RepH-pheS-R	TTTAGTAACGTGTAACTTTCGTGAATTTTTCAACTTGCC	32
ermB-traJ-F	CACCGACGAGCAAGGCAAGCTTAAGAGTGTGTTGATAGTG	34
ermB-repH-R	GCTTCTTATTTTTATGCAAATTTACAAAAGCGACTCATAG	35

[0159] The same strategy and plasmid can also be applied to *C. ljungdahlii* or *C. ragsdalei.* Transformation protocols have been described (WO2012/053905) (Leang, Ueki, Nevin, & Lovley, 2012).

[0160] The invention has been described herein, with reference to certain preferred embodiments, in order to enable the reader to practice the invention without undue experimentation. However, a person having ordinary skill in the art will readily recognise that many of the components and parameters may be varied or modified to a certain extent or substituted for known equivalents without departing from the scope of the invention. It should be appreciated that such modifications and equivalents are herein incorporated as if individually set forth.

[0161] Throughout this specification and any claims which follow, unless the context requires otherwise, the words "comprise", "comprising" and the like, are to be construed in an inclusive sense as opposed to an exclusive sense, that is to say, in the sense of "including, but not limited to".

REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- WO2009064200A [0049]
- US5593886A [0049]
- US6368819B [0049] [0049]
- WO2008028055A [0049] [0050] [0052] [0053] [0053]
- US20110229947A [0049] [0050] [0052]
- NZ2011000203W [0107] [0107]
- WO2012053905A [0107] [0107] [0125] [0126] [0146] [0154] [0158] [0159]

Non-patent literature cited in the description

- Production of Bioethanol from Synthesis Gas Using Clostridium ljungdahlii**TIRADO-ACEVEDO O.**PhD thesisNorth Carolina State University20100000 [0049]
- **KAST et al.**J. Mol. Biol, 1991, vol. 222, 99-124 [0059] [0059]
- **BROCKENBROUGH et al.**Nucl Med Biol., 2007, vol. 34, 6619-23 [0059]
- **JONSSONMCIVOR**Anal Biochem., 1991, vol. 199, 2232-7 [0059]
- **HEAP et al.**J Microbial Methods, 2009, vol. 78, 178-85 [0095]
- **SAMBROOK et al.**Molecular Cloning: A laboratory manualCold Spring Harbor Laboratory Press19890000 [0096] [0101]
- **SAMBROOK JFRITSCH EFMANIATIS T**Molecular Cloning: A laboratory ManualCold Spring Harbour Laboratory Press19890000 [0106]
- **KÖPKE et al.**Poc. Nat. Acad. Sci. U.S.A., 2010, vol. 107, 13087-92 [0107]
- **LEANG et al.**Appl. Environ. Microbiol., 2012, [0107]
- **MERMELSTEIN et al.**Biotechnology, 1992, vol. 10, 190-195 [0107]
- **JENNERT et al.**Microbiology, 2000, vol. 146, 3071-3080 [0107]
- **TYURIN et al.**Appl. Environ. Microbiol., 2004, vol. 70, 883-890 [0107]
- **TYURINKIRIUKHIN**J Biotech, 2012, 1-12 [0108]
- **HERBERT et al.**FEMS Microbiol. Lett., 2003, vol. 229, 103-110 [0110]

- **WILLIAMS et al.**J. Gen. Microbiol., 1990, vol. 136, 819-826 [0110]

Patentkrav

1. Modificeret phenylalanin-tRNA-syntetase (PheS), hvor den modificerede PheS har sekvensen ifølge SEQ ID No. 21, som omfatter mindst en ændring i aminosyrepositionerne 306 til 313.

2. Modificeret PheS ifølge krav 1, hvor den modificerede PheS aminoacylerer tRNA med en phenylalaninanalog, der er valgt fra gruppen bestående af chlorphenylalanin, fluorphenylalanin og bromphenylalanin.

3. Modificeret PheS ifølge krav 2, hvor phenylalaninanalogen er valgt fra gruppen bestående af DL-4-chlorphenylalanin, p-chlorphenylalanin, p-fluor-L-phenylalanin, p-fluor-DL-phenylalanin og p-brom-L-phenylalanin.

4. Modificeret PheS ifølge krav 1, hvor den modificerede PheS omfatter en ændring i aminosyrepositionen 311 i SEQ ID No. 21.

5. Modificeret PheS ifølge krav 4, hvor den modificerede PheS omfatter glycin i stedet for alanin i aminosyrepositionen 311 i SEQ ID No. 21.

6. Nukleinsyre, der koder for den modificerede PheS ifølge krav 1.

7. Fremgangsmåde til fremstilling af en rekombinant mikroorganisme, hvilken fremgangsmåde omfatter at indføre en vektor, som omfatter en nukleinsyre, der koder for den modificerede PheS ifølge krav 1, i en forældremikroorganisme.

8. Fremgangsmåde ifølge krav 7, hvor fremgangsmåden endvidere omfatter selektion i forhold til ekspression af den modificerede PheS.

9. Fremgangsmåde ifølge krav 8, hvor selektionen i forhold til ekspression af den modificerede PheS indebærer at dyrke den rekombinante mikroorganisme i nærvær af en phenylalaninanalog, der er valgt fra gruppen bestående af chlorphenylalanin, fluorphenylalanin og bromphenylalanin.

10. Fremgangsmåde ifølge krav 9, hvor phenylalaninanalogen er valgt fra gruppen bestående af DL-4-chlorphenylalanin, p-chlorphenylalanin, p-fluor-L-phenylalanin, p-fluor-DL-phenylalanin og p-brom-L-phenylalanin.

11. Fremgangsmåde ifølge krav 7, hvor vektoren endvidere omfatter homologiarme, der tillader homolog rekombination mellem vektoren og forældremikroorganismens genom, en nukleinsyre af interesse og/eller en positiv selektionsmarkør.

12. Fremgangsmåde ifølge krav 7, hvor fremgangsmåden endvidere omfatter selektion for ekspression af den positive selektionsmarkør, fortrinsvis hvor den positive selektionsmarkør er valgt fra gruppen bestående af CatP, ErmB og TetA.

13. Anvendelse af PheS ifølge krav 1 som en modselektionsmarkør.

14. Fremgangsmåde ifølge krav 9, hvor den modificerede PheS omfatter en ændring i aminosyrepositionen 311 i SEQ ID No. 21.

15. Fremgangsmåde ifølge krav 14, hvor den modificerede PheS omfatter glycin i stedet for alanin i aminosyrepositionen 311 i SEQ ID No. 21.

DRAWINGS

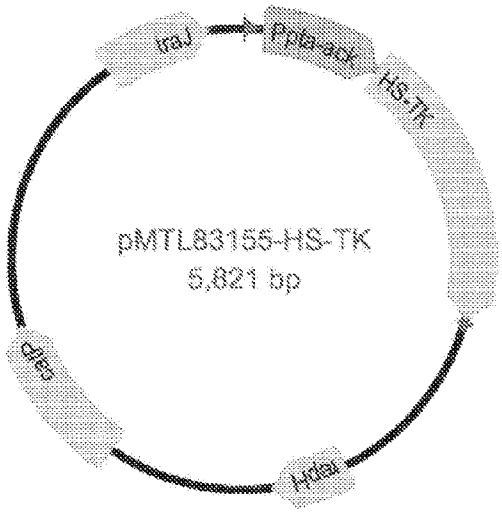

FIG. 1

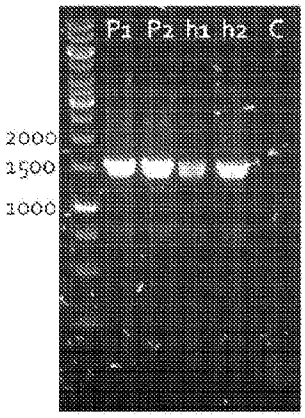

FIG. 2

FIG. 3

***E. coli* MG1655 (SEQ ID 20):**

MSHLAELVASAKAAISQASDVAALDNVRVEYLGKKGHLTLQMTTLRELPPEERPAAGAVIN
EAKEQVQQALNARKAELESAALNARLAAETIDVSLPGRRIENGGLHPVTRTIDRIESFFGELGF
TVATGPEIEDDYHNFDALNIPGHHPARADHDTFWFDTTRLLRTQTSGVQIRTMKAQQPPIRII
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EPSAEVDVMGKNGKWLEVLGCGMVHPNVLRNVGIDPEVYS GFAFG
MGMERLTMLRYGVTDLRSFFENDLRFLKQFK

***C. autoethanogenum* (SEQ ID 21):**

VKGEFKMKEELKQIKENAFNELKNKKLDIEDIRVKYLGKKGELTKILRGMKDLSKEERPAIG
KLANEVRSTLENAIEEASKKIKSSAIQAKLQNETIDITMPGIKQTVGKRHPLEQTLEEMKQIFIS
MGFTIEEGPEVEKDYYNFEALNIPKNHPARGEQDTFYINDNVVLRTQTSPIQVRTMEKQKPPI
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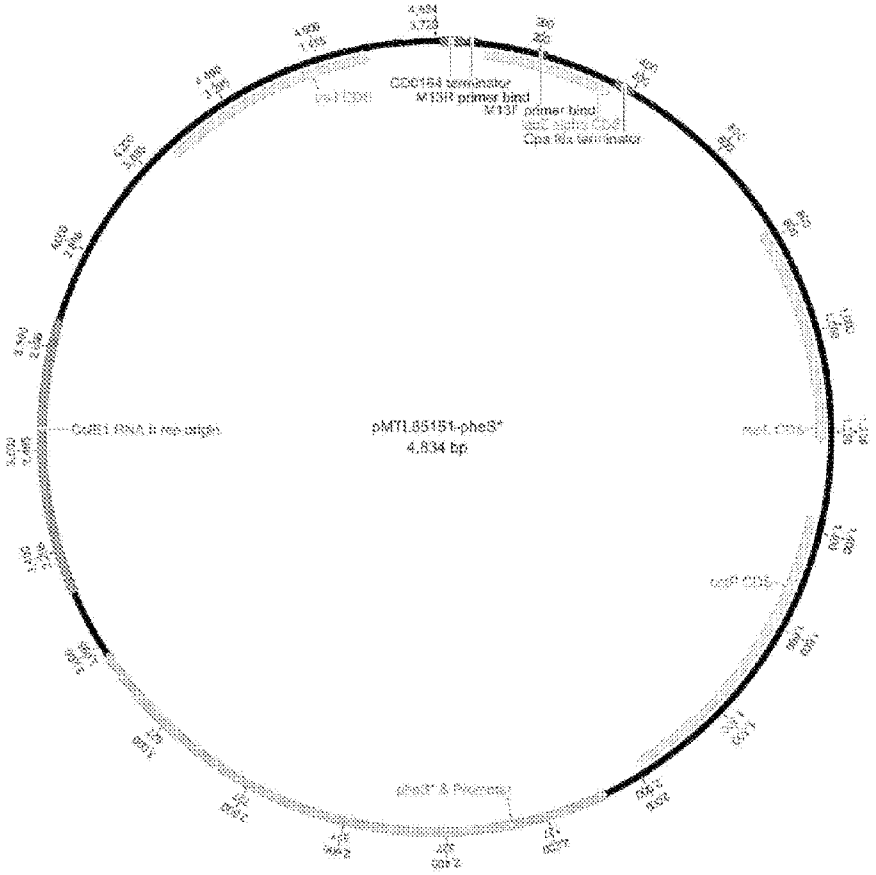

FIG. 4

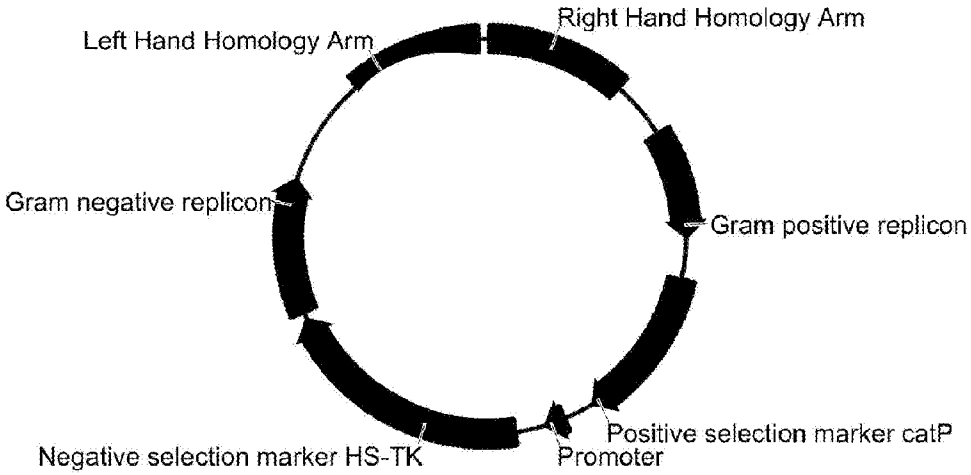
Right Hand Homology Arm
Left Hand Homology Arm
Gram negative replicon
Gram positive replicon
Positive selection marker catP
Promoter
Negative selection marker HS-TK

FIG. 5

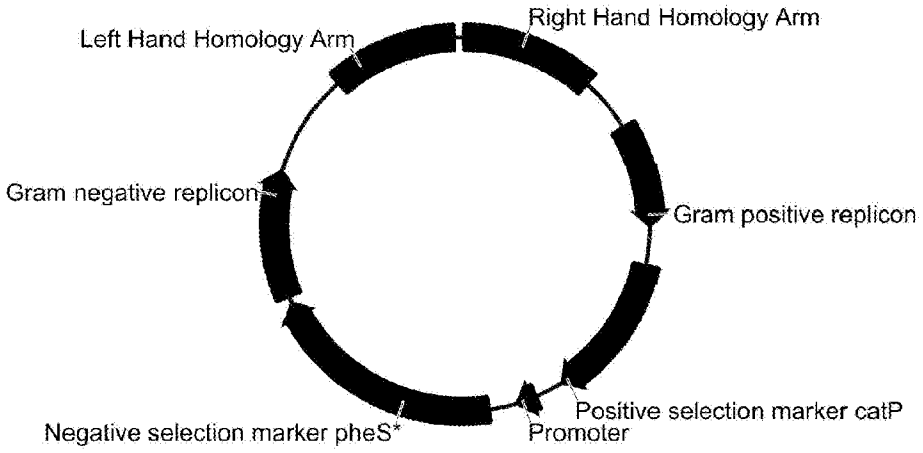
Right Hand Homology Arm
Left Hand Homology Arm
Gram negative replicon
Gram positive replicon
Positive selection marker catP
Promoter
Negative selection marker pheS*

FIG. 6

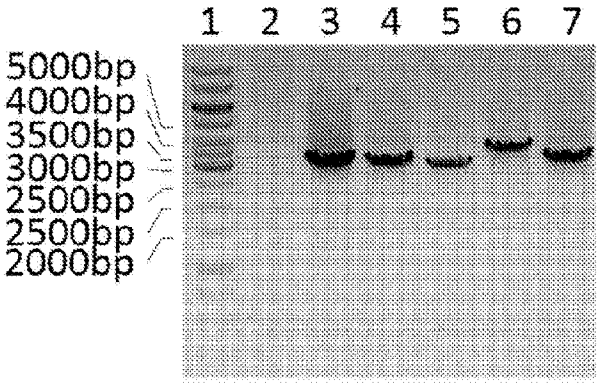
1 2 3 4 5 6 7
5000bp
4000bp
3500bp
3000bp
2500bp
2500bp
2000bp

FIG. 7

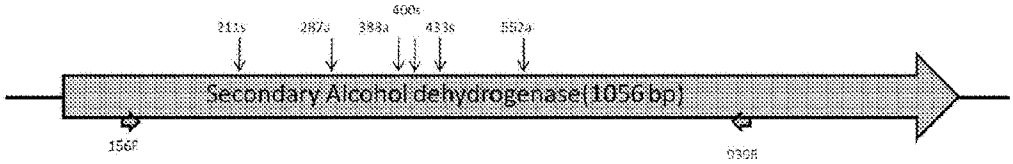
211s
287s
388a
433s
552a
Secondary Alcohol dehydrogenase(1056 bp)
156F

FIG. 8

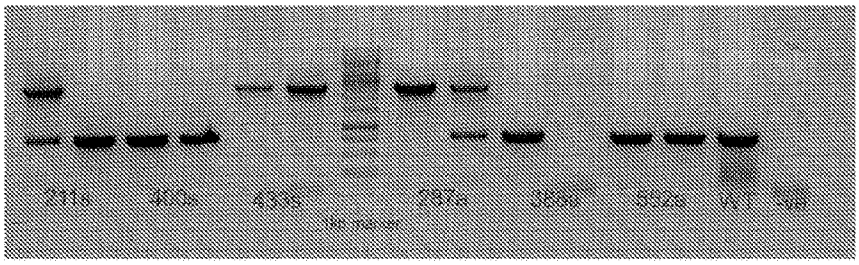

FIG. 9A

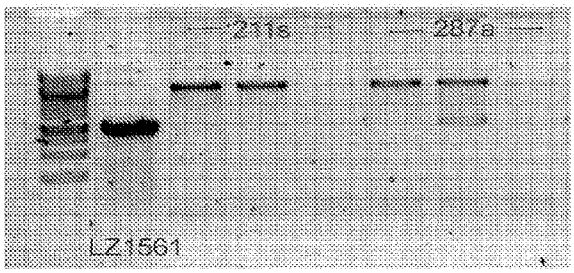

FIG. 9B

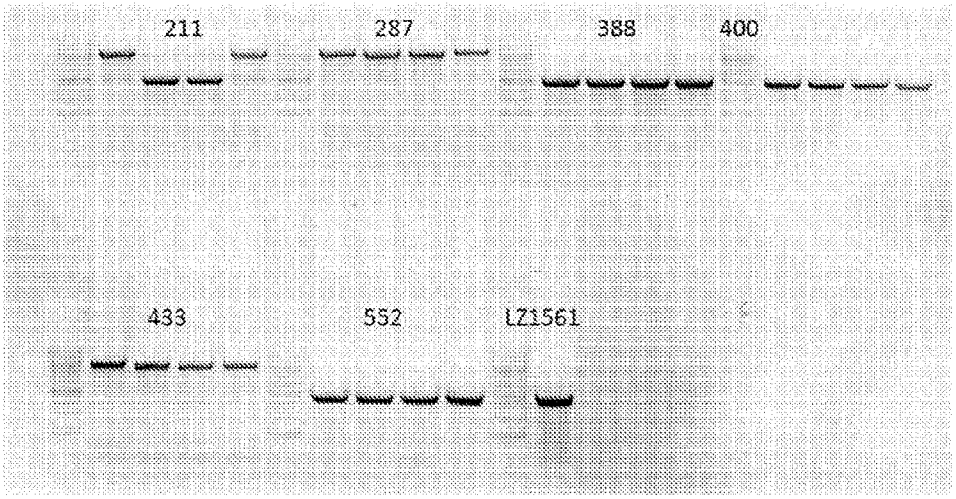

FIG. 9C