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Description

Technical Field

[0001] The present invention relates to a recombinant microorganism which may be used to produce useful proteins or polypeptides, as well as to such proteins and polypeptides.

Technical Background

[0002] Microorganisms are widely used for industrially producing a broad range of useful substances, including alcoholic beverages, certain types of foods such as *miso* and *shoyu*, amino acids, organic acids, nucleic-acid-related substances, antibiotics, sugars, lipids, and proteins. These substances also find diversified uses, including foods, pharmaceuticals, detergents, products for daily use such as cosmetics, and a variety of chemical raw materials.

[0003] In industrial production of useful substances by use of microorganisms, improvement of productivity is one major topic of interest, and one approach therefor is breeding of microorganisms through mutagenesis or other genetic means. Recently, in particular, with advancement of microbial genetics and biotechnology, more efficient breeding of useful microorganisms is performed through gene recombination techniques, and in association therewith, host microorganisms for obtaining recombinant genes are under development. For example, *Bacillus subtilis* Marburg No. 168, which has already been confirmed to be safe and have excellent characteristics as a host microorganism, has been further improved.

[0004] However, microorganisms inherently possess diversified genes so that they can cope with environmental changes in the natural world, and thus, they do not necessarily exhibit high production efficiency of proteins or similar substances in industrial production, where only limited production media are employed.

Disclosure of the Invention

[0005] The present invention is characterized by the embodiments as defined in the claims. Thus, it relates to the following items:

1. A recombinant microorganism prepared by transferring, to a mutant strain of a microorganism from which any of *Bacillus subtilis* genes cspB, sigL, glcT, rocR and ccpA have been deleted or knocked out, a gene encoding a heterologous protein or polypeptide,
wherein the microorganism is *Bacillus subtilis*, wherein three regions constituted by a transcription initiation regulatory region, a translation initiation regulatory region, and a secretion signal region are ligated to an upstream region of the gene encoding a heterologous protein or polypeptide, wherein the transcription initiation regulatory region and the translation initiation regulatory region are each derived from a 0.6 to 1 kb region upstream of the cellulase gene of a bacterium belonging to the genus Bacillus, and wherein the three regions constituted by the transcription initiation regulatory region, the translation initiation regulatory region, and the secretion signal region are

    (a) a nucleotide sequence of base numbers 1 to 659 of a cellulase gene of SEQ ID NO: 1;
    (b) a nucleotide sequence of base numbers 1 to 696 of a cellulase gene of SEQ ID NO: 3; or
    (c) a DNA fragment having a nucleotide sequence having 80% homology with either of the nucleotide sequences of (a) or (b).

2. A method for producing a protein or polypeptide by use of a recombinant microorganism as defined in item 1.

[0006] The present disclosure provides a recombinant microorganism prepared by transferring, to a mutant strain of microorganism from which any of *Bacillus subtilis* genes *comA, yopO, treR, yvbA, cspB, yvaN, yttP, yurK, yozA, licR, sigL, mntR, glcT, yvdE, ykvE, slr, rocR, ccpA, yaaT, yyaA, yycH; yacP, hprK, rsiX, yhdK,* and *ylbO,* or one or more genes functionally equivalent to any of these genes have been deleted or knocked out, a gene encoding a heterologous protein or polypeptide.

Brief Description of the Drawings

[0007] Fig. 1 schematically shows a method for preparing a DNA fragment for deleting a gene through SOE-PCR (SOE: splicing by overlap extension) (see Gene, 77, 61 (1989), and a method for deleting a target gene (replacing the target gene with a drug resistance gene) through use of the DNA.

Modes for Carrying out the Invention

[0008] The present invention is directed to a recombinant microorganism obtained by transferring, into a host microorganism capable of producing protein or polypeptide with increased productivity, a gene encoding a protein or polypeptide, and to a method for producing a protein or polypeptide by use of the recombinant microorganism.

[0009] The present inventors have conducted extensive studies on, among many different genes encoded on the genome of a microorganism, genes which are not needed in or which are detrimental to the production of useful proteins or polypeptides, and have found that, when a gene encoding a target protein or polypeptide is transferred to the microorganism *Bacillus subtilis* after a specific gene is deleted or knocked out from the genome of the microorganism, productivity of the target protein or polypeptide is enhanced as compared with the case before the deletion or knocking out.

[0010] In the microorganism of the present invention, since genes which are unnecessary in or detrimental to the production or a target protein or polypeptide are deleted or knocked out, waste of culture media, including energy loss, production of byproducts, and reduced specific production rate, is significantly reduced, and in addition, protein and polypeptide can be produced over a prolonged period, whereby a target product can be produced with high efficiency.

[0011] In the present invention, homology between amino acid sequences and that between nucleic acid sequences are both determined by use of the Lipman-Pearson method (Science, 227, 1935 (1985)). Specifically, calculation is performed by use of a homology analysis program (Search Homology) developed by genetic information processing software, Genetyx-Win (Software Development Co., Ltd.), with ktup (the unit size to be compared) being set 2.

[0012] The parent microorganism for constructing the microorganism of the present invention is *Bacillus subtilis* having genes or genes functionally equivalent thereto as shown in Table 1, wherein the gene may be of wild-type or a mutant.

[0013] *Bacillus subtilis* is used, from the viewpoint that complete genomic information of this microorganism has already been obtained, and thus genetic engineering techniques and genomic engineering techniques have been established, and that the microorganism has ability to secrete the produced protein extracellularly.

[0014] Examples of the target protein or polypeptide to be produced by use of the microorganism of the present invention include enzymes, physiologically active substances, and other proteins and polypeptides which find utility in foods, pharmaceuticals, cosmetics, detergents, fiber-treating agents, clinical assay agents, etc.

[0015] Taking *Bacillus subtilis,* which is known to have 4,106 genes on the genome, as an example, one or more genes which are to be deleted or knocked out are any of the *Bacillus subtilis* genes shown in Table 1, or are selected from among the genes functionally equivalent thereto. The present inventors have found that such genes do not directly participate in production of the target protein or polypeptide and are unnecessary for the growth of microorganism in ordinary industrial production media.

[0016] The names, numbers, and functions of respective genes in the Tables contained herein conform with the *Bacillus subtilis* genome data reported in Nature, 390, 249-256 (1997) and made public by JAFAN (Japan Functional Analysis Network for *Bacillus subtilis;* BSORF DB) on the Internet (http://bacillus.genome.ad.jp/, renewed June 17, 2003).

Table 1

Name of the gene	Gene ID	Functions or other information of the gene
ComA	BG10381	two-component response regulator
yopO	BG13648	deduced transcriptional regulator, spβ prophage protein
treR	BG11011	trehalose operon transcriptional repressor (GntR family)
yvbA	BG14078	deduced transcriptional regulator (ArsR family)
cspB	BG10824	cold shock-related major factor
yvaN	BG14069	deduced transcriptional regulator
yttP	BG13927	deduced transcriptional regulator (TetR family)
yurK	BG13997	deduced transcriptional regulator (GntR family)
yozA	BG13748	deduced transcriptional regulator (ArsR family)
licR	BG11346	transcriptional regulator (antiterminator), lichenan operon (*licBCAH*) regulation
sigL	BG10748	RNA polymerase σ factor (o54)
mntR	BG11702	manganese transport regulator

(continued)

Name of the gene	Gene ID	Functions or other information of the gene
glcT	BG12593	transcriptional regulator essential to expression of *ptsGHI* operon (BglG family, antiterminator)
yvdE	BG12414	deduced transcriptional regulator (LacI family)
ykvE	BG13310	deduced transcriptional regulator (MarR family)
slr	BG11858	transcriptional activator for competence- or sporulation-related genes
rocR	BG10723	transcriptional activator for arginine-assimilating operon (NtrC family)
ccpA	BG10376	carbon source catabolism reppression-related transcriptional regulator (LacI family)
yaaT	BG10096	type-II signal peptidase-like protein
yyaA	BG10057	DNA-binding protein SpoOJ-like protein
yycH	BG11462	Function unknown (homologous gene has been found in other organisms)
yacP	BG10158	Function unknown (homologous gene has been found in other organisms)
hprK	BG14125	Hpr protein Ser residue phosphoenzyme/dephosphoenzyme
rsiX	BG10537	anti σX factor
yhdK	BG13017	Function unknown, related to repression of σM factor expression
ylbO	BG13367	expression regulator for gene in σE-related metrocytein

[0017] Genes derived from other microorganisms, preferably from bacteria belonging to the genus *Bacillus,* which have the same functions as any of the *Bacillus subtilis* genes shown in Table 1, or have 70% or more homology with the nucleotide sequence of any of the genes shown in Table 1, preferably 80% or more homology, more preferably 90% or more, further preferably 95% or more, yet more preferably 98% or more, should be interpreted to be functionally equivalent to the genes shown in Table 1, and thus to constitute the genes which are to be deleted or knocked out according to the present invention. In this connection, homology of nucleotides is computed by use of the Lipman-Pearson method (Science, 227, 1435, 1985).

[0018] Many of the genes shown in Table 1 which encode *Bacillus subtilis* are regulatory genes participating in activation or suppression of expression of a variety of genes, or genes deduced to be such regulatory genes. The present invention has been attained on the basis of this finding; i.e., the presence of regulatory genes unnecessary in or detrimental to production of protein or polypeptide has now been unveiled in the present invention.

[0019] Notably, attention is drawn to the fact that many of the listed "unnecessary" or "detrimental" genes are regulatory genes participating in sugar intake or metabolism, as exemplified by the *glcT* gene, which acts as an anti-terminator for a glucose PTS intake operon; the *licT* gene, which acts as an anti-terminator for a lichenan hydrolysis operon; the *treR* gene, which acts as a repressor of trehalose intake and metabolism; and the *hprK* gene and *ccpA* gene, which relate to glucose catabolite repression.

[0020] Also, in addition to the regulatory genes involved in sugar intake and metabolism, the *rocR* gene participating in activation of arginine assimilation, and competence-related *comA* gene and *slr* gene, which are also regulatory genes, may be deleted or knocked out, to thereby improve productivity of protein or polypeptide.

[0021] The genes shown in Table 1 include the *yhdK* gene, and the *rsiX* gene encoding the anti-EC F sigma factor which suppresses expression of an ECF sigma factor, sigma x. The *yhdK* gene has been reported to participate in suppression of sigma M (Mol. Microbiol., 32, 41, 1999). The *sigL* gene, which encodes sigma L, is also included in the genes of Table 1. This suggests that expression of a gene under regulation by sigma X or sigma M is favorable for production of protein, and conversely, some gene expression under regulation by sigma L is unfavorable.

[0022] By deleting or knocking out one or more genes selected from the above-mentioned genes, expression which is unnecessary in or harmful to the production of protein or polypeptide can be prevented, leading to enhanced productivity in such production of protein or polypeptide.

[0023] The number of gene(s) to be deleted or knocked out is one or more, preferably two or more, more preferably three or more, even more preferably 5 or more. When a microorganism of the present invention is constructed, deletion or inactivation of a gene or genes other than those mentioned above is possible. In such a case, a more improved effect is expected. An alternative method for achieving the present invention is inactivation, or knocking out, of a target gene

by inserting thereto a DNA fragment of another origin or introducing a mutation to the transcription/translation-initiation region of the gene. Preferably, however, the target genes are physically deleted.

[0024] In an example procedure for deleting or knocking out the genes, any of the target genes shown in Table 1 is deleted or knocked out according to a plan which has been set up in advance. Alternatively, randomized deletion of genes or mutation by way of knocking out is performed, followed by evaluation on protein productivity and gene analysis.

[0025] The target gene may be deleted or knocked out through homologous recombination. That is, a DNA fragment containing a portion of the target gene is cloned with an appropriate plasmid vector to thereby obtain a ring-shaped recombinant plasmid, and the resultant plasmid is transferred into cells of a parent microorganism. Thereafter, through homologous recombination effected in a partial region of the target gene, the target gene on the genome of the parent microorganism is cleaved, thereby completing inactivation of the target gene. Alternatively, the target gene is knocked out by substitution or insertion of a base, or a linear DNA fragment containing a region outside the target gene sequence but not containing the target gene may be constituted through PCR or a similar method, and the thus-engineered gene or fragment is transferred into a cell of a parent microorganism. At two sites outside the mutation within the target gene in the genome of the parent microorganism genome, or at two regions outside the target gene sequence, double crossing-over homologous recombination is caused to occur, to thereby attain substitution with a gene fragment in which the target gene on the genome is deleted or knocked out.

[0026] Particularly when the parent microorganism used to construct the microorganism of the present invention is *Bacillus subtilis,* since several reports have already described methods for deleting or knocking out the target gene (see, for example, Mol. Gen. Genet., 223, 268 1990), repetition of any of such methods may be followed, to thereby produce a host microorganism of the present invention.

[0027] Randomized gene deletion or inactivation may be performed through use of a method similar to the above-described method for inducing homologous recombination by use of a randomly cloned DNA fragment, or by way of irradiation of a parent microorganism with gamma rays or similar rays.

[0028] Next will be described in more detail a deletion method employing double crossing over by use of a DNA fragment designed for the deletion purpose, the DNA fragment being prepared through SOE-PCR (Gene, 77, 61, 1989). However, in the present invention, the method for deleting genes is not limited to only the below-described method.

[0029] The DNA fragment use for the deletion purpose is a fragment constructed such that a drug resistant marker gene is inserted between a ca. 0.5 to 3 kb upstream sequence which flanks and is upstream of the gene to be deleted, and a ca. 0.5 to 3 kb downstream sequence which flanks and is downstream of the same gene. In the first cycle of PCR, the following three fragments are prepared: the upstream and the downstream fragments, which are to be deleted, and the drug resistant marker gene. The primers to be used in this step may, for example, be those specifically designed so that an upstream 10-30 base pair sequence of a drug resistance gene is added to the lower end of the upstream fragment, and a downstream 10-30 base pair sequence of the drug resistance marker gene is added to the upper end of the downstream fragment (Fig. 1).

[0030] Next, using three PCR fragments prepared in the first cycle as templates, the second cycle of PCR is performed by use of an upper primer of the upstream fragment and a lower primer of the downstream fragment. This step causes annealing with the drug resistance marker gene fragment in the sequence of the above-engineered drug resistance marker gene, and through PCR amplification, there can be obtained a DNA fragment with the drug resistance marker gene inserted between the upstream fragment and the downstream fragment (Fig. 1).

[0031] When a chloramphenicol-resistant gene is employed as a drug resistance marker gene, a DNA fragment for deleting a gene can be obtained through SOE-PCR under typical conditions described in literature (see, for example, PCR Protocols. Current Methods and Applications, Edited by B. A. White, Humana Press, pp. 251 (1993), Gene, 77, 61, 1989), by use of a primer set such as that shown in Table 2 and a conventional enzyme kit for PCR (e.g., Pyrobest DNA Polymerase (product of Takara Shuzo)).

[0032] When the thus-obtained DNA fragment for effecting gene deletion is introduced into cells through the competent method or a similar method, intracellular genetic recombination occurs in homologous regions which are present upstream and downstream of the gene to be deleted. Thus, cells in which the target gene has been substituted by a drug resistance gene can be selectively separated through employment of a drug resistance marker (Fig. 1). Specifically, when a DNA fragment for gene deletion prepared by use of a primer set shown in Table 2 is introduced into cells, colonies which have grown on an agar culture medium containing chloramphenicol are separated, and deletion of the target gene by way of substitution by the chloramphenicol-resistant gene is confirmed through an appropriate method such as PCR employing a genome as a template.

[0033] Subsequently, when a gene encoding a target protein or polypeptide is transferred to a host mutant microorganism strain from which any of the *Bacillus subtilis* genes shown in Table 1, or one or more genes selected from among the genes corresponding thereto has been deleted or knocked out, the microorganism of the present invention can be obtained.

[0034] No particular limitation is imposed on the gene encoding the target protein or polypeptide. Examples of the protein and polypeptide include physiologically-active peptides and enzymes for industrial purposes such as detergents,

foods, fibers, feeds, chemicals, medicine, and diagnostic agents. Industrial enzymes may be functionally grouped into oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases/synthetases. Preferably, hydrolases such as cellulase, α-amylase, and protease may be used. Specific examples include cellulase belonging to family 5 in the classification of hydrolase (Bioche M. J., 280, 309, 1991); in particular, cellulase derived from a microorganism, more particularly cellulase derived from the genus *Bacillus.* Other specific examples of the types of industrial enzymes include alkaline cellulase which is derived from the genus *Bacillus* and has an amino-acid of SEQ ID NOs: 2 or 4, and cellulase which has an another amino-acid sequence having 70% homology with said amino-acid sequence, preferably 80% homology, more preferably 90%, further preferably 95%, still further preferably 98% or more.

[0035] Specific examples of α-amylase include α-amylase derived from a microorganism, preferably liquefied amylase derived from the genus *Bacillus.* More specific examples include alkaline amylase which is derived from the genus *Bacillus* and has an amino-acid sequence of SEQ ID NO: 6, and amylase which has another amino-acid sequence having 70% homology with said amino-acid sequence, preferably 80% homology, more preferably 90%, further preferably 95%, particularly preferably 98% or more. The homology of the amino-acid sequence is calculated by the Lipman-Pearson method (Science, 227, 1435 (1985)). Specific examples of protease include serine protease and metallo-protease which are derived from microorganisms, particularly those belonging to the genus *Bacillus.*

[0036] Preferably, a gene coding for a target protein or polypeptide has, on its upstream region thereof, one or more regulatory regions relating to transcription, translation, or secretion of the gene (specially, one or more regions selected from among a transcription initiation regulatory region including a promoter and a transcription initiation site; a translation initiation region including a ribosome-binding site and a start codon; and a secretion signal peptide region) properly ligated thereto. Preferably, it is preferred that three regions consisting of the transcription initiation regulatory region, the translation initiation regulatory region, and the secretion signal region be ligated to the target gene. Further preferably, the secretion signal peptide region is one that originates from the cellulase gene of a microorganism belonging to the genus *Bacillus,* and the transcription initiation region and the translation initiation region is a 0.6 to 1 kb region upstream of the cellulase gene. In one preferred example, a transcription initiation regulatory region, a translation initiation region, and a secretion signal peptide region of a cellulase gene derived from a microorganism belonging to the genus *Bacillus* disclosed in, for example, Japanese Patent Application Laid-Open (*kokai*) Nos. 2000-210081 and 190793/1990; i.e., a cellulase gene derived from KSM-S237 strain (FERM BP-7875) or KSM-64 strain (FERM BP-2886), is properly ligated to a structural gene of the target protein or polypeptide. More specifically, preferred DNA fragments to be ligated include a nucleotide sequence of base numbers 1 to 659 of SEQ ID NO: 1; a nucleotide sequence of base numbers 1 to 696 of a cellulase gene of SEQ ID NO: 3; a DNA fragment having a nucleotide sequence having 70% homology with any one of said nucleotide sequences, preferably 80% homology, more preferably 90%, further preferably 95%, even more preferably 98% or more; or a DNA fragment having a nucleotide sequence lacking a portion of any one of said nucleotide sequences. Preferably, one of these DNA fragments is properly ligated to a structural gene of the target protein or polypeptide. As used herein, a DNA fragment having a nucleotide sequence lacking a portion of any one of the above-mentioned nucleotide sequences is intended to mean a DNA fragment which has functions relating to transcription, translation, and secretion of the gene, without having a portion of any one of the above-mentioned nucleotide sequences.

[0037] The recombinant microorganism of the present invention can be obtained by a conventional transformation technique in which a recombinant plasmid containing a DNA fragment which includes a gene encoding the target protein or polypeptide, and is ligated to a proper plasmid vector is transferred into a host microorganism cell. Alternatively, the recombinant microorganism may be obtained making use of a DNA fragment prepared by ligating the above DNA fragment to a proper region which is homologous with a certain portion of the host microorganism genome, and inserted directly into a host microorganism genome.

[0038] The target protein or polypeptide obtained by use of the recombinant microorganism of the present invention may be produced in such a manner that a corresponding cell strain is inoculated onto a culture medium containing assimilable carbon sources and nitrogen sources, and other essential components; the cell strain is cultured through a conventional microorganism culturing method; and subsequently, protein or polypeptide is collected and purified.

[0039] Through the aforementioned procedure, a host mutant microorganism strain in which any of the *Bacillus subtilis* genes shown in Table 1 or one or more genes selected from genes functionally equivalent thereto have been deleted or knocked out can be engineered. In addition, by use of such a mutant strain, a recombinant microorganism can be produced. Thus, a useful protein or polypeptide can be effectively produced through employment of the mutant strain or the recombinant microorganism.

[0040] The method for constructing a recombinant microorganism according to the present invention, and the method for producing cellulase and α-amylase by use of the recombinant microorganism will next be described in detail, centering on working examples for constructing recombinant strain belonging to *Bacillus subtilis* from which the ccpA gene (BG10376) of *Bacillus subtilis* has been deleted.

Examples

Example 1

[0041] A genome DNA sample, serving as a template, extracted from *Bacillus subtilis* 168 strain and two primer sets (ccpA-AF and ccpA-A/CmR; and ccpA-B/CmF and ccpA-BR) shown in Table 2 were used to prepare a 0.6 kb fragment (A) flanking the upstream side of the *ccpA* gene on the genome and a 0.6 kb fragment (B) flanking the downstream side of the *ccpA* gene. A chloramphenicol-resistant gene of plasmid pC194 (J. Bacteriol. 150 (2), 815 (1982))) was inserted into the XbaI-BamHI cleavages site of plasmid pUC18, to thereby prepare a recombinant plasmid pCBB 31. The recombinant plasmid pCBB and a primer set consisting of CmF and CmR shown in Table 2 were used to prepare a 1 kb fragment (C) containing the chloramphenicol-resistant gene. Subsequently, SOE-PCR was performed by use of the primers ccpA-AF and ccpA-BR shown in Table 2, and by use of the thus-prepared three fragments (A), (B), and (C) in combination as templates, a 2.2 kb DNA fragment in which the fragments (A), (B), and (C) were ligated in this sequence was prepared (see Fig. 1). By use of the thus-prepared DNA fragment, *Bacillus subtilis* 168 strain was transformed through the competent method. Colonies grown in an LB agar medium containing chloramphenicol were collected as transformants. The genome of the above-obtained transformant was extracted, and PCR performed thereon confirmed that the *ccpA* gene had been deleted and substituted by a chloramphenicol-resistant gene.

Table 2-1

Primer	Nucleotide sequence	SEQ ID NO:
comA-AF	AAGGATGATAATCCGTCCCGTG	7
comA-A/CmR	GTTATCCGCTCACAATTCGGATGGTCATCAATCACTAG	8
comA-B/CmF	CGTCGTGACTGGGAAAACTGCGAAATCAGACGGTGTAC	9
comA-BR	CGTCGCCTATCGGCGGGCAC	10
yopO-AF	ATGTATATAGGAGGTTGGTGGTATG	11
yopO-A/CmR	GTTATCCGCTCACAATTCGCTCTGACATGTCAACCTCC	12
yopO-B/CmF	CGTCGTGACTGGGAAAACAGATGAGAAAGGAGGAGAAG	13
yopO-BR	ATAACTGTTACTATATAATGGCC	14
treR-AF	GCTGGGGATGACGAATCCGA	15
treR-A/CmR	GTTATCCGCTCACAATTCTCACCTTCATTATGGACCAC	16
treR-B/CmF	CGTCGTGACTGGGAAAACCACCGTCTCGACAAATTCCG	17
treR-BR	GTTGCCAAGCGCGATATAGG	18
yvbA-AF	TATACAGGGATTATCAGTATTGAGC	19
yvbA-A/CmR	GTTATCCGCTCACAATTCTTTTCTCCTTGTTGGATCTG	20
yvbA-B/CmF	CGTCGTGACTGGGAAAACGGGGATAACGATTTATGAAG	21
yvbA-BR	TTTTGTAATAATGATATGAAGCTAGTGTTG	22
cspB-AF	ATATCCAGCCCTGCCTCTTC	23
cspB-A/CmR	CTGTGTGAAATTGTTATCCGCTCACAATTCGAAATTTCCTCCTAA AGCGATCATAACG	24
cspB-B/CmF	GTCGTTTTACAACGTCGTTGACTGGGAAAACCCACAAGCTGCTAA CGTTAC	25
cspB-BR	TCCTGTTTGGGCTCCTGTTG	26
yvaN-AF	TGTTTATGTATGGCGGCCTGCGGGAC	27
yvaN-A/CmR	GTTATCCGCTCACAATTCAGCTTTCCATATATCTCACC	28
yvaN-B/CmF	CGTCGTGACTGGGAAAACACGGTCTGCTGATGACTGAC	29
yvaN-BR	GCGTTTACTTAAGATGTCGA	30

(continued)

Primer	Nucleotide sequence	SEQ ID NO:
yttP-AF	TTTCTAGCGTTTCGGCAAATTGAGTTAAG	31
yttP-A/CmR	GTTATCCGCTCACAATTCCTTACTTTCATACGGCTCAC	32
yttP-B/CmF	CGTCGTGACTGGGAAAACGAGACGTGGCGCTCACCAAC	33
yttP-BR	CGGATTAAAAAAAGAATATCGCGGACAGC	34
yurK-AF	TGCCGCTGCCCGCCGGAGAG	35

Table 2-2

yurK-A/CmR	GTTATCCGCTCACAATTCAAGGTGTAGAACTTCCGTTG	36
yurK-B/CmF	CGTCGTGACTGGGAAAACACCATCAACAGCCCCTACAC	37
yurK-BR	TCAAATAAAGGCGGCATTCAGTCC	38
yozA-AF	ATAATGGTATCCAAATCCACGC	39
yozA-A/CmR	GTTATCCGCTCACAATTCATTCAGTCATATGTATCACC	40
yozA-B/CmF	CGTCGTGACTGGGAAAACGATCCATCATACACAGCATG	41
yozA-BR	CACTTCTCAACGGAGGGGATTTCACATC	42
licR-AF	TAATGGAGGAGAGAAGGCCG	43
licR-A/CmR	GTTATCCGCTCACAATTCAGTCGCCCATGAAGCATGAG	44
licR-B/CmF	CGTCGTGACTGGGAAAACACCAAAAAATGCTGAGCTGACAGC	45
licR-BR	TTGCCAATGATGAGGAAAAAGGAACC	46
sigL-AF	CTGAACGTCTTGAATAAAAAAGCAGG	47
sigL-A/CmR	GTTATCCGCTCACAATTCGCTGAAGTTTCATATCCATC	48
sigL-B/CmF	CGTCGTGACTCGGAAAACATTCCGTCATCGGCAGCGAG	49
sigL-BR	AGCGGTTTACAAGTTGGAGG	50
mntR-AF	ATTTCAGAAGGCATACTTCAAG	51
rnntR-A/CmR	GTTATCCGCTCACAATTCCATACTTGGTGTTGTCATCG	52
mntR-B/CmF	CGTCGTGACTGGGAAAACCATAATCAGTAAAAAGGCGGTC	53
mntR-BR	TTCTGACCGCTCTGGCAACC	54
glcT-AF	ATAATGCCCGCTTCCCAACC	55
glcT-A/CmR	GTTATCCGCTCACAATTCCGATCCTCAGCTCCTTTGTC	56
glcT-B/CmF	CGTCGTGACTGGGAAAACTCATCTGATACCGATTAACC	57
glcT-BR	CAACTGAATCCGAAGGAATG	58
yvdE-AF	TCGGGGTCATGCCGAGCGGT	59
yvdE-A/CmR	GTTATCCGCTCACAATTCCAATGTTGCCATTTTCATCC	60
yvdE-B/CmF	CGTCGTGACTGGGAAAACTTGTACGAGAATCAACGCTG	61
yvdE-BR	CACGGCAATGCATTCTTCGG	62
ykvE-AF	AGATCTGTCGGCCAGGTTTAC	63
ykvE-A/CmR	GTTATCCGCTCACAATTCTGATTTTTCTGTCATGTCTC	64
ykvE-B/CmF	CGTCGTGACTGGGAAAACGGTAGAGATGTGCACCGAAA	65

(continued)

ykvE-BR	GAGTCAGACGGCATCGATGA	66
slr-AF	TTCTGATTCATTTTCACTGCTGG	67
slr-A/CmR	GTTATCCGCTCACAATTCAACGGATAATTCTTCCAATC	68
slr-B/CmF	CGTCGTGACTGGGAAAACTGTCCATGAAGTCAAATCC	69
slr-BR	CGCTGAAATATTCTCTCGCA	70
rocR-AF	CGCCGCTTTCACCGCGGATTC	71
rocR-A/CmR	GTTATCCGCTCACAATTCCTTTGACCACTGTATGAACC	72

Table 2-3

rocR-B/CmF	CGTCGTGACTGGGAAAACACTCGTCTAACGAATAATCC	73
rocR-BR	TGTCATCACGGAATTTGACG	74
ccpA-AF	CCAAATTATCCTTTGTGAGCGCGGAATCAG	75
ccpA-A/CmR	GTTATCCGCTCACAATTCCGTAGATCGTAATATTGCTC	76
ccpA-B/CmF	CGTCGTGACTGGGAAAACAGCTTAGAAAGTCAACCAAG	77
ccpA-BR	TTTGAGCATCAGCACAAGCC	78
yaaT-AF	TGTAGCAGAAGCAGTCGAATT	79
yaaT-A/Cm2R	CTAATGGGTGCTTTAGTTGACAATTACGCAGCTGTCATGT	80
yaaT-B/Cm2F	CTGCCCCGTTAGTTGAAGAACTGATAAACCGTGAAAAAGTG	81
yaaT-RV	CCTTTGAAAAAGGCTCCCGT	82
yyaA-AF	GTTTTCCAAGTCTGCCGATAAAAATATGC	83
yyaA-A/CmR	GTTATCCGCTCACAATTCATGCTTCATGTACCTACACC	84
yyaA-B/CmF	CGTCGTGACTGGGAAAACCAATTAACGATTCGCATACC	85
yyaA-BR	AAAAAGAAGAAGTCACAGTACAGAACGTGG	86
yycH-AF	ATTTTTCGCCATCTTGAATTTTC	87
yycH-A/Cm2R	CTAATGGGTGCTTTAGTTGGATGATCCTCTCGTTGAACTG	88
yycH-B/Cm2F	CTGCCCCGTTAGTTGAAGGGATGAGCCTTCAGAAAAGTT	89
yycH-BR	GCCGGACAGAGATCTGTATG	90
yacP-B/Cm4F	GAAGAAGGTTTTTATGTTGACGCTTTTTTGCCCAATACTGTATAA	91
yacP-B/Cm4R	CAAAAAAGCGTCAACATAAAAACCTTCTTCAACTAACGGGGCAGG	92
yacP-BR	AAGACGAGTACTTTTCTCTCTAAATCACTT	93
yacP-AF	AACTCGATCAAATGGTGACAGGACAGCATC	94
yacP-A/Cm4F	GGAGAATAAAGACCCTCTTCAACTAAAGCACCCATTAGTTCAACA	95
yacP-A/Cm4R	TGCTTTAGTTGAAGAGGGTCTTTATTCTCCCACAGGGTTTCGTTT	96
hprK-B/Cm4F	TTTTTATATTACAGCGAGTTGGCGTTAAATGAATGAAGCGATAGA	97
hprK-B/Cm4R	ATTTAACGCCAACTCGCTGTAATATAAAAACCTTCTTCAACTAAC	98
hprK-BR	TTGATTGATGATAAATTCAGGCAGGTGCAG	99
hprK-AF	CAAAGCTTGAGAAATGTTCCCATGCTCTTG	100
hprK-A/Cm4F	CAGGAGGAACATATCTCTTCAACTAAAGCACCCATTAGTTCAACA	101

(continued)

hgrK-A/Cm4R	TGCTTTAGTTGAAGAGATATGTTCCTCCTGTTCCGGGCTGCCCCG	102
rsiX-AF	ATTCCAGTTACTCGTAATATAGTTG	103
rsiX-A/CmR	GTTATCCGCTCACAATTCACTTCATCATCCATTAGCTC	104
rsiX-B/CmF	CGTCGTGACTGGGAAAACCTGCTCCAAATCCGATTTCC	105
rsiX-BR	GTCCTGCATTTTTCGAAGTCTGG	106
yhdK-AF	TACACATCCTTCAAACAAGTCTGAACAAAC	107

Table 2-4

yhdK-A/Cm4R	TGCTTTAGTTGAAGATTACCAGTTCCATAATTCCACCTCGCCGAC	108
yhdK-B/Cm4F	TTTTTATATTACAGCGTGTGTATACCATTGTATCTGTAGATACGA	109
yhdK-BR	GCTATGATCATTGTAACGAAAGGAAAGGGG	110
yhdK-A/Cm4F	TTATGGAACTGGTAATCTTCAACTAAAGCACCCATTAGTTCAACA	111
yhdK-B/Cm4R	CAATGGTATACACACGCTGTAATATAAAAACCTTCTTCAACTAAC	112
ylbO-AF	AATCTGAACAAGAAAAAGGAGCTGCTCCTC	113
ylb0-A/Cm4R	TGCTTTAGTTGAAGAATTCAATCTCCCTCCATGTCAGCTTATTTA	114
ylb0-B/Cm4F	TTTTTATATTACAGCAGAAACGCCTGAAATGAACCGGCCCTATAG	115
ylbO-BR	TGTTTGACAAAGGTAGAACGTCTGCTTATC	116
ylbO-A/Cm4F	GGAGGGAGATTGAATTCTTCAACTAAAGCACCCATTAGTTCAACA	117
ylbO-B/Cm4R	ATTTCAGGCGTTTCTGCTGTAATATAAAAACCTTCTTCAACTAAC	118
CmF	GAATTGTGAGCGGATAAC	119
CmR	GTTTTCCCAGTCACGACG	120
Cm2F	CAACTAAAGCACCCATTAG	121
Cm2R	CTTCAACTAACGGGGCAG	122

Example 2

[0042] In a manner similar to that described in Example 1, sporulation gene-deleted strains into which a chloramphenicol-resistant gene had been introduced by way of substitution in place of the below-described deleted genes were separated through use of a DNA fragment for effecting deletion prepared from an adequate primer set selected from among various primer sets shown in Table 2; i.e., gene-AF, gene-A/CmR, gene-B/CmF, gene-BR, CmF, and CmR. The gene deleted from the genome was *comA, yopO, treR, yvbA, yvaN, yttP, yurK, yozA, licR, sigL, mntR, glcT, ykvE, slr, rocR, yyaA,* or *rsiX*.

Example 3

[0043] In a manner similar to that described in Example 2, a DNA fragment for deletion was prepared by use of an adequate primer set selected from among the gene-AF, gene-A/Cm2R, gene-B/Cm2F, gene-BR, Cm2F, and Cm2R, which are shown in Table 2. By use of the thus-prepared DNA fragment, sporulation gene-deleted strains into which a chloramphenicol-resistant gene had been introduced by way of substitution in place of the below-described deleted genes were separated. The gene deleted from the genome was *cspB, yvdE, yaaT, yycH,* or *ylbO.*

Example 4

[0044] In a manner similar to that described in Example 2, a DNA fragment for effecting deletion was prepared from an adequate primer set selected from among the gene-AF, gene-A/Cm4R, gene-B/Cm4F, gene-BR, Cm4F, and Cm4R,

which are shown in Table 2. By use of the thus-prepared DNA fragment, sporulation gene-deleted strains into which a chloramphenicol-resistant gene had been introduced by way of substitution in place of deleted genes; *yacP, hprK,* and yhdK, were separated.

Example 5

[0045] To each of the gene-deleted strains obtained in Examples 1 to 4 and to *Bacillus subtilis* 168 strain serving as a control, a recombinant plasmid pHY-S237 was introduced through the protoplast transformation method. The recombinant plasmid pHY-S237 was prepared by inserting a DNA fragment (3.1 kb) encoding an alkaline cellulase derived from *Bacillus* sp. KSM-S237 strain (Japanese Patent Application Laid-Open *(kokai)* No. 2000-210081) into the restriction enzyme *Bam*HI cleavage site of a shuttle vector pHY300 PLK. Each of the thus-obtained cell strains was shake-cultured in an LB medium (5 mL) overnight at 30°C. The culture broth (0.03 mL) was inoculated to a 2 × L-maltose medium (2% tryptone, 1% yeast extract, 1% NaCl, 7.5% maltose, 7.5 ppm manganese sulfate 4-5 hydrate, and 15 ppm tetracycline), followed by shake culturing at 30°C for three days. After completion of culturing, cells were removed through centrifugation, and alkaline cellulase activity of the supernatant obtained from the culture was determined, thereby calculating the amount of the alkaline cellulase secreted from the cells during culturing; i.e., the amount of the extracellularly produced alkaline cellulase. As is clear from Table 3, more effective production, or secretion, of alkaline cellulase has been confirmed in the case where a gene-deleted strain was employed as a host, as compared with the control 168 strain (wild type strain).

Table 3

Name of deleted gene	Gene ID	Gene size (bp)	Size of deleted fragment (bp)	Amount of produced (secreted) alkaline cellulase (relative value)
ComA	BG10381	645	588	160
yopO	BG13648	213	169	154
treR	BG11011	717	656	139
yvbA	BG14078	273	210	137
cspB	8G10824	204	171	132
yvaN	BG14069	408	379	124
yttP	BG13927	624	590	121
yurK	BG13997	729	677	118
yozA	BG13748	324	289	117
licR	BG11346	1926	1889	116
sigL	BG10748	1311	1256	114
mntR	BG11702	429	399	114
glcT	BG12593	858	811	110
yvdE	BG12414	951	916	109
ykvE	BG13310	438	356	108
slr	BG11858	459	394	105
rocR	BG10723	1386	1359	128
ccpA	BG10376	1005	957	205
yaaT	BG10096	828	828	127
yyaA	BG10057	852	816	113
yycH	BG11462	1368	1368	146
yacP	BG10158	513	513	156
hprK	BG14125	933	933	196

(continued)

Name of deleted gene	Gene ID	Gene size (bp)	Size of deleted fragment (bp)	Amount of produced (secreted) alkaline cellulase (relative value)
rsiX	BG10537	1107	1068	125
yhdK	BG13017	291	228	114
ylb0	BG13367	582	582	136
None (Wild type)	-	-	-	100

Example 6

[0046] To each of the gene-deleted strains obtained in Examples 1 to 4 and to *Bacillus subtilis* 168 strain serving as a control, recombinant plasmid pHSP-K38 was introduced through the protoplast transformation method. The recombinant plasmid pHSP-K38 was prepared by inserting, into the restriction enzyme BagII-XbaI cleavage site of a shuttle vector pHY300 PLK, a 2.1 kb fragment (SEQ ID No: 5) prepared by ligating an upstream 0.6 kb fragment (SEQ ID NO: 3) including portions of a promoter region and a signal sequence region of an alkaline cellulase gene with an upstream side of a DNA fragment (1.5 kb) encoding a mature enzyme region (Asp1-Gln480) of an alkaline amylase gene derived from *Bacillus* sp. KSM-K38 strain (Japanese Patent Application Laid-Open (*kokai*) No. 2000-1884882, Eur. J. Biochem., 268, 2974 (2001)). Each of the thus-obtained cell strains was shake-cultured in an LB medium (5 mL) overnight at 30°C. The culture broth (0.03 mL) was inoculated to a 2 × L-maltose medium (2% tryptone, 1% yeast extract, 1% NaCl, 7.5% maltose, 7.5 ppm manganese sulfate 4-5 hydrate, and 15 ppm tetracycline), followed by shake culturing at 30°C for three to six days. After completion of culturing, cells were removed through centrifugation, and alkaline amylase activity of the supernatant obtained from the culture was determined, thereby calculating the amount of the alkaline amylase secreted from the cells during culturing; i.e., the amount of the extracellularly produced alkaline amylase. As is clear from Table 3, more effective production, or secretion, of alkaline amylase has been confirmed in the case where a gene-deleted strain was employed as a host, as compared with the control 168 strain (wild type strain).

Table 4

Name of deleted gene	Gene ID	Gene size (bp)	Size of deleted fragment (bp)	Amount of produced (secreted) alkaline amylase (relative value)
Cultured for 3 days				
slr	BG11858	459	394	178
treR	BG11011	717	656	124
yop0	BG13648	213	169	364
yvaN	BG14069	408	379	148
yvbA	BG14078	273	210	171
None (Wild type)	-	-	-	100
Culture for 5 days (Wild type)				
cspB	BG10824	204	171	195
rocR	BG10723	1386	1359	215
sigL	BG10748	1311	1256	204
glcT	BG12593	858	811	132
yvdE	BG12414	951	916	127
yacP	BG10158	513	513	110

(continued)

Culture for 5 days (Wild type)				
None (Wild type)	-	-	-	100
Cultured for 6days				
yycH	BG11462	1368	1368	120
licR	BG11346	1926	1889	122
None (Wild type)	-	-	-	100

SEQUENCE LISTING

[0047]

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                                                    -25

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        -20                 -15                 -10

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Phe Pro Ala Ala Leu Ala Ala Glu Gly Asn Thr Arg Glu Asp Asn Phe
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cat gga gaa aaa att caa tta cgt gga atg agt aca cac gga tta cag      833
His Gly Glu Lys Ile Gln Leu Arg Gly Met Ser Thr His Gly Leu Gln
        45                  50                  55

tgg ttt cct gag atc ttg aat gat aac gca tac aaa gct ctt tct aac      881
Trp Phe Pro Glu Ile Leu Asn Asp Asn Ala Tyr Lys Ala Leu Ser Asn
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Asp Trp Asp Ser Asn Met Ile Arg Leu Ala Met Tyr Val Gly Glu Asn
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Val His Ala Pro Gly Asp Pro Arg Asp Pro Val Tyr Ala Gly Ala Lys
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Asp Phe Phe Arg Glu Ile Ala Ala Leu Tyr Pro Asn Asn Pro His Ile
    140                 145                 150

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Ile Tyr Glu Leu Ala Asn Glu Pro Ser Ser Asn Asn Asn Gly Gly Ala
155                 160                 165                 170

ggg att ccg aat aac gaa gaa ggt tgg aaa gcg gta aaa gaa tat gct     1217
Gly Ile Pro Asn Asn Glu Glu Gly Trp Lys Ala Val Lys Glu Tyr Ala
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Pro Asn Ser Glu Arg Gly Asn Val Met Ser Asn Thr Arg Tyr Ala Leu
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Glu Asn Gly Val Ala Val Phe Ala Thr Glu Trp Gly Thr Ser Gln Ala
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Lys Asn Glu Val Ser Gly Ala Phe Thr Pro Phe Glu Leu Gly Lys Ser
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Asn Ala Thr Asn Leu Asp Pro Gly Pro Asp His Val Trp Ala Pro Glu
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Asn Tyr Glu Pro Ile Asp Arg Thr Lys Tyr Thr Lys Val Leu Trp Asp
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Phe Asn Asp Gly Thr Lys Gln Gly Phe Gly Val Asn Ser Asp Ser Pro
    380                 385                 390

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Asn Lys Glu Leu Ile Ala Val Asp Asn Glu Asn Asn Thr Leu Lys Val
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tcg gga tta gat gta agt aac gat gtt tca gat ggc aac ttc tgg gct     1937
Ser Gly Leu Asp Val Ser Asn Asp Val Ser Asp Gly Asn Phe Trp Ala
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aat gct cgt ctt tct gcc aac ggt tgg gga aaa agt gtt gat att tta     1985
Asn Ala Arg Leu Ser Ala Asn Gly Trp Gly Lys Ser Val Asp Ile Leu
            430                 435                 440

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Gly Ala Glu Lys Leu Thr Met Asp Val Ile Val Asp Glu Pro Thr Thr
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Val Ala Ile Ala Ala Ile Pro Gln Ser Ser Lys Ser Gly Trp Ala Asn
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Pro Glu Arg Ala Val Arg Val Asn Ala Glu Asp Phe Val Gln Gln Thr
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                495                 500                 505

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Asp Phe Tyr Leu Asp Pro Val Arg Ala Thr Glu Gly Ala Met Asn Ile
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Phe Glu Gly Ala Ala Thr Thr Glu Pro Val Glu Pro Glu Pro Val Asp
                735                 740                 745

cct ggc gaa gag acg cca cct gtc gat gag aag gaa gcg aaa aaa gaa      2945
Pro Gly Glu Glu Thr Pro Pro Val Asp Glu Lys Glu Ala Lys Lys Glu
            750                 755                 760

caa aaa gaa gca gag aaa gaa gag aaa gaa gca gta aaa gaa gaa aag      2993
Gln Lys Glu Ala Glu Lys Glu Glu Lys Glu Ala Val Lys Glu Glu Lys
        765                 770                 775

aaa gaa gct aaa gaa gaa aag aaa gca gtc aaa aat gag gct aag aaa      3041
Lys Glu Ala Lys Glu Glu Lys Lys Ala Val Lys Asn Glu Ala Lys Lys
    780                 785                 790

aaa taatctatta aactagttat agggttatct aaaggtctga tgtagatctt          3094
Lys
795

ttagataacc tttttcttgc ataactggac acagagttgt tattaaagaa agtaag       3150
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Leu Val Leu Leu Leu Ser Leu Phe Pro Ala Ala Leu Ala Ala Glu Gly
            -10                 -5                  -1  1

Asn Thr Arg Glu Asp Asn Phe Lys His Leu Leu Gly Asn Asp Asn Val
    5                   10                  15

Lys Arg Pro Ser Glu Ala Gly Ala Leu Gln Leu Gln Glu Val Asp Gly
20                  25                  30                  35

Gln Met Thr Leu Val Asp Gln His Gly Glu Lys Ile Gln Leu Arg Gly
                40                  45                  50

Met Ser Thr His Gly Leu Gln Trp Phe Pro Glu Ile Leu Asn Asp Asn
            55                  60                  65

Ala Tyr Lys Ala Leu Ser Asn Asp Trp Asp Ser Asn Met Ile Arg Leu
        70                  75                  80

Ala Met Tyr Val Gly Glu Asn Gly Tyr Ala Thr Asn Pro Glu Leu Ile
    85                  90                  95

Lys Gln Arg Val Ile Asp Gly Ile Glu Leu Ala Ile Glu Asn Asp Met
100                 105                 110                 115

Tyr Val Ile Val Asp Trp His Val His Ala Pro Gly Asp Pro Arg Asp
                120                 125                 130

Pro Val Tyr Ala Gly Ala Lys Asp Phe Phe Arg Glu Ile Ala Ala Leu
            135                 140                 145

Tyr Pro Asn Asn Pro His Ile Ile Tyr Glu Leu Ala Asn Glu Pro Ser
        150                 155                 160

Ser Asn Asn Asn Gly Gly Ala Gly Ile Pro Asn Asn Glu Glu Gly Trp
    165                 170                 175

Lys Ala Val Lys Glu Tyr Ala Asp Pro Ile Val Glu Met Leu Arg Lys
180                 185                 190                 195

Ser Gly Asn Ala Asp Asp Asn Ile Ile Ile Val Gly Ser Pro Asn Trp
                200                 205                 210

Ser Gln Arg Pro Asp Leu Ala Ala Asp Asn Pro Ile Asp Asp His His
            215                 220                 225
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Thr Met Tyr Thr Val His Phe Tyr Thr Gly Ser His Ala Ala Ser Thr
230 235 240

Glu Ser Tyr Pro Ser Glu Thr Pro Asn Ser Glu Arg Gly Asn Val Met
245 250 255

Ser Asn Thr Arg Tyr Ala Leu Glu Asn Gly Val Ala Val Phe Ala Thr
260 265 270 275

Glu Trp Gly Thr Ser Gln Ala Ser Gly Asp Gly Gly Pro Tyr Phe Asp
280 285 290

Glu Ala Asp Val Trp Ile Glu Phe Leu Asn Glu Asn Asn Ile Ser Trp
295 300 305

Ala Asn Trp Ser Leu Thr Asn Lys Asn Glu Val Ser Gly Ala Phe Thr
310 315 320

Pro Phe Glu Leu Gly Lys Ser Asn Ala Thr Asn Leu Asp Pro Gly Pro
325 330 335

Asp His Val Trp Ala Pro Glu Glu Leu Ser Leu Ser Gly Glu Tyr Val
340 345 350 355

Arg Ala Arg Ile Lys Gly Val Asn Tyr Glu Pro Ile Asp Arg Thr Lys
360 365 370

Tyr Thr Lys Val Leu Trp Asp Phe Asn Asp Gly Thr Lys Gln Gly Phe
375 380 385

Gly Val Asn Ser Asp Ser Pro Asn Lys Glu Leu Ile Ala Val Asp Asn
390 395 400

Glu Asn Asn Thr Leu Lys Val Ser Gly Leu Asp Val Ser Asn Asp Val
405 410 415

Ser Asp Gly Asn Phe Trp Ala Asn Ala Arg Leu Ser Ala Asn Gly Trp
420 425 430 435

Gly Lys Ser Val Asp Ile Leu Gly Ala Glu Lys Leu Thr Met Asp Val
440 445 450

Ile Val Asp Glu Pro Thr Thr Val Ala Ile Ala Ala Ile Pro Gln Ser
455 460 465

Ser Lys Ser Gly Trp Ala Asn Pro Glu Arg Ala Val Arg Val Asn Ala
470 475 480

Glu Asp Phe Val Gln Gln Thr Asp Gly Lys Tyr Lys Ala Gly Leu Thr
485 490 495

Ile Thr Gly Glu Asp Ala Pro Asn Leu Lys Asn Ile Ala Phe His Glu
500 505 510 515

Glu Asp Asn Asn Met Asn Asn Ile Ile Leu Phe Val Gly Thr Asp Ala
520 525 530

Ala Asp Val Ile Tyr Leu Asp Asn Ile Lys Val Ile Gly Thr Glu Val
535 540 545

Glu Ile Pro Val Val His Asp Pro Lys Gly Glu Ala Val Leu Pro Ser
550 555 560

Val Phe Glu Asp Gly Thr Arg Gln Gly Trp Asp Trp Ala Gly Glu Ser
565 570 575

Gly Val Lys Thr Ala Leu Thr Ile Glu Glu Ala Asn Gly Ser Asn Ala
580 585 590 595

Leu Ser Trp Glu Phe Gly Tyr Pro Glu Val Lys Pro Ser Asp Asn Trp
600 605 610

Ala Thr Ala Pro Arg Leu Asp Phe Trp Lys Ser Asp Leu Val Arg Gly
615 620 625

Glu Asn Asp Tyr Val Ala Phe Asp Phe Tyr Leu Asp Pro Val Arg Ala
630 635 640

Thr Glu Gly Ala Met Asn Ile Asn Leu Val Phe Gln Pro Pro Thr Asn
645 650 655

Gly Tyr Trp Val Gln Ala Pro Lys Thr Tyr Thr Ile Asn Phe Asp Glu
660 665 670 675

Leu Glu Glu Ala Asn Gln Val Asn Gly Leu Tyr His Tyr Glu Val Lys
680 685 690

Ile Asn Val Arg Asp Ile Thr Asn Ile Gln Asp Asp Thr Leu Leu Arg
695 700 705

Asn Met Met Ile Ile Phe Ala Asp Val Glu Ser Asp Phe Ala Gly Arg
710 715 720

Val Phe Val Asp Asn Val Arg Phe Glu Gly Ala Ala Thr Thr Glu Pro
725 730 735

Val Glu Pro Glu Pro Val Asp Pro Gly Glu Glu Thr Pro Pro Val Asp
740 745 750 755

Glu Lys Glu Ala Lys Lys Glu Gln Lys Glu Ala Glu Lys Glu Glu Lys
760 765 770

Glu Ala Val Lys Glu Glu Lys Lys Glu Ala Lys Glu Glu Lys Lys Ala
775 780 785

Val Lys Asn Glu Ala Lys Lys Lys
790 795

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cttatattta gagggaattt ctttttaaat tgaatacgga ataaaatcag gtaaacaggt     120

cctgatttta ttttttttgaa ttttttttgag aactaaagat tgaaatagaa gtagaagaca    180

acggacataa gaaaattgta ttagttttaa ttatagaaaa cgcttttcta taattattta     240

tacctagaac gaaaatactg tttcgaaagc ggtttactat aaaaccttat attccggctc     300

ttttttttaaa caggggggtga aaattcactc tagtattcta atttcaacat gctataataa    360

atttgtaaga cgcaatatac atctttttttt tatgatattt gtaagcggtt aaccttgtgc     420

tatatgccga tttaggaagg gggtagattg agtcaagtag tcataattta gataacttat     480

aagttgttga gaagcaggag agaatctggg ttactcacaa gtttttttaaa acattatcga    540

aagcactttc ggttatgctt atgaatttag ctatttgatt caattacttt aataatttta     600

ggaggtaat atg atg tta aga aag aaa aca aag cag ttg att tct tcc att      651
          Met Met Leu Arg Lys Lys Thr Lys Gln Leu Ile Ser Ser Ile
                          -25                 -20

ctt att tta gtt tta ctt cta tct tta ttt ccg aca gct ctt gca gca      699
Leu Ile Leu Val Leu Leu Leu Ser Leu Phe Pro Thr Ala Leu Ala Ala
-15                 -10                 -5                  -1  1

gaa gga aac act cgt gaa gac aat ttt aaa cat tta tta ggt aat gac      747
Glu Gly Asn Thr Arg Glu Asp Asn Phe Lys His Leu Leu Gly Asn Asp
            5                   10                  15

aat gtt aaa cgc cct tct gag gct ggc gca tta caa tta caa gaa gtc      795
Asn Val Lys Arg Pro Ser Glu Ala Gly Ala Leu Gln Leu Gln Glu Val
        20                  25                  30

gat gga caa atg aca tta gta gat caa cat gga gaa aaa att caa tta      843
Asp Gly Gln Met Thr Leu Val Asp Gln His Gly Glu Lys Ile Gln Leu
    35                  40                  45
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cgt gga atg agt aca cac gga tta caa tgg ttt cct gag atc ttg aat      891
Arg Gly Met Ser Thr His Gly Leu Gln Trp Phe Pro Glu Ile Leu Asn
50                  55                  60                  65

gat aac gca tac aaa gct ctt gct aac gat tgg gaa tca aat atg att      939
Asp Asn Ala Tyr Lys Ala Leu Ala Asn Asp Trp Glu Ser Asn Met Ile
                70                  75                  80

cgt cta gct atg tat gtc ggt gaa aat ggc tat gct tca aat cca gag      987
Arg Leu Ala Met Tyr Val Gly Glu Asn Gly Tyr Ala Ser Asn Pro Glu
            85                  90                  95

tta att aaa agc aga gtc att aaa gga ata gat ctt gct att gaa aat     1035
Leu Ile Lys Ser Arg Val Ile Lys Gly Ile Asp Leu Ala Ile Glu Asn
        100                 105                 110

gac atg tat gtc atc gtt gat tgg cat gta cat gca cct ggt gat cct     1083
Asp Met Tyr Val Ile Val Asp Trp His Val His Ala Pro Gly Asp Pro
    115                 120                 125

aga gat ccc gtt tac gct gga gca gaa gat ttc ttt aga gat att gca     1131
Arg Asp Pro Val Tyr Ala Gly Ala Glu Asp Phe Phe Arg Asp Ile Ala
130                 135                 140                 145

gca tta tat cct aac aat cca cac att att tat gag tta gcg aat gag     1179
Ala Leu Tyr Pro Asn Asn Pro His Ile Ile Tyr Glu Leu Ala Asn Glu
                150                 155                 160

cca agt agt aac aat aat ggt gga gct ggg att cca aat aat gaa gaa     1227
Pro Ser Ser Asn Asn Asn Gly Gly Ala Gly Ile Pro Asn Asn Glu Glu
            165                 170                 175

ggt tgg aat gcg gta aaa gaa tac gct gat cca att gta gaa atg tta     1275
Gly Trp Asn Ala Val Lys Glu Tyr Ala Asp Pro Ile Val Glu Met Leu
        180                 185                 190

cgt gat agc ggg aac gca gat gac aat att atc att gtg ggt agt cca     1323
Arg Asp Ser Gly Asn Ala Asp Asp Asn Ile Ile Ile Val Gly Ser Pro
    195                 200                 205

aac tgg agt cag cgt cct gac tta gca gct gat aat cca att gat gat     1371
Asn Trp Ser Gln Arg Pro Asp Leu Ala Ala Asp Asn Pro Ile Asp Asp
210                 215                 220                 225

cac cat aca atg tat act gtt cac ttc tac act ggt tca cat gct gct     1419
His His Thr Met Tyr Thr Val His Phe Tyr Thr Gly Ser His Ala Ala
                230                 235                 240

tca act gaa agc tat ccg cct gaa act cct aac tct gaa aga gga aac     1467
Ser Thr Glu Ser Tyr Pro Pro Glu Thr Pro Asn Ser Glu Arg Gly Asn
            245                 250                 255

gta atg agt aac act cgt tat gcg tta gaa aac gga gta gca gta ttt     1515
Val Met Ser Asn Thr Arg Tyr Ala Leu Glu Asn Gly Val Ala Val Phe
        260                 265                 270

gca aca gag tgg gga act agc caa gca aat gga gat ggt ggt cct tac     1563
Ala Thr Glu Trp Gly Thr Ser Gln Ala Asn Gly Asp Gly Gly Pro Tyr
    275                 280                 285

ttt gat gaa gca gat gta tgg att gag ttt tta aat gaa aac aac att     1611
Phe Asp Glu Ala Asp Val Trp Ile Glu Phe Leu Asn Glu Asn Asn Ile
290                 295                 300                 305

agc tgg gct aac tgg tct tta acg aat aaa aat gaa gta tct ggt gca     1659
Ser Trp Ala Asn Trp Ser Leu Thr Asn Lys Asn Glu Val Ser Gly Ala
                310                 315                 320
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ttt aca cca ttc gag tta ggt aag tct aac gca aca agt ctt gac cca      1707
Phe Thr Pro Phe Glu Leu Gly Lys Ser Asn Ala Thr Ser Leu Asp Pro
            325                 330                 335

ggg cca gac caa gta tgg gta cca gaa gag tta agt ctt tct gga gaa      1755
Gly Pro Asp Gln Val Trp Val Pro Glu Glu Leu Ser Leu Ser Gly Glu
        340                 345                 350

tat gta cgt gct cgt att aaa ggt gtg aac tat gag cca atc gac cgt      1803
Tyr Val Arg Ala Arg Ile Lys Gly Val Asn Tyr Glu Pro Ile Asp Arg
    355                 360                 365

aca aaa tac acg aaa gta ctt tgg gac ttt aat gat gga acg aag caa      1851
Thr Lys Tyr Thr Lys Val Leu Trp Asp Phe Asn Asp Gly Thr Lys Gln
370                 375                 380                 385

gga ttt gga gtg aat gga gat tct cca gtt gaa gat gta gtt att gag      1899
Gly Phe Gly Val Asn Gly Asp Ser Pro Val Glu Asp Val Val Ile Glu
                390                 395                 400

aat gaa gcg ggc gct tta aaa ctt tca gga tta gat gca agt aat gat      1947
Asn Glu Ala Gly Ala Leu Lys Leu Ser Gly Leu Asp Ala Ser Asn Asp
            405                 410                 415

gtt tct gaa ggt aat tac tgg gct aat gct cgt ctt tct gcc gac ggt      1995
Val Ser Glu Gly Asn Tyr Trp Ala Asn Ala Arg Leu Ser Ala Asp Gly
        420                 425                 430

tgg gga aaa agt gtt gat att tta ggt gct gaa aaa ctt act atg gat      2043
Trp Gly Lys Ser Val Asp Ile Leu Gly Ala Glu Lys Leu Thr Met Asp
    435                 440                 445

gtg att gtt gat gag ccg acc acg gta tca att gct gca att cca caa      2091
Val Ile Val Asp Glu Pro Thr Thr Val Ser Ile Ala Ala Ile Pro Gln
450                 455                 460                 465

ggg cca tca gcc aat tgg gtt aat cca aat cgt gca att aag gtt gag      2139
Gly Pro Ser Ala Asn Trp Val Asn Pro Asn Arg Ala Ile Lys Val Glu
                470                 475                 480

cca act aat ttc gta ccg tta gga gat aag ttt aaa gcg gaa tta act      2187
Pro Thr Asn Phe Val Pro Leu Gly Asp Lys Phe Lys Ala Glu Leu Thr
            485                 490                 495

ata act tca gct gac tct cca tcg tta gaa gct att gcg atg cat gct      2235
Ile Thr Ser Ala Asp Ser Pro Ser Leu Glu Ala Ile Ala Met His Ala
        500                 505                 510

gaa aat aac aac atc aac aac atc att ctt ttt gta gga act gaa ggt      2283
Glu Asn Asn Asn Ile Asn Asn Ile Ile Leu Phe Val Gly Thr Glu Gly
    515                 520                 525

gct gat gtt atc tat tta gat aac att aaa gta att gga aca gaa gtt      2331
Ala Asp Val Ile Tyr Leu Asp Asn Ile Lys Val Ile Gly Thr Glu Val
530                 535                 540                 545

gaa att cca gtt gtt cat gat cca aaa gga gaa gct gtt ctt cct tct      2379
Glu Ile Pro Val Val His Asp Pro Lys Gly Glu Ala Val Leu Pro Ser
                550                 555                 560

gtt ttt gaa gac ggt aca cgt caa ggt tgg gac tgg gct gga gag tct      2427
Val Phe Glu Asp Gly Thr Arg Gln Gly Trp Asp Trp Ala Gly Glu Ser
            565                 570                 575

ggt gtg aaa aca gct tta aca att gaa gaa gca aac ggt tct aac gcg      2475
Gly Val Lys Thr Ala Leu Thr Ile Glu Glu Ala Asn Gly Ser Asn Ala
        580                 585                 590
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tta tca tgg gaa ttt gga tac cca gaa gta aaa cct agt gat aac tgg      2523
Leu Ser Trp Glu Phe Gly Tyr Pro Glu Val Lys Pro Ser Asp Asn Trp
    595                 600                 605

gca aca gct cca cgt tta gat ttc tgg aaa tct gac ttg gtt cgc ggt      2571
Ala Thr Ala Pro Arg Leu Asp Phe Trp Lys Ser Asp Leu Val Arg Gly
610                 615                 620                 625

gaa aat gat tat gta act ttt gat ttc tat cta gat cca gtt cgt gca      2619
Glu Asn Asp Tyr Val Thr Phe Asp Phe Tyr Leu Asp Pro Val Arg Ala
                630                 635                 640

aca gaa ggc gca atg aat atc aat tta gta ttc cag cca cct act aac      2667
Thr Glu Gly Ala Met Asn Ile Asn Leu Val Phe Gln Pro Pro Thr Asn
            645                 650                 655

ggg tat tgg gta caa gca cca aaa acg tat acg att aac ttt gat gaa      2715
Gly Tyr Trp Val Gln Ala Pro Lys Thr Tyr Thr Ile Asn Phe Asp Glu
        660                 665                 670

tta gag gaa gcg aat caa gta aat ggt tta tat cac tat gaa gtg aaa      2763
Leu Glu Glu Ala Asn Gln Val Asn Gly Leu Tyr His Tyr Glu Val Lys
    675                 680                 685

att aac gta aga gat att aca aac att caa gat gac acg tta cta cgt      2811
Ile Asn Val Arg Asp Ile Thr Asn Ile Gln Asp Asp Thr Leu Leu Arg
690                 695                 700                 705

aac atg atg atc att ttt gca gat gta gaa agt gac ttt gca ggg aga      2859
Asn Met Met Ile Ile Phe Ala Asp Val Glu Ser Asp Phe Ala Gly Arg
                710                 715                 720

gtc ttt gta gat aat gtt cgt ttt gag ggg gct gct act act gag ccg      2907
Val Phe Val Asp Asn Val Arg Phe Glu Gly Ala Ala Thr Thr Glu Pro
            725                 730                 735

gtt gaa cca gag cca gtt gat cct ggc gaa gag acg ccg cct gtc gat      2955
Val Glu Pro Glu Pro Val Asp Pro Gly Glu Glu Thr Pro Pro Val Asp
        740                 745                 750

gag aag gaa gcg aaa aaa gaa caa aaa gaa gca gag aaa gaa gag aaa      3003
Glu Lys Glu Ala Lys Lys Glu Gln Lys Glu Ala Glu Lys Glu Glu Lys
    755                 760                 765

gaa gca gta aaa gaa gaa aag aaa gaa gct aaa gaa gaa aag aaa gca      3051
Glu Ala Val Lys Glu Glu Lys Lys Glu Ala Lys Glu Glu Lys Lys Ala
770                 775                 780                 785

atc aaa aat gag gct acg aaa aaa taatctaata aactagttat agggttatct      3105
Ile Lys Asn Glu Ala Thr Lys Lys
                790

aaaggtctga tgcagatctt ttagataacc ttttttttgca taactggaca tagaatggtt      3165

attaaagaaa gcaaggtgtt tatacgatat taaaaaggta gcgattttaa attgaaacct      3225

ttaataatgt cttgtgatag aatgatgaag taatttaaga gggggaaacg aagtgaaaac      3285

ggaaatttct agtagaagaa aaacagacca agaaatactg caagctt                    3332
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Leu Val Leu Leu Leu Ser Leu Phe Pro Thr Ala Leu Ala Ala Glu Gly
            -10                 -5                  -1  1

Asn Thr Arg Glu Asp Asn Phe Lys His Leu Leu Gly Asn Asp Asn Val
    5                   10                  15

Lys Arg Pro Ser Glu Ala Gly Ala Leu Gln Leu Gln Glu Val Asp Gly
20                  25                  30                  35

Gln Met Thr Leu Val Asp Gln His Gly Glu Lys Ile Gln Leu Arg Gly
                40                  45                  50

Met Ser Thr His Gly Leu Gln Trp Phe Pro Glu Ile Leu Asn Asp Asn
            55                  60                  65

Ala Tyr Lys Ala Leu Ala Asn Asp Trp Glu Ser Asn Met Ile Arg Leu
        70                  75                  80

Ala Met Tyr Val Gly Glu Asn Gly Tyr Ala Ser Asn Pro Glu Leu Ile
    85                  90                  95

Lys Ser Arg Val Ile Lys Gly Ile Asp Leu Ala Ile Glu Asn Asp Met
100                 105                 110                 115

Tyr Val Ile Val Asp Trp His Val His Ala Pro Gly Asp Pro Arg Asp
                120                 125                 130

Pro Val Tyr Ala Gly Ala Glu Asp Phe Phe Arg Asp Ile Ala Ala Leu
            135                 140                 145

Tyr Pro Asn Asn Pro His Ile Ile Tyr Glu Leu Ala Asn Glu Pro Ser
        150                 155                 160

Ser Asn Asn Asn Gly Gly Ala Gly Ile Pro Asn Asn Glu Glu Gly Trp
    165                 170                 175

Asn Ala Val Lys Glu Tyr Ala Asp Pro Ile Val Glu Met Leu Arg Asp
180                 185                 190                 195

Ser Gly Asn Ala Asp Asp Asn Ile Ile Ile Val Gly Ser Pro Asn Trp
                200                 205                 210

Ser Gln Arg Pro Asp Leu Ala Ala Asp Asn Pro Ile Asp Asp His His
            215                 220                 225

Thr Met Tyr Thr Val His Phe Tyr Thr Gly Ser His Ala Ala Ser Thr
        230                 235                 240
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Glu Ser Tyr Pro Pro Glu Thr Pro Asn Ser Glu Arg Gly Asn Val Met
245 250 255

Ser Asn Thr Arg Tyr Ala Leu Glu Asn Gly Val Ala Val Phe Ala Thr
260 265 270 275

Glu Trp Gly Thr Ser Gln Ala Asn Gly Asp Gly Gly Pro Tyr Phe Asp
280 285 290

Glu Ala Asp Val Trp Ile Glu Phe Leu Asn Glu Asn Asn Ile Ser Trp
295 300 305

Ala Asn Trp Ser Leu Thr Asn Lys Asn Glu Val Ser Gly Ala Phe Thr
310 315 320

Pro Phe Glu Leu Gly Lys Ser Asn Ala Thr Ser Leu Asp Pro Gly Pro
325 330 335

Asp Gln Val Trp Val Pro Glu Glu Leu Ser Leu Ser Gly Glu Tyr Val
340 345 350 355

Arg Ala Arg Ile Lys Gly Val Asn Tyr Glu Pro Ile Asp Arg Thr Lys
360 365 370

Tyr Thr Lys Val Leu Trp Asp Phe Asn Asp Gly Thr Lys Gln Gly Phe
375 380 385

Gly Val Asn Gly Asp Ser Pro Val Glu Asp Val Val Ile Glu Asn Glu
390 395 400

Ala Gly Ala Leu Lys Leu Ser Gly Leu Asp Ala Ser Asn Asp Val Ser
405 410 415

Glu Gly Asn Tyr Trp Ala Asn Ala Arg Leu Ser Ala Asp Gly Trp Gly
420 425 430 435

Lys Ser Val Asp Ile Leu Gly Ala Glu Lys Leu Thr Met Asp Val Ile
440 445 450

Val Asp Glu Pro Thr Thr Val Ser Ile Ala Ala Ile Pro Gln Gly Pro
455 460 465

Ser Ala Asn Trp Val Asn Pro Asn Arg Ala Ile Lys Val Glu Pro Thr
470 475 480

Asn Phe Val Pro Leu Gly Asp Lys Phe Lys Ala Glu Leu Thr Ile Thr
485 490 495

Ser Ala Asp Ser Pro Ser Leu Glu Ala Ile Ala Met His Ala Glu Asn
500 505 510 515

Asn Asn Ile Asn Asn Ile Ile Leu Phe Val Gly Thr Glu Gly Ala Asp
520 525 530

Val Ile Tyr Leu Asp Asn Ile Lys Val Ile Gly Thr Glu Val Glu Ile
535 540 545

Pro Val Val His Asp Pro Lys Gly Glu Ala Val Leu Pro Ser Val Phe
550 555 560

Glu Asp Gly Thr Arg Gln Gly Trp Asp Trp Ala Gly Glu Ser Gly Val
565 570 575

Lys Thr Ala Leu Thr Ile Glu Glu Ala Asn Gly Ser Asn Ala Leu Ser
580 585 590 595

Trp Glu Phe Gly Tyr Pro Glu Val Lys Pro Ser Asp Asn Trp Ala Thr
600 605 610

Ala Pro Arg Leu Asp Phe Trp Lys Ser Asp Leu Val Arg Gly Glu Asn
615 620 625

Asp Tyr Val Thr Phe Asp Phe Tyr Leu Asp Pro Val Arg Ala Thr Glu
630 635 640

Gly Ala Met Asn Ile Asn Leu Val Phe Gln Pro Pro Thr Asn Gly Tyr
645 650 655

Trp Val Gln Ala Pro Lys Thr Tyr Thr Ile Asn Phe Asp Glu Leu Glu
660 665 670 675

Glu Ala Asn Gln Val Asn Gly Leu Tyr His Tyr Glu Val Lys Ile Asn
680 685 690

Val Arg Asp Ile Thr Asn Ile Gln Asp Asp Thr Leu Leu Arg Asn Met
695 700 705

Met Ile Ile Phe Ala Asp Val Glu Ser Asp Phe Ala Gly Arg Val Phe
710 715 720

Val Asp Asn Val Arg Phe Glu Gly Ala Ala Thr Thr Glu Pro Val Glu
725 730 735

Pro Glu Pro Val Asp Pro Gly Glu Glu Thr Pro Pro Val Asp Glu Lys
740 745 750 755

Glu Ala Lys Lys Glu Gln Lys Glu Ala Glu Lys Glu Glu Lys Glu Ala
760 765 770

Val Lys Glu Glu Lys Lys Glu Ala Lys Glu Glu Lys Lys Ala Ile Lys
775 780 785

Asn Glu Ala Thr Lys Lys
790

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aactaaagat tgaaatagaa gtagaagaca acggacataa gaaaattgta ttagttttaa    180

ttatagaaaa cgcttttcta taattattta tacctagaac gaaaatactg tttcgaaagc    240

ggtttactat aaaaccttat attccggctc tttttttaaa caggggggtga aaattcactc   300

tagtattcta atttcaacat gctataataa atttgtaaga cgcaatatac atctttttt     360

tatgatattt gtaagcggtt aaccttgtgc tatatgccga tttaggaagg gggtagattg    420

agtcaagtag tcataattta gataacttat aagttgttga gaagcaggag agaatctggg    480

ttactcacaa gtttttaaa acattatcga aagcactttc ggttatgctt atgaatttag     540

ctatttgatt caattacttt aataatttta ggaggtaat atg atg tta aga aag        594
                                          Met Met Leu Arg Lys
                                              -15

aaa aca aag cag ttg ggt cga cca gca caa gcc gat gga ttg aac ggt       642
Lys Thr Lys Gln Leu Gly Arg Pro Ala Gln Ala Asp Gly Leu Asn Gly
    -10                 -5                  -1  1                   5

acg atg atg cag tat tat gag tgg cat ttg gaa aac gac ggg cag cat       690
Thr Met Met Gln Tyr Tyr Glu Trp His Leu Glu Asn Asp Gly Gln His
                10                  15                  20

tgg aat cgg ttg cac gat gat gcc gca gct ttg agt gat gct ggt att       738
Trp Asn Arg Leu His Asp Asp Ala Ala Ala Leu Ser Asp Ala Gly Ile
            25                  30                  35

aca gct att tgg att ccg cca gcc tac aaa ggt aat agt cag gcg gat       786
Thr Ala Ile Trp Ile Pro Pro Ala Tyr Lys Gly Asn Ser Gln Ala Asp
        40                  45                  50

gtt ggg tac ggt gca tac gat ctt tat gat tta gga gag ttc aat caa       834
Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu Gly Glu Phe Asn Gln
    55                  60                  65
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aag ggt act gtt cga acg aaa tac gga act aag gca cag ctt gaa cga      882
Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys Ala Gln Leu Glu Arg
70                  75                  80                  85

gct att ggg tcc ctt aaa tct aat gat atc aat gta tac gga gat gtc      930
Ala Ile Gly Ser Leu Lys Ser Asn Asp Ile Asn Val Tyr Gly Asp Val
                90                  95                 100

gtg atg aat cat aaa atg gga gct gat ttt acg gag gca gtg caa gct      978
Val Met Asn His Lys Met Gly Ala Asp Phe Thr Glu Ala Val Gln Ala
            105                 110                 115

gtt caa gta aat cca acg aat cgt tgg cag gat att tca ggt gcc tac     1026
Val Gln Val Asn Pro Thr Asn Arg Trp Gln Asp Ile Ser Gly Ala Tyr
        120                 125                 130

acg att gat gcg tgg acg ggt ttc gac ttt tca ggg cgt aac aac gcc     1074
Thr Ile Asp Ala Trp Thr Gly Phe Asp Phe Ser Gly Arg Asn Asn Ala
    135                 140                 145

tat tca gat ttt aag tgg aga tgg ttc cat ttt aat ggt gtt gac tgg     1122
Tyr Ser Asp Phe Lys Trp Arg Trp Phe His Phe Asn Gly Val Asp Trp
150                 155                 160                 165

gat cag cgc tat caa gaa aat cat att ttc cgc ttt gca aat acg aac     1170
Asp Gln Arg Tyr Gln Glu Asn His Ile Phe Arg Phe Ala Asn Thr Asn
                170                 175                 180

tgg aac tgg cga gtg gat gaa gag aac ggt aat tat gat tac ctg tta     1218
Trp Asn Trp Arg Val Asp Glu Glu Asn Gly Asn Tyr Asp Tyr Leu Leu
            185                 190                 195

gga tcg aat atc gac ttt agt cat cca gaa gta caa gat gag ttg aag     1266
Gly Ser Asn Ile Asp Phe Ser His Pro Glu Val Gln Asp Glu Leu Lys
        200                 205                 210

gat tgg ggt agc tgg ttt acc gat gag tta gat ttg gat ggt tat cgt     1314
Asp Trp Gly Ser Trp Phe Thr Asp Glu Leu Asp Leu Asp Gly Tyr Arg
    215                 220                 225

tta gat gct att aaa cat att cca ttc tgg tat aca tct gat tgg gtt     1362
Leu Asp Ala Ile Lys His Ile Pro Phe Trp Tyr Thr Ser Asp Trp Val
230                 235                 240                 245

cgg cat cag cgc aac gaa gca gat caa gat tta ttt gtc gta ggg gaa     1410
Arg His Gln Arg Asn Glu Ala Asp Gln Asp Leu Phe Val Val Gly Glu
                250                 255                 260

tat tgg aag gat gac gta ggt gct ctc gaa ttt tat tta gat gaa atg     1458
Tyr Trp Lys Asp Asp Val Gly Ala Leu Glu Phe Tyr Leu Asp Glu Met
            265                 270                 275

aat tgg gag atg tct cta ttc gat gtt cca ctt aat tat aat ttt tac     1506
Asn Trp Glu Met Ser Leu Phe Asp Val Pro Leu Asn Tyr Asn Phe Tyr
        280                 285                 290

cgg gct tca caa caa ggt gga agc tat gat atg cgt aat att tta cga     1554
Arg Ala Ser Gln Gln Gly Gly Ser Tyr Asp Met Arg Asn Ile Leu Arg
    295                 300                 305

gga tct tta gta gaa gcg cat ccg atg cat gca gtt acg ttt gtt gat     1602
Gly Ser Leu Val Glu Ala His Pro Met His Ala Val Thr Phe Val Asp
310                 315                 320                 325

aat cat gat act cag cca ggg gag tca tta gag tca tgg gtt gct gat     1650
Asn His Asp Thr Gln Pro Gly Glu Ser Leu Glu Ser Trp Val Ala Asp
                330                 335                 340
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tgg ttt aag cca ctt gct tat gcg aca att ttg acg cgt gaa ggt ggt      1698
Trp Phe Lys Pro Leu Ala Tyr Ala Thr Ile Leu Thr Arg Glu Gly Gly
            345                 350                 355

tat cca aat gta ttt tac ggt gat tac tat ggg att cct aac gat aac      1746
Tyr Pro Asn Val Phe Tyr Gly Asp Tyr Tyr Gly Ile Pro Asn Asp Asn
        360                 365                 370

att tca gct aaa aaa gat atg att gat gag ctg ctt gat gca cgt caa      1794
Ile Ser Ala Lys Lys Asp Met Ile Asp Glu Leu Leu Asp Ala Arg Gln
    375                 380                 385

aat tac gca tat ggc acg cag cat gac tat ttt gat cat tgg gat gtt      1842
Asn Tyr Ala Tyr Gly Thr Gln His Asp Tyr Phe Asp His Trp Asp Val
390                 395                 400                 405

gta gga tgg act agg gaa gga tct tcc tcc aga cct aat tca ggc ctt      1890
Val Gly Trp Thr Arg Glu Gly Ser Ser Ser Arg Pro Asn Ser Gly Leu
                410                 415                 420

gcg act att atg tcg aat gga cct ggt ggt tcc aag tgg atg tat gta      1938
Ala Thr Ile Met Ser Asn Gly Pro Gly Gly Ser Lys Trp Met Tyr Val
            425                 430                 435

gga cgt cag aat gca gga caa aca tgg aca gat tta act ggt aat aac      1986
Gly Arg Gln Asn Ala Gly Gln Thr Trp Thr Asp Leu Thr Gly Asn Asn
        440                 445                 450

gga gcg tcc gtt aca att aat ggc gat gga tgg ggc gaa ttc ttt acg      2034
Gly Ala Ser Val Thr Ile Asn Gly Asp Gly Trp Gly Glu Phe Phe Thr
    455                 460                 465

aat gga gga tct gta tcc gtg tac gtg aac caa taacaaaaag ccttgagaag    2087
Asn Gly Gly Ser Val Ser Val Tyr Val Asn Gln
470                 475                 480

ggattcctcc ctaactcaag gctttcttta tgtcgcttag ctttacgctt ctacgacttt    2147

gaagcttggg gatccgtcga gacaaggtaa aggataaaac agcacaattc caagaaaaac    2207

acgatttaga acctaaaaag aacgaatttg aactaactca taaccgagag gtaaaaaaag    2267

aacgaagtcg agatcaggga atgagtttat aaaataaaaa aagcacctga aaaggtgtct    2327

ttttttgatg tctaga                                                   2343
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Asp Gly Leu Asn Gly Thr Met Met Gln Tyr Tyr Glu Trp His Leu Glu
1               5                   10                  15

Asn Asp Gly Gln His Trp Asn Arg Leu His Asp Asp Ala Ala Ala Leu
            20                  25                  30

Ser Asp Ala Gly Ile Thr Ala Ile Trp Ile Pro Pro Ala Tyr Lys Gly
        35                  40                  45
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Asn Ser Gln Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu
50 55 60

Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys
65 70 75 80

Ala Gln Leu Glu Arg Ala Ile Gly Ser Leu Lys Ser Asn Asp Ile Asn
85 90 95

Val Tyr Gly Asp Val Val Met Asn His Lys Met Gly Ala Asp Phe Thr
100 105 110

Glu Ala Val Gln Ala Val Gln Val Asn Pro Thr Asn Arg Trp Gln Asp
115 120 125

Ile Ser Gly Ala Tyr Thr Ile Asp Ala Trp Thr Gly Phe Asp Phe Ser
130 135 140

Gly Arg Asn Asn Ala Tyr Ser Asp Phe Lys Trp Arg Trp Phe His Phe
145 150 155 160

Asn Gly Val Asp Trp Asp Gln Arg Tyr Gln Glu Asn His Ile Phe Arg
165 170 175

Phe Ala Asn Thr Asn Trp Asn Trp Arg Val Asp Glu Glu Asn Gly Asn
180 185 190

Tyr Asp Tyr Leu Leu Gly Ser Asn Ile Asp Phe Ser His Pro Glu Val
195 200 205

Gln Asp Glu Leu Lys Asp Trp Gly Ser Trp Phe Thr Asp Glu Leu Asp
210 215 220

Leu Asp Gly Tyr Arg Leu Asp Ala Ile Lys His Ile Pro Phe Trp Tyr
225 230 235 240

Thr Ser Asp Trp Val Arg His Gln Arg Asn Glu Ala Asp Gln Asp Leu
245 250 255

Phe Val Val Gly Glu Tyr Trp Lys Asp Asp Val Gly Ala Leu Glu Phe
260 265 270

Tyr Leu Asp Glu Met Asn Trp Glu Met Ser Leu Phe Asp Val Pro Leu
275 280 285

Asn Tyr Asn Phe Tyr Arg Ala Ser Gln Gln Gly Gly Ser Tyr Asp Met
290 295 300

Arg Asn Ile Leu Arg Gly Ser Leu Val Glu Ala His Pro Met His Ala
305 310 315 320

Val Thr Phe Val Asp Asn His Asp Thr Gln Pro Gly Glu Ser Leu Glu
325 330 335

Ser Trp Val Ala Asp Trp Phe Lys Pro Leu Ala Tyr Ala Thr Ile Leu
340 345 350

Thr Arg Glu Gly Gly Tyr Pro Asn Val Phe Tyr Gly Asp Tyr Tyr Gly
355 360 365

Ile Pro Asn Asp Asn Ile Ser Ala Lys Lys Asp Met Ile Asp Glu Leu
370 375 380

Leu Asp Ala Arg Gln Asn Tyr Ala Tyr Gly Thr Gln His Asp Tyr Phe
385 390 395 400

Asp His Trp Asp Val Val Gly Trp Thr Arg Glu Gly Ser Ser Ser Arg
405 410 415

Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asn Gly Pro Gly Gly Ser
420 425 430

Lys Trp Met Tyr Val Gly Arg Gln Asn Ala Gly Gln Thr Trp Thr Asp
435 440 445

Leu Thr Gly Asn Asn Gly Ala Ser Val Thr Ile Asn Gly Asp Gly Trp
450 455 460

Gly Glu Phe Phe Thr Asn Gly Gly Ser Val Ser Val Tyr Val Asn Gln
465 470 475 480

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<400> 7
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<210> 8
<211> 38
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<400> 8
gttatccgct cacaattcgg atggtcatca atcactag 38

<210> 9
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<212> DNA
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<220>
<223> Oligonucleotide as PCR primer comA-B/CmF

<400> 9
cgtcgtgact gggaaaactg cgaaatcaga cggtgtac 38

<210> 10
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<400> 11
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<400> 13
cgtcgtgact gggaaaacag atgagaaagg aggagaag 38

<210> 14
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<400> 14
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<210> 15
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<210> 17
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<210> 18
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<400> 18
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<400> 19
tatacaggga ttatcagtat tgagc 25

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<400> 20
gttatccgct cacaattctt ttctccttgt tggatctg 38

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<400> 21
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<400> 22
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<210> 23
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<400> 23
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<220>
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<400> 24
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<210> 25
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<400> 27
tgtttatgta tggcggcctg cgggac 26

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gttatccgct cacaattcag ctttccatat atctcacc 38

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tgccgctgcc cgccggagag 20

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<400> 39
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<400> 51
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<400> 63
agatctgtcg gccaggttta c 20

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<400> 70
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cgccgctttc accgcggatt c 21

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<220>

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ccaaattatc ctttgtgagc gcggaatcag 30

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cttcaactaa cggggcag 18

Patentkrav

1. Rekombinant mikroorganisme fremstillet ved overførsel, til en mutant stamme af en mikroorganisme fra hvilken et hvilket som helst af *Bacillus subtilis*-generne cspB, sigL, glcT, rocR og ccpA er blevet deleteret eller knocked out, et gen der koder for et heterologt protein eller polypeptid,
hvor mikroorganismen er *Bacillus subtilis* ,hvor tre regioner sammensat af en transkriptionsinitieringsregulatorisk region, en translationsinitieringsregulatorisk region og en sekretions-signalregion er ligeret til en opstrømsregion af genet der koder for et heterologt protein eller polypeptid, hvor den transkriptionsinitierings-regulatoriske region og den translationsinitieringsregulatoriske region hver er afledt fra en 0,6 til 1 kb region opstrøms for cellulasegenet fra en bakterie hørende til slægten Bacillus, og hvor de tre regioner udgøres af den transkriptionsinitieringsregulatoriske region, den translationsinitieringsregulatoriske region, og sekretions-signalregionen er

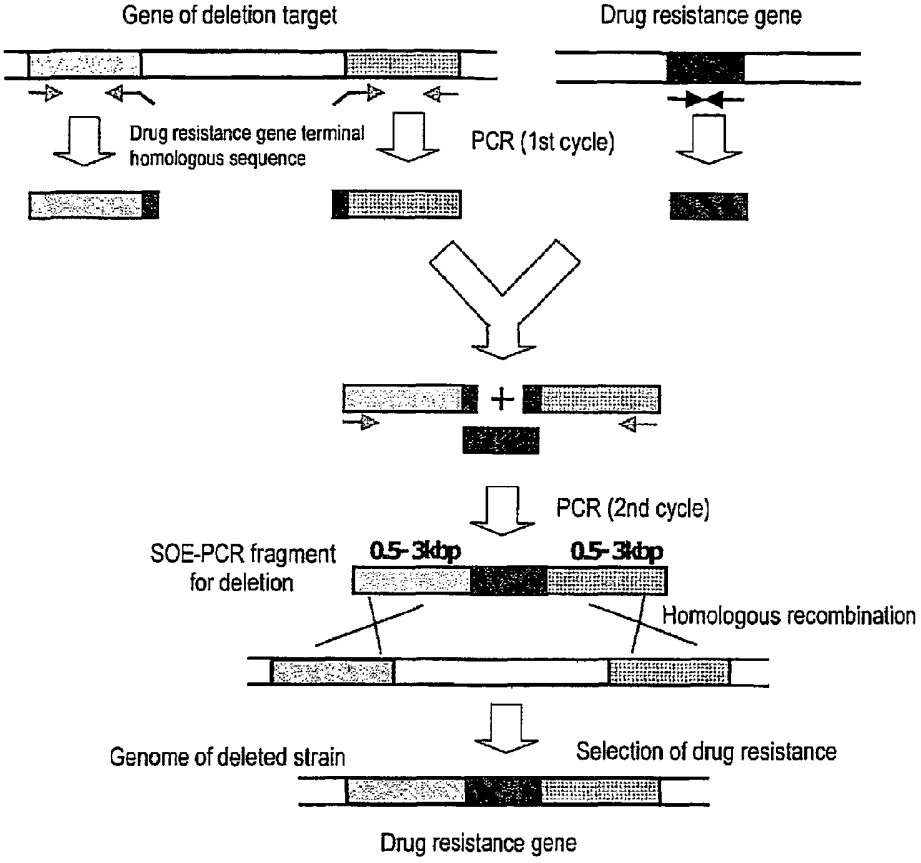
(a) en nukleotidsekvens af basenumrene 1 til 659 fra et cellulasegen ifølge SEQ ID NO: 1;

(b) en nukleotidsekvens af basenumrene 1 til 696 fra et cellulasegen ifølge SEQ ID NO: 3; eller

(c) a DNA fragment der har en nukleotidsekvens med 80% homologi med hver af nukleotidsekvenserne fra (a) eller (b).

2. Fremgangsmåde til fremstilling af et protein eller polypeptid ved anvendelse af rekombinant mikroorganisme som defineret i krav 1.

Fig. 1


Gene of deletion target
Drug resistance gene
Drug resistance gene terminal homologous sequence
PCR (1st cycle)
PCR (2nd cycle)
SOE-PCR fragment for deletion
0.5-3kbp
0.5-3kbp
Homologous recombination
Genome of deleted strain
Selection of drug resistance
Drug resistance gene