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(54) Title: GENETIC MODIFICATION OF HOMOLACTIC THERMOPHILIC BACILLI

(57) Abstract: The present invention relates to genetic modification for industrial applications of moderately thermophilic *Bacillus* species that are facultative anaerobic and homolactic. The present invention comprises a method for modifying moderately thermophilic *Bacillus* species that are facultative anaerobic and homolactic by genetic engineering comprising: introducing a DNA cloned in a thermosensitive plasmid system containing a pSH71 replicon or a homologue thereof into cells of a moderately thermophilic *Bacillus* species that is facultative anaerobic and homolactic; culturing the cells on a selective medium at a permissive temperature for plasmid replication to select transformed cells capable of growing on said selective medium at said permissive temperature; culturing said transformed cells on a selective medium at a non-permissive temperature for plasmid replication to select transformed cells capable of growing on said selective medium at said non-permissive temperature. The method according to the invention can be used to modify the *Bacilli* for R-lactic acid production, production of other organic acids than lactic acid, alcohol, enzymes, amino acids, and vitamins. In particular, the invention provides a method wherein the *Bacillus* species is modified by replacing the gene encoding S-lactate dehydrogenase by a DNA construct comprising a DNA sequence encoding R-lactate dehydrogenase.

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Genetic modification of homolactic thermophilic Bacilli

The present invention relates to genetic modification for industrial applications of moderately thermophilic *Bacillus* species that are facultative anaerobic and homolactic.

Lactic acid and its salts, known as lactate, are commercially viable products useful in various

fields including medicine, biodegradable polymers and food processing. Moderately

thermophilic *Bacillus* species that are facultative anaerobic and homolactic are ideal

5 organisms for the industrial manufacture of lactic acid. They are capable of growing at temperatures between 30-65°C and allow anaerobic industrial fermentation at temperatures above 50°C. This high temperature has several advantages when fermenting on industrial scale: less risk of infections and thus higher enantiomeric purity, faster reactions etcetera. The homolactic nature allows the production of lactic acid from hydrocarbon sources

10 (including hexose and pentose sugars; See WO 04/063382) without the formation of more than 15 wt% side products such as formic acid and acetic acid. The facultative anaerobic nature of the bacilli allows fermentation under anaerobic conditions, or at least under a low partial pressure of oxygen, which for Industrial scale is desirable because it allows for relatively inexpensive equipment and processing. Furthermore, the nutrient requirements of

15 these bacteria are less demanding than those of lactic acid bacteria such as *Lactobacillus* species, which also allows for relatively inexpensive Industrial processes. One disadvantage of the known moderately thermophilic bacilli which are facultative anaerobic and homolactic is the fact that they do not or do virtually not produce R-lactate. Since successful application of biodegradable lactic acid polymers will depend on the availability of both inexpensive S-

20 lactic acid and inexpensive R-lactic acid, a cost-effective production of both enantiomers is required. Presently known R-lactate-producing bacteria are either mesophilic (e.g. *Bacillus laevolacticus*) or have demanding nutrient requirements (e.g. *Lactobacillus delbrueckii*), which makes the manufacture of R-lactate much more expensive than that of S-lactate.

25 Thus, one object of the present invention is to avail of a moderately thermophilic *Bacillus* strain which is facultative anaerobic and produces R-lactic acid by homolactic fermentation. Another object of the invention is to avail of a method to produce genetically engineered moderately thermophilic bacilli which are facultative anaerobic and homolactic.

The genus *Bacillus* comprises over 200 different species (See Sneath, P.H.A., 1986: Endospore-forming Gram-positive rods and cocci. in Bergey's manual of systematic 5 bacteriology. Vol 2. Sneath, P.H.A., Mair, N.S., Sharpe, M.E., Holt, J.G. (eds) Williams & Wilkins, Baltimore). Only a small fraction of these are known to be genetically accessible. For instance, protoplast transformation functions for a number of different *Bacillus* species, but transformation of competent cells has generally only been shown to work satisfactory for 10 cells of the strictly aerobic and mesophilic *Bacillus subtilis* 168. Industrial strains are often more resistant to genetic modification as is known from *Bacillus licheniformis*. This facultative-anaerobic moderately thermophilic *Bacillus* species is used for the industrial production of enzymes. However, because of its heterolactic nature it cannot be used for the production of lactic acid (See Bulthuis, B. A., C. Rommens, G. M. Koningstein, A. H. 15 Stouthamer, H. W. van Verseveld, 1991: Formation of fermentation products and extracellular protease during anaerobic growth of *Bacillus licheniformis* in chemostat and batch-culture, Antonie van Leeuwenhoek 60:355-371). In general, high-frequency competence transformation procedures are not available for industrial strains (See Outrup, H., and S. T. Jørgensen, 2002: The importance of *Bacillus* species in the production of industrial enzymes in Applications and systematics of *Bacillus* and relatives, R. Berkeley, M. 20 Heyndrickx, N. Logan, and P. de Vos (eds.), pp. 206-218, Blackwell Publishing, Malden, USA). Such strains may be modified by protoplast transformation as disclosed in US 6,083,718.

To date, no methods demonstrating genetic engineering of moderately thermophilic *Bacillus* 25 species that are facultative anaerobic and homolactic are described. Although one report has been published that claims plasmid transfer from *B. subtilis* to *Bacillus coagulans* by protoplast fusion (See Ball, A.S., and C. Edwards, 1989: Properties of protoplasts from the thermophile *Bacillus coagulans* and their significance for genetic studies, Lett. Appl. Microbiol. 9:141-144), it provides no evidence that the *B. coagulans* indeed harbours the 30 plasmid. The claim is only based on the observation of growth of antibiotic-resistant colonies, which could very well have been spontaneous antibiotic-resistant mutants. We have observed such spontaneous antibiotic-resistant mutants of *B. coagulans* frequently for various types of antibiotics. In another publication: Transformation of *Bacillus* spp.: An Examination of the transformation of *Bacillus* protoplasts by plasmids pUB110 and pHV33,

Current Microbiology, Vol 13 (1986), pp 191-195, the protoplast transformation in various bacilli was described. However the transformation in *B. coagulans* was reported as unsuccessful

5 Electroporation is widely used for bacteria but requires species-specific (or even strain-specific) optimisation of the growth medium and electroporation buffer. Successful electroporation of *Bacillus* species often requires *in vivo* or *in vitro* methylation of plasmid DNA to prevent its restriction after transformation. WO 02/29030 discloses introduction of *in vivo* methylated plasmid in cells of a thermophilic *Bacillus* strain TN by electroporation. The
10 plasmid used is based on the thermosensitive plasmid pUB110. However, we found that this plasmid did not yield transformed cells when used to transform moderately thermophilic *Bacillus* species that are facultative anaerobic and homolactic.

We have now found that genetic modification of moderately thermophilic *Bacillus* species
15 that are facultative anaerobic and homolactic by genetic engineering is possible. Thus, the present invention for the first time shows the genetic engineering of moderately-thermophilic facultative-anaerobic homolactic *Bacillus* species by transformation. In addition to engineering said moderately thermophilic bacilli which are facultative anaerobic and homolactic for R-lactic acid production, the bacilli can also be genetically engineered for
20 production of industrial compounds, including other organic acids than lactic acid, alcohol, enzymes, amino acids, and vitamins.

According to the invention, it is important that the moderately thermophilic *Bacillus* species is homolactic because this ensures that the newly introduced functionality will result in high
25 yield production with a small amount of side products. Furthermore, the use of homolactic *Bacillus* species enables that only a few modifications have to be applied in order to obtain an industrially applicable micro-organism.

Moderately thermophilic *Bacillus* species are defined as bacteria that are capable of growing
30 at temperatures between 30-65 °C. Examples of moderately thermophilic facultative-anaerobic homolactic species are *Bacillus coagulans* and *Bacillus smithii*. Homolactic Bacilli can naturally produce S-lactate by homolactic fermentation. Which specific strains can produce lactic acid by homolactic fermentation can easily be determined by the person skilled in the art. The present invention also encompasses strains derived from moderately

thermophilic facultative-anaerobic *Bacillus* species, wherein the homolactic phenotype is modified. Preferably a strain or derivative is chosen which is sporulation deficient.

As no literature was available on transformation of moderately thermophilic facultative-anaerobic homolactic Bacilli, the inventors needed to find a plasmid able to replicate in these Bacilli, needed to optimize the method for introduction of DNA into the cells and needed to find a suitable selectable marker for the defined group of *Bacillus* species, in order to be able to genetically engineer these Bacilli by transformation..

10 The inventors decided to use electroporation as the method to find out which plasmids could replicate in these Bacilli. Plasmids tested were pIL253 (See Simon, D., and A. Chopin, 1988: Construction of a vector plasmid family and its use for molecular cloning in *Streptococcus lactis*, Biochimie 70:559-566), pMV158 (See Burdett, V., 1980: Identification of tetracycline resistant R-plasmids in *Streptococcus agalactiae* (group B), Antimicrob. Agents Chemother. 18: 753-766.), pHP13 (See Haima, P., S. Bron, G. Venema, 1987: The effect of restriction on shotgun cloning and plasmid stability in *Bacillus subtilis* Marburg, Mol. Gen. Genet. 209:335-342.), pUB110 (See Keggins, K.M., P. S. Lovett, E. J. Duvall, 1978: Molecular cloning of genetically active fragments of *Bacillus* DNA in *Bacillus subtilis* and properties of the vector plasmid pUB110, Proc. Natl. Acad. Sci. USA 75:1423-1427), pAMS100 (See 15 Kiewiet, R., J. Kok, J.F.M.L. Seegers, G. Venema, S. Bron, 1993: The mode of replication is a major factor in segregational plasmid instability in *Lactococcus lactis*, Appl. Environ. Microbiol. 59:358-364), pWCFS105 (See Van Kranenburg, R., N. Golic, R. Bongers, R.J. Leer, W.M. de Vos, R.J. Siezen, M. Kleerebezem, 2005: Functional analysis of three plasmids from *Lactobacillus plantarum*, Appl. Environ. Microbiol. 71: 1223-1230), pNZ280 20 (See Platteeuw, C., F. Michiels, H. Joos, J. Seurinck, and W.M. de Vos, 1995: Characterization and heterologous expression of the *tetL* gene and identification of iso-ISS1 elements from *Enterococcus faecalis* plasmid pJH1, Gene 160: 89-93.), pNZ124 (See 25 Platteeuw, C., G. Simons, and W. M. de Vos. 1994: Use of the *Escherichia coli* β -glucuronidase (*gusA*) gene as a reporter gene for analyzing promoters in lactic acid bacteria, Appl. Environ. Microbiol. 60:587-593), and pNW33n (See Zeigler, D.R. 2001: The genus *Geobacillus*; introduction and strain catalog, 7th ed., vol. 3. *Bacillus* Genetic Stock Center. www.bpsc.org). After various trials, it was found that the latter three plasmids could replicate and yielded transformants.

Once a plasmid was identified that could replicate in these *Bacilli*, other process parameters relating to the transformation protocol could conveniently be optimized. In addition, other methods for the introduction of DNA, like natural transformation or conjugation, could be tested for their feasibility.

5

Tests with antibiotic resistance markers demonstrated that at least chloramphenicol resistance, tetracycline resistance and kanamycin resistance could be used. Care should be taken to avoid too low concentrations, since these could give rise to spontaneous antibiotic resistant colonies. Introduction of the erythromycin resistance gene from pIL253 cloned in pNZ124 or pNW33n yielded no transformants.

Thus, in a first aspect, the present invention discloses a method for genetic modification of moderately thermophilic *Bacillus* species that are facultative anaerobic and homolactic by means of genetic engineering. The method comprises the steps of:

15 introducing DNA cloned in a thermosensitive plasmid system containing a pSH71 replicon or a homologue thereof into cells of a moderately thermophilic *Bacillus* species that is facultative anaerobic and homolactic;

culturing the cells on a selective medium at a permissive temperature for plasmid replication to select transformed cells capable of growing on said selective medium at said permissive

20 temperature;

culturing said transformed cells on a selective medium at a non-permissive temperature for plasmid replication to select transformed cells capable of growing on said selective medium at said non-permissive temperature.

25 The culturing of the cells at the permissive temperature on a selective medium allows for selection of transformants, i.e. cells that have taken up the transforming DNA. Preferably, transformed colonies are isolated prior to culturing the transformed cells at a non-permissive temperature, to allow checking the integrity of the transforming DNA. Cells of one or a few individual colonies are then cultured at a non-permissive temperature, to allow the selection 30 of integrants.

According to the invention, a DNA of interest is cloned in a thermosensitive plasmid system containing a pSH71 replicon (GenBank Accession Number A09338) or a homologue thereof. A pSH71 replicon is a replicon providing a thermosensitive replication functionality.

A thermosensitive replication functionality provides replication of a plasmid containing the replicon at a permissive temperature and lack of replication of said plasmid at a non-permissive temperature. This thermosensitive replication functionality of the pSH71 replicon is provided by an origin of replication and a replication protein (RepA) that is encoded by the 5 replicon.

In the context of this invention, a "pSH71 replicon or a homologue thereof" is defined as a DNA comprising a DNA sequence encoding a polypeptide having a thermosensitive replication functionality (the RepA protein) and having an amino acid sequence of SEQ ID 10 NO: 1 or an amino acid sequence that is 80% identical to SEQ ID NO:1, preferably 90% identical, more preferably 95, 96%, 97%, 98%, 99% identical. The pSH71 replicon or a homologue thereof further comprises an origin of replication where the RepA protein as defined above is capable to act upon. An example of a homologue of the pSH71 replicon is pWV01 (GenBank Accession Number X56954). The pSH71 replicon or a homologue 15 thereof may further comprise a regulatory protein (RepC).

For the purpose of the present invention, the degree of identity between two amino acid sequences refers to the percentage of amino acids that are identical between the two sequences. The degree of identity is determined using the BLAST algorithm, which is 20 described in Altschul, et al., J. Mol. Biol. 215: 403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology 25 Information (<http://www.ncbi.nlm.nih.gov/>). The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (see Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA 89: 10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

When chromosomal integration of the transforming DNA is intended, the availability of an 30 integration plasmid with a conditional replicon is desirable. Such a plasmid can be introduced under permissive conditions after which the growth conditions can be changed (e.g. by a shift to a non-permissive temperature) making the plasmid non-replicating and allowing for selection for chromosomal integration events caused by homologous or non-homologous recombination. As a conditional cloning vector, a thermosensitive replicon can be used such as pSH71 present in pNZ124, or plasmids essentially identical (homologous)

thereto or a derivative thereof having retained the plasmid's thermosensitive properties. Permissive temperatures for this replicon in moderately thermophilic *Bacillus* species that are facultative anaerobic and homolactic are preferably between 37 and 50°C. Non-permissive temperatures are preferably above 50°C. Permissive and non-permissive temperatures may not only depend on the replicon, but also on the host *Bacillus* species. The latter is a known phenomenon from the prior art, exemplified by pG+host plasmids for which 37°C is a permissive temperature in *Lactobacillus delbrueckii* but a non-permissive temperature for *Lactococcus lactis* (See US 5919678).

10 Conditional cloning vectors can also be obtained as thermosensitive derivatives of pNW33n or other plasmids able to replicate in moderately thermophilic *Bacillus* species that are facultative anaerobic and homolactic.

15 A DNA of interest cloned in a thermosensitive plasmid system containing a pSH71 replicon or a homologue thereof is introduced into cells of moderately thermophilic *Bacillus* species that are facultative anaerobic and homolactic by:

1. protoplast transformation or protoplast fusion,
2. electroporation,
3. biolistic transformation
- 20 4. conjugation, or
5. transformation of natural competent cells.

25 Transformation of these *Bacillus* species by electroporation can be achieved by a high-voltage discharge through a suspension containing a moderately thermophilic *Bacillus* species that is facultative anaerobic and homolactic and a suitable transforming DNA comprising the desired functionality and/or DNA sequences homologous to genomic sequences of the specific *Bacilli*.

30 Transformation of these *Bacillus* species by conjugation can be achieved by contacting (a population of) the moderately thermophilic *Bacillus* species that is facultative anaerobic and homolactic with (population of) a donor cell containing self-transmissible or mobilizable plasmid having the desired functionality. Self-transmissible plasmids encode all the functions they need to move among cells, and sometimes they also aid in the transfer of chromosomal DNA and mobilizable plasmids. Mobilizable plasmids do not encode all of the

proteins required for transfer and consequently need these functions to be provided by the donor genome (chromosome or plasmid-encoded). Mobilizable plasmids at least contain an origin of transfer (*oriT*) region. Any donor cell which can be co-cultured with the moderately thermophilic *Bacillus* to be modified is in principle suitable to serve as a donor cell.

5 Examples of suitable donor cells are those of *Bacillus* species, including *B. alkalophilus*, *B. amyloliquefaciens*, *B. brevis*, *B. cereus*, *B. circulans*, *B. coagulans*, *B. lautus*, *B. lentus*, *B. licheniformis*, *B. megaterium*, *B. smithii*, *B. subtilis*, *B. thermoamylovorans*, *B. thuringiensis*, *Geobacillus stearothermophilus*, *E. coli*, *Enterococcus faecalis*, *Lactobacillus* species, including *L. acidophilus*, *L. amylophilus*, *L. amylovorus*, *L. casei*, *L. coryniformis*, *L. crispatus*, *L. curvatus*, *L. delbrueckii*, *L. gasseri*, *L. helveticus*, *L. johnsonii*, *L. plantarum*, *L. reuteri*, *L. rhamnosus*, *L. sakei*, *L. sanfranciscensis*, *Streptococcus* species, including *S. agalactiae*, *S. mutans*, *S. oralis*, *S. pneumoniae*, *S. salivarius*, *S. sobrinus*, and *S. thermophilus*. Suitable self-transmissible plasmids include pRK24, pLS20, pAMβ1, or a plasmid essentially identical thereto or a derivative hereof having retained the plasmid's self-transmissible capability. Suitable mobilizable plasmids include pAT18, pAT28, pJS28, or a plasmid essentially identical thereto or a derivative hereof having retained the plasmid's mobilizing capability.

20 The inventors further managed to develop a natural transformation protocol for these *Bacillus* species. This required determining the right medium composition for growth, starvation, and transformation, the right timing for developing and harvesting competent cells, and the right transformation procedure. While natural transformation is not known to be wide-spread among *Bacillus* species the inventors discovered that moderately thermophilic bacilli that are facultative anaerobic and homolactic can be made naturally 25 competent.

For electroporation and protoplast transformation, the source of the transforming DNA may influence the transformation outcome. The source of the DNA to be transformed (isolated from either *Lactococcus lactis* MG 1363, *Escherichia coli* DH5 α , *E. coli* JM109, or *E. coli* 30 JM110) did not affect transformation efficiencies in the present *Bacillus* species, indicating that the methylation state of the DNA is not important. This is in contrast to other *Bacillus* species (see e.g. WO 02/29030).

In a preferred embodiment of the invention, the transforming DNA for introduction into

Bacillus is isolated from *Lactococcus lactis*. More preferably, cloning steps to construct the transforming DNA are also performed in *Lactococcus lactis*. This, because cloning in *E. coli* often appeared to result in deletions and/or rearrangements in the cloned DNA.

5 With the present invention, methods for transformation by preferably induction of natural competence, transformation of electrocompetent cells, and conjugation are provided, the use of a a thermosensitive plasmid is demonstrated, and application of these elements for the production of genetically engineered moderately thermophilic *Bacillus* species that are facultative anaerobic and homolactic, for instance for the production of R-lactate, is
10 demonstrated.

The transforming DNA contains a pSH71 replicon or a homologue thereof and a DNA of interest capable of providing a desired functionality to the *Bacillus* cells.

15 Chromosomal modification is the preferred modification of moderately thermophilic *Bacillus* species that are facultative anaerobic and homolactic, since chromosomal modification will ensure a stable distribution of said functionality over the progeny cells. Introduction of a desired functionality in the chromosome can be done with non-homologous as well as with homologous recombination. Homologous recombination is preferred, as it opens the
20 opportunity to introduce, to remove or to simultaneously introduce and remove a functionality.

In the context of the invention, a functionality may be a gene encoding a desired polypeptide to be produced by the cell and/or a gene encoding a polypeptide involved in production of a
25 primary or secondary metabolite by the cell and/or a DNA sequence enabling deletion of a DNA sequence from the chromosome of the cell.

Genes encoding polypeptides are provided with regulatory sequences functional in the cell, for instance a promoter sequence. The regulatory sequences may be sequences that are
30 natively associated with the coding sequence, or may be heterologous thereto.

A gene encoding a polypeptide may be fused to any regulatory or promoter sequence functioning in the *Bacillus* species of choice. Suitable promoter sequences include promoters obtainable from the *Bacillus* species of choice, hybrid promoters derived from

different native *Bacillus* promoters and artificial promoters. A preferred promoter is the promoter of a *Bacillus* gene to be inactivated by homologous recombination. Especially preferred is the *ldhL* promoter from *Bacillus coagulans*, or the promoter of the amylase gene as disclosed in US 5171673.

5

To enable selection of transformed *Bacillus* cells from the majority of untransformed cells, a selection marker is part of the transforming DNA. The selection marker may be present on the same DNA fragment or plasmid as the functionality of interest or on a separate DNA fragment or plasmid. A preferred selection marker is the *cat* gene coding for

10 chloramphenicol acetyltransferase from pMH3.

Desired functionalities that can be introduced are R-lactic acid production as described below, and other functionalities that provide the production of compounds that are metabolizable from pyruvate. Examples of these compounds are pyruvate, acetolactate, 15 diacetyl, acetoin, 2,3-butanediol, 1,2-propanediol, acetate, formate, acetaldehyde, ethanol, L-alanine, oxaloacetate, S-malate, succinate, fumarate, 2-oxoglutarate, oxalosuccinate, isocitrate, citrate, glyoxylate.

When homologous recombination is intended, the transforming DNA further contains a DNA 20 sequence that is homologous to a genomic target sequence of the specific *Bacillus* to be engineered. The skilled person will understand that no 100% identity is required to obtain homologous recombination. A percentage identity of about 90% will also suffice. Generally, the DNA sequence of interest to be inserted in the chromosome by homologous recombination is flanked by homologous sequences with a sufficient length to enable 25 homologous recombination. Such a length may be at least about 200 bp, for instance between about 200 and about 1500 bp, preferably between about 200 and about 1000 bp.

The present invention is directed to the modification by means of genetic engineering of 30 moderately thermophilic *Bacillus* species that are facultative anaerobic and homolactic wherein a desired functionality is introduced, preferably by homologous recombination.

The present invention is also directed to the modification by means of genetic engineering of moderately thermophilic *Bacillus* species that are facultative anaerobic and homolactic wherein an undesired functionality is removed by homologous recombination.

The invention is further directed to the modification by means of genetic engineering of moderately thermophilic *Bacillus* species that are facultative anaerobic and homolactic wherein a desired functionality is introduced in the chromosome and at the same time an 5 undesired functionality is removed by homologous recombination.

The genetically engineered moderately thermophilic *Bacillus* strains that are facultative anaerobic and homolactic or derivatives of a parent strain that is homolactic, that are obtainable by said modification by means of genetic engineering form a further aspect of this 10 invention. The modification by means of genetic engineered includes introduction of a functionality in, removal of a functionality from or simultaneous introduction of a functionality in and removal of a functionality from the bacterial chromosome.

In a preferred embodiment, the modification by means of genetic engineered occurs by 15 homologous recombination.

In one preferred embodiment, the genetically engineered derivative of a moderately thermophilic *Bacillus* species that is facultative anaerobic and homolactic is a strain wherein the conversion of pyruvate to lactate is blocked and pyruvate accumulates, or wherein 20 additional modifications are applied to redirect pyruvate towards production of other products including acetolactate, diacetyl, acetoin, 2,3-butanediol, 1,2-propanediol, acetate, formate, acetaldehyde, ethanol, L-alanine, oxaloacetate, S-malate, succinate, fumarate, 2-oxoglutarate, oxalosuccinate, isocitrate, citrate, glyoxylate.

25 In another preferred embodiment, the genetically engineered derivative of a moderately thermophilic *Bacillus* species that is facultative anaerobic and homolactic is a strain wherein the *ldhL* gene coding for S-lactate dehydrogenase activity is removed by homologous recombination.

30 In another preferred embodiment, the genetically engineered derivative of a moderately thermophilic *Bacillus* species that is facultative anaerobic and homolactic is a strain wherein the *ldhL* gene coding for S-lactate dehydrogenase activity is replaced with a gene coding for an NADH-dependent 2-hydroxyacid dehydrogenase having R-lactate dehydrogenase activity by homologous recombination.

In these embodiment, the construct that is used for homologous recombination contains a defective *ldhL* gene that does not encode a functional S-lactate dehydrogenase enzyme. A defective *ldhL* gene can be provided by a construct wherein part or all of the *ldhL* coding sequence is deleted.

A defective *ldhL* gene may be created by replacing part or all of the *ldhL* gene with another gene, for instance a gene encoding a selection marker or a gene of interest. Preferably, the *ldhL* gene coding for S-lactate dehydrogenase activity is replaced by a construct containing a gene coding for an NADH-dependent 2-hydroxyacid dehydrogenase having R-lactate dehydrogenase activity, including enzymes with EC number EC 1.1.1.28..

Suitable genes coding for R-lactate dehydrogenase activity are those genes that are able to complement an *E. coli* *ldhA*⁻ mutant, such as *E. coli* FMJ144 as described by Bernard et al.

(See Bernard, N., T. Ferain, D. Garmyn, P. Hols, and J. Delcour, 1991: Cloning of the D-lactate dehydrogenase gene from *Lactobacillus delbrueckii* subsp. *bulgaricus* by complementation in *Escherichia coli*. FEBS Lett. 290:61-64). A suitable *ldhA* gene coding for R-lactate dehydrogenase activity for instance is the *ldhA* gene from *Lactobacillus delbrueckii* subsp. *bulgaricus* (see also Bernard et al.) or the *hddD* gene from the same species

(Bernard, N., K. Johnsen, T. Ferain, D. Garmyn, P. Hols, J.J. Holbrook, and J. Delcour, 1994. NAD⁺-dependent D-2-hydroxyisocaproate dehydrogenase of *Lactobacillus delbrueckii* subsp. *bulgaricus*. Eur. J. Biochem. 224:439-446). The amino acid sequence of the R-lactate dehydrogenase encoded by the *ldhA* gene is depicted in SEQ ID NO: 2. It appears that polypeptides encoded by genes capable of the complementation of the above *E. coli* mutant substantially differ in amino acid sequence. A percentage identity as low as 30% with the amino acid sequence of SEQ ID NO: 2 is feasible. The *ldhA* and the *hddD* genes appear to encode proteins with a degree of identity of about 50%.

Suitable genes coding for R-lactate dehydrogenase activity are those genes that encode an amino acid sequence of SEQ ID NO:2 or homologous genes that encode an amino acid sequence that displays a degree of identity of at least 30%, more preferably at least 40%, even more preferably at least 50%, 60%, 70%, 80%, 90%, to the amino acid sequence of SEQ ID NO: 2 and that are able to complement an *E. coli* *ldhA*⁻ mutant, such as *E. coli*

FMJ144. Such homologous sequences may encompass polymorphisms that may exist in cells from different populations or within a population due to natural allelic or intra-strain variation. A homologue may further be derived from a *Bacillus* species other than the species where the specified DNA or amino acid sequence originates from, or may be 5 artificially designed and synthesized.

In addition to introducing desired functionalities, genetic engineering can be used to optimize the industrial fermentation, e.g. to enable the fermentation of cheap substrates like lignocellulose-derived sugars including xylose and arabinose and/or to remove undesired 10 functionalities.

In a further aspect, the present invention provides a process for the production of a compound of interest comprising culturing the genetically engineered strain of the previous aspect under conditions conducive to production of said compound.

15

Description of the Figures

Figure 1 shows restriction analyses of pNW33N isolated from three *E. coli* isolates that were transformed with plasmid material isolated from three transformed *B. coagulans* DSM 1 20 isolates. Lanes 1, 4, and 7. pNW33N digested with *Eco*RI; 4217-bp fragment. Lanes 2, 5, and 8. pNW33N digested with *Eco*RI-*Stu*I; 333-bp and 3884-bp fragments. Lanes 3 and 6. Kb DNA ladder (Stratagene) 250 bp, 500 bp, 750 bp, 1.0 kb, 1.5 kb, 2.0 kb, 3.0 kb, 4.0 kb, 5.0 kb, 6.0 kb, 7.0 kb, 8.0 kb, 9.0 kb, 10.0 kb, and 12.0 kb.

25 Figure 2 shows a plasmid map of pJS28. The replication gene (repB) and the chloramphenicol resistance gene (cat) are depicted by arrows. The IncP plasmid RK2 origin of transfer (oriT) and the *E. coli* origin of replication (ori) are depicted as boxed regions.

30 Figure 3 shows the nucleotide sequence of synthetic *B. coagulans* ATCC 23498 amylase promoter region based on the sequence disclosed in US 5171673. *Bgl*II (AGATCT), *Bam*HI (GGATCC), and *Nco*I (CCATGG) cloning sites are underlined. The *Nco*I site allows translational fusion to the amylase promoter. The ATG start codon of the amylase gene is depicted boldface.

Figure 4 shows a plasmid map of pJS25. Replication genes (*repA* and *repC*), the chloramphenicol resistance gene without *N*coI site (*cm**) are indicated by arrows. The *B. coagulans* promoter region (P) is depicted as boxed region.

5 Figure 5 shows a plasmid map of pJS26. Replication genes (*repA* and *repC*), the chloramphenicol resistance gene without *N*coI site (*cm**), and the LMG 6901 *ldhA* gene (*ldhA* 6901) are indicated by arrows. The *B. coagulans* promoter region (P) is boxed.

10 Figure 6 shows a plasmid map of pJS27. The replication gene (*repB*), the chloramphenicol resistance gene (*cat*), and the *L. bulgaricus* LMG 6901 *ldhA* gene (*ldhA* 6901) are depicted by arrows. The *B. coagulans* promoter region (P) and the *E. coli* origin of replication (ori) are depicted as boxed regions.

15 Figure 7 shows a plasmid map of pRK1. The replication genes (*repA* and *repC*), the chloramphenicol resistance gene (*cat*), and the *L. bulgaricus* *ldhA* gene (*ldhA*) are depicted by arrows. The *B. coagulans* promoter region (Pamy ATTC) and *B. coagulans* *ldhL* upstream and downstream regions are depicted as boxed regions.

20 The present invention is further illustrated by means of the following non-limitative Examples.

EXAMPLES

25 MATERIALS AND METHODS

Plasmids and strains

Plasmid pNZ124 (Platteeuw, C., G. Simons, and W. M. de Vos. 1994. Use of the Escherichia coli β -glucuronidase (*gusA*) gene as a reporter gene for analyzing promoters in lactic acid bacteria.

30 *Appl. Environ. Microbiol.* 60:587-593) was obtained from NIZO food research. It is based on the cloning vector pNZ12 disclosed in EP 0228726 B1.

Plasmid pNW33N (Zeigler, D.R. 2001: The genus Geobacillus; introduction and strain catalog, 7th ed., vol. 3. *Bacillus* Genetic Stock Center. www.bpsc.org) was obtained from the *Bacillus* Genetic Stock Center, Ohio State University, Columbus, Ohio, USA, and propagated in *Escherichia coli* DH5 α (Invitrogen Life Technologies). The nucleotide

sequence of pNW33N is available under GenBank accession number AY237122.

Plasmid pAT Δ S28 containing the IncP plasmid RK2 origin of transfer (*oriT*) (Namy, O., M.

Mock, A. Fouet, 1999: Co-existence of *c/pB* and *c/pC* in the Baciliceae. FEMS Microbiol.

Lett. 173: 297-302) was obtained from Institute Pasteur.

5 *B. coagulans* DSM 1 was obtained from DSMZ, Braunschweig, Germany.

Lactococcus lactis subsp. *cremoris* MG1363 was described by Gasson (M. J. Gasson. 1983:

Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. 154:1-9).

Lactobacillus delbrueckii subsp. *bulgaricus* LMG 6901 was obtained from the BCCM/LMG

10 bacteria collection, Gent, Belgium.

E. coli HB101 harbouring pRK24 was described by Trieu-Cuot, et al. (Trieu-Cuot, P., C.

Carlier, P. Martin, and P. Courvalin, 1987: Plasmid transfer by conjugation from *Escherichia coli* to Gram-positive bacteria. FEMS Microbiol. Lett. 48:289-294).

15 *Culture conditions*

B. coagulans was routinely grown at 45°C under aerobic conditions in BC-broth (BC for *B. coagulans*) containing 10 g/L yeast extract, 2 g/L di-ammoniumphosphate, 3.5 g/L di-ammoniumsulphate, 10 g/L Bis-Tris buffer (bis[2-hydroxymethyl]iminotris[hydroxymethyl]-methane), 3 mg/L CaCl₂, 5 mg/L MgCl₂; if appropriate the medium was supplemented with

20 50 g/L sucrose; pH was adjusted to 6.6 - 6.7 and medium was autoclaved (20 min 121°C) before use. For plates the medium was supplemented with 10 g/L Gelrite and 1 g/L MgCl₂. Filter sterilised trace elements were added separately. Final concentrations were: 0.2 mg/L CoCl₂ · 6 H₂O, 0.01 mg/L CuCl₂ · 2 H₂O, 0.3 mg/L H₃BO₃, 0.03 mg/L Na₂MoO₄ · 2 H₂O, 0.02 mg/L NiSO₄ · 6 H₂O, 0.03 mg/L MnCl₂ · 4 H₂O, 0.05 mg/L ZnCl₂. If appropriate, the media

25 were supplemented with filter sterilised chloramphenicol at 7 mg/L. Competence medium (C-broth) contained 0.05 g/L yeast extract, 2 g/L di-ammoniumphosphate, 3.5 g/L di-ammoniumsulphate, 10 g/L glucose, 10 mg/L CaCl₂, 0.5 g/L KCl, 25 mg/L MgCl₂; pH was adjusted to 6.8 and medium was autoclaved (20 min 121°C) before use. Filter sterilised trace elements and vitamins were added separately. Final concentrations were: 2.4 mg/L

30 CoCl₂, 3,6 mg/L FeCl₃, 3 mg/L MnCl₂, 1.2 mg/L ZnCl₂, 0.024 mg/L biotine, 0.012 mg/L thiamine, 20 mg/L methionine. Transformation medium (T-broth) was C-broth with 0.025 g/L yeast extract instead of 0.05 g/L.

E. coli was routinely cultured in LB broth (Molecular Cloning, a laboratory manual. 3rd edition.

J. Sambrook and D. W. Russell. 2001. Cold Spring Harbor Laboratory Press, New York) at 37°C under aerobic conditions. When appropriate chloramphenicol and/or ampicillin were used at concentrations of 5 mg/L and 100 mg/L, respectively.

L. bulgaricus was routinely cultured in MRS broth ® (BD Biosciences) at 37°C under

5 anaerobic conditions.

L. lactis was routinely cultured in M17 broth ® (BD Biosciences) supplemented with 0.5% glucose at 30°C under anaerobic conditions. When appropriate chloramphenicol was used at a concentration of 5 mg/L.

10 *DNA manipulation techniques*

Standard DNA manipulation techniques were performed as described by Sambrook and Russell (J. Sambrook and D. W. Russell. 2001: Molecular Cloning, a laboratory manual. 3rd edition. Cold Spring Harbor Laboratory Press, New York).

15 Construction of pNZ124 and pNW33N derivatives was performed in *L. lactis* and *E. coli*, respectively.

Large-scale *E. coli* plasmid DNA isolation from 100 mL culture was performed using the Jetstar 2.0 Plasmid Maxiprep Kit ® (Genomed) following the instructions of the manufacturer. Small-scale *E. coli* plasmid DNA isolation from 1 mL culture was performed using the Nucleospin Plasmid Quick Pure ® (Macherey-Nagel) kit following the instructions of the manufacturer.

20 Large-scale *B. coagulans* plasmid DNA isolation was performed using equilibrium centrifugation in cesium chloride-ethidium bromide gradients. Two overnight cultures of 300 mL grown under aerobic conditions (170 rpm) at 45°C were harvested by centrifugation. The cell pellets were pooled and resuspended in 7 mL of a buffer containing 2 mg/mL lysozyme, 25 30 mM Tris/HCl, pH 8.0, 3 mM MgCl₂, and 25% sucrose, and was incubated for 15 minutes on ice. Cells were lysed by addition of 16 mL of a solution containing 0.2 M NaOH and 1% SDS. After 5 min. incubation on ice the sample was neutralized by addition of 12 mL of 3M KAc and mixing. Precipitates were removed by centrifugation. The DNAs in the supernatants were precipitated by addition of 20 mL of isopropanol. The DNAs were 30 pelleted by centrifugation, dried and dissolved in TE buffer containing 1.0 g/mL CsCl and 0.4 mg/mL ethidiumbromide. Chromosomal and plasmid DNAs were separated by cesiumchloride density gradients using a vertical rotor (Stepsaver 65 V13 ®; Sorvall) at 45000 rpm for 16 h. Plasmid DNA was collected and ethidium bromide was removed as

described elsewhere (Molecular Cloning, a laboratory manual. 3rd edition. J. Sambrook and D. W. Russell. 2001. Cold Spring Harbor Laboratory Press, New York).

Small-scale *L. lactis* and *B. coagulans* plasmid DNA isolation from 10 mL culture was performed following a mini-prep protocol for Gram-positive bacteria (Rapid mini-prep

5 isolation of high quality plasmid DNA from *Lactococcus* and *Lactobacillus* spp. D. J. O'Sullivan and T. R. Klaenhammer. 1993. *Appl. Environ. Microbiol.* 59:2730-2733).

E. coli competent cells were prepared using calcium chloride and transformed by heat shock as described by Sambrook and Russell (Molecular Cloning, a laboratory manual. 3rd edition. J. Sambrook and D. W. Russell. 2001. Cold Spring Harbor Laboratory Press, New York).

10 *L. lactis* was transformed by electroporation as described by Holo and Nes (High-frequency transformation by electroporation of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. 1989. Holo, H., and I. F. Nes. *Appl. Environ. Microbiol.* 55:3119-3123).

PCR reactions for cloning purposes were performed with the high-fidelity Pwo polymerase 15 (Roche) following the instructions of the manufacturer.

Colony-PCR analysis was used to demonstrate the presence of pNW33N in the chloramphenicol resistant colonies. PCR primers were designed for identification of the pNW33N replication gene *repB* with the sequences 5'-TCGCCTTCTTCTGTGTCATC-3' and 5'-CTGGAGGAGAGCAATGAAAC-3'. Colonies were picked with a tooth pick and a little cell 20 material was transferred to a 0.5 mL PCR reaction tube. The cells were disrupted by 1 min incubation at 1000 W in a microwave oven. PCR reaction mixtures of 50 µL with rTaq polymerase (Amersham Biosciences) and 0.5 µg of each primer were prepared as recommended by the manufacturer and added to the reaction tubes with the disrupted cells. 25 PCR reactions were performed in a RoboCycler ® (Stratagene). 4 min of incubation at 94°C was followed by 25 cycles of 30 sec at 94°C for denaturation, 1 min at 58°C for primer annealing, and 1 min at 72°C for elongation. After the final cycle the reaction mixture was incubated at 72°C for another 5 min.

Electroporation

30 Preparation of electrocompetent cells required determination of the proper culture conditions, time of harvest, and composition of wash and electroporation buffers. Although *B. coagulans* DSM 1 is able to grow on LB broth, the developed BC broth increased electroporation efficiencies. Glycine was added to weaken the cell wall, and the concentrations were optimized. Glycine concentrations were between 0.5 and 2.0% and

preferably between 1.0 and 1.5%. Cells were harvested at early to mid-log phase for optimal results. The electroporation buffer had a pH between 4.3 and 6.0 and preferably between 4.3 and 5.0. The optimal electroporation settings (voltage, resistance, capacitance) also needed to be determined. Voltage preferably was between 1.0 and 2.5 kV, and more preferably between 1.25 and 2.0 kV. Resistance was preferably between 100 and 800 Ω , and more preferably between 200 and 600 Ω . Recovery before plating on a selective medium is important and was at least 2 hours and preferably 3 hours.

5 Electrocompetent cells of *B. coagulans* were prepared as follows. An overnight culture was used to inoculate (5% volume/volume) 50 mL of medium supplemented with 1% glycine

10 (resulting in a turbidity at 600nm of approximately 0.13-0.14). After 2.5 hrs of aerobic incubation at 45°C cells were harvested by centrifugation. Cell pellets were washed twice with 50 mL and 25 mL, respectively, of ice-cold electroporation buffer (5 mM KH₂PO₄, 0.4 M sorbitol, 10% glycerol, 4 mM MgCl₂ adjusted to pH 4.5), and resuspended in 1 mL of ice-cold electroporation buffer. For electroporation, 100 μ L of the cell suspension was mixed with 1

15 μ g plasmid DNA and transferred to a 0.2 cm electroporation cuvet (Bio-Rad ®) that was precooled on ice. The sample was subjected to a 1.6 kV pulse at 200 Ω and 25 μ F using a Gene Pulser and a Pulse Controller apparatus (Bio-Rad®). Immediately after electroporation 1 mL of medium was added and the cells were incubated for 3 hours at 45°C in a

15 Thermomixer ® (Eppendorf) at 900 rpm, after which they were plated on plates

20 supplemented with chloramphenicol. The plates were incubated at 45°C for 1 to 2 days under aerobic conditions.

Conjugation

Filter matings were used for conjugal transfer of recombinant plasmids from *E. coli* to *B.*

25 *coagulans*. Logarithmic growing cells of donor (2 mL) and recipient (2 mL) were pooled and using a syringe harvested on a 0.45 μ m cellulose acetate filter (Schleiger & Schuell) sterilized in a plastic filter holder (Schleiger & Schuell). Cells were washed with 10 mL of BC-broth and the filters were dried by forcing air through the filter. Filters were placed on the surface of BC-plates without antibiotics and incubated overnight at 45°C. After the mating

30 the cells were resuspended from the filter in BC-broth and dilution series were plated on BC-plates containing 7 mg/L chloramphenicol and incubated at 55°C aerobically for 1-2 days.

Enzyme analyses

Enzyme overproduction in *B. coagulans* using the *B. coagulans* promoter was determined in exponentially growing cultures. Cells were harvested by centrifugation. Cell-free extracts were prepared using a FastPrep FP120 ® apparatus (Qbiogene) in two runs of 30 sec at

5 speed 4. Cells were cooled on ice for 1 min between the runs. Protein content was determined by the method of Bradford (Bio-Rad) using Bovine Serum Albumine as a standard. SDS-PAGE (12.5%) was performed as described by Sambrook and Russell (Molecular Cloning, a laboratory manual. 3rd edition. J. Sambrook and D. W. Russell. 2001. Cold Spring Harbor Laboratory Press, New York) using the Protean II electrophoresis

10 system (Bio-Rad). R-lactate dehydrogenase-specific activities were determined spectrophotometrically at 340 nm and carried out at 50°C, using 1 ml of an assay mix containing 0.3 M glycylglycine buffer pH10, 0.25% (v/v) Triton X-100, 5 mM NAD, and 1% R-lactate. The reaction was started by the addition of 40 or 50 µL of cell-free extract. Specific activities were expressed as $\Delta A \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ where ΔA is the increase in absorbance at 340 nm for 1 cm path length and mg the amount of protein.

Fermentations

Batch fermentations were performed in a bioreactor (7 L Applikon ®) with 4 L of BC broth

20 without Bis-Tris buffer and supplemented with 30 g/L glucose. An overnight aerobic culture (40 mL) grown at 50°C and inoculated from glycerolstock was transferred to 360 mL fresh BC broth and incubated for another 4-5 hrs. This 400 mL was used to inoculate the bioreactor. The pH maintenance was achieved by the automatic addition of a lime solution at 20% (w/v). The fermentation was performed at 54°C, pH 6.5 and agitation speed of 250-300 rpm. The temperature control was performed with a water bath (Lauda) while the pH reading/control was performed by ADI 1020 Bio-Processor (Applikon ®). All the data (pH and base consumption) were processed by online data acquisition (Applikon FM V5.0 ®). Samples were withdrawn before inoculation and at the end of fermentation for measurement of R- and S-lactic acid, and possible by-products. Samples were centrifuged and remaining debris was removed by filtration using a Millex GP 0.22 µm filter ® (Millipore). Filtrate was stored at -21°C until further analysis.

Organic acids (lactic acid, acetic acid, formic acid, succinic acid) were measured using a derivatisation and GLC. R- and S-lactates were methylated to methyl-lactate and measured

by headspace analysis on a chiral column.

RESULTS

5

EXAMPLE 1. Transformation of *B. coagulans* with plasmid pNW33N using natural competent cells

1) Preparation of competent cells of *B. coagulans* DSM 1

In three independent experiments the following procedures were used. *Bacillus coagulans*

10 DSM 1 was cultured overnight at 45°C in 5 ml BC-broth under aerobic conditions. This culture was used to inoculate 25 ml of prewarmed C-broth resulting in a turbidity at 600 nm of 0.15. The fresh culture was incubated aerobically at 45°C until a turbidity at 600 nm between 0.9 – 1.2 was reached (Table 1).

15 2) Transformation of *B. coagulans* DSM 1 with pNW33N

A 0.5 mL portion of the competent cells was pelleted and the pellet was resuspended in 0.1 mL of T-broth and mixed with 5 µg of pNW33N plasmid DNA. After 1.5 h incubation at 45°C in a Thermomixer (Eppendorf) at 900 rpm, 0.3 mL of prewarmed BC broth was added and the incubation was continued for 2 h, after which the cells were plated on BC plates

20 supplemented with 7 mg/L chloramphenicol. The plates were incubated aerobically at 45°C. After 3 days of incubation colonies had appeared (Table 1).

TABLE 1. Natural transformation of *B. coagulans* DSM 1

Turbidity at 600 nm	Number of colonies
0.92	24
0.97	3
1.16	2

3) Plasmid isolation from *B. coagulans* DSM 1

25 The colonies were striped to fresh BC plates supplemented with 7 mg/L chloramphenicol for overnight incubation at 45°C. Colony PCR was used to detect the presence of the *repB* gene from plasmid pNW33N. All PCR reactions yielded a product of the expected size. One

colony from each experiment was transferred to BC-broth for O/N incubation and miniprep plasmid isolation. Although no plasmid DNA could be visualized by agarose gel electrophoresis, transformation of *E. coli* DH5 α with this miniprep DNA resulted in transformants from which pNW33N could be retrieved. Plasmid DNA was digested with 5 *Eco*RI-*S*taI to confirm the integrity of pNW33N. The restriction patterns were as expected (Fig. 1) demonstrating that plasmid pNW33N was transformed to *B. coagulans* DSM 1.

EXAMPLE 2. Transformation of *B. coagulans* with plasmid pNW33N using electroporation

10 *B. coagulans* DSM 1 was routinely transformed with 1 μ g plasmid pNW33N by electroporation. Typically, after 1 day of incubation at 45°C more than 20 colonies appeared on chloramphenicol-containing plates. In one experiment 15 colonies were transferred to fresh chloramphenicol-containing plates after 2 days of incubation. After 24 h of incubation at 45°C transformation was confirmed by colony-PCR analysis using *repB*-specific primers. 15 PCR products of the expected size were obtained for all transformants tested, demonstrating that the *repB* gene of pNW33N was present. *B. coagulans* DSM 1 was used as negative control and yielded no PCR products. Plasmid DNA of a single transformant was isolated from a cesium-chloride gradient. Plasmid DNA was digested with *Eco*RI and with *Eco*RI-*S*taI to confirm the integrity of pNW33N. The restriction patterns were as 20 expected (333-bp and 3884-bp fragments) demonstrating that plasmid pNW33N was transformed to *B. coagulans* DSM 1.

EXAMPLE 3. Transformation of *B. coagulans* with plasmid pNW33N using conjugation

25 1) Construction of pNW33N-derivative containing an origin of transfer. The origin of transfer (*oriT*) of the IncP plasmid RK2 was cloned into pNW33N. This allows effective comobilization by any self-transferable IncP plasmid co-resident in *E. coli* donors (Plasmid transfer by conjugation from *Escherichia coli* to Gram-positive bacteria, Trieu-Cuot, P., Carlier, C., Martin, P., and Courvalin, P. 1987. FEMS Microbiol. Lett. 48:289-294). The 30 RK2 *oriT* region was cloned as a blunted 0.5-kb *Acc*I-*Av*I fragment derived from pAT Δ S28 in pNW33N digested with *Sma*I. The resulting plasmid, pJS28 (Fig. 2), was transformed to *E. coli* HB101 harbouring pRK24. This strain was used as donor in plate matings with *B. coagulans* DSM 1.

2) Conjugal transfer of pJS28 from *E. coli* to *B. coagulans*.

An overnight culture of *E. coli* harbouring pJS28 and pRK24 grown aerobically at 37°C in LB

5 containing ampicillin and chloramphenicol was transferred 1/50 to fresh LB broth with antibiotics and cultured to a turbidity at 600 nm of 0.56. An overnight culture of *B. coagulans* DSM 1 grown aerobically at 45°C in BC broth was transferred 1/50 to fresh BC broth and cultured to a turbidity at 600 nm of 0.52. Equal volumes (2 mL) of *E. coli* and *B. coagulans* were harvested on a 0.45 µm filter. The filter containing the cells was transferred to BC 10 plates without antibiotics and incubated overnight at 45°C. The cells were removed from the filter and dilution series were plated on BC plates and incubated at 55°C aerobically. After 1-2 days colonies appeared. The presence of pJS28 was confirmed by plasmid DNA isolation and the integrity was confirmed by digestion with *N*col yielding the expected digestion pattern (1444-bp and 3299-bp fragments). The efficiency of conjugation was approximately 15 9.3 10⁷ per recipient.

EXAMPLE 4. Construction of a *B. coagulans*-derived expression system

1) Construction of *B. coagulans* expression system

A *B. coagulans* ATCC 23498 nucleotide sequence fragment (disclosed in US 5171673)

20 having promoter activity was produced as a synthetic DNA fragment (Fig. 3) and cloned as *Bg*II-*Bam*HI fragment into pMH3 digested with the same enzymes. The resulting cloning vector, pJS25 (Fig. 3), allows translational fusion to this promoter by the use of an *N*col site overlapping the start codon. To enable the use of this *N*col site, first plasmid pMH3 was constructed from plasmid pNZ124 by removing the *N*col site from the *cat* gene using a 25 megaprimer. Primers with the sequences 5'-CTATTATTCCGTGGACTTC-3' and 5'-CAGCTGAGATCTTGGAG-3' were used to generate the megaprimer, which was used in a second PCR reaction in combination with a primer having the sequence 5'-GACGAAAGTCGACGGCAATAGTTAC-3'. The resulting PCR product encompassed the complete *cat* gene and was digested with *Bg*II-*S*alI to replace the pNZ124 *cat* gene 30 generating plasmid pMH3. Plasmid pJS25 (Fig. 4) can be used in various mesophilic Gram-positive organisms including *Bacillus subtilis*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactococcus lactis*, and *Leuconostoc lactis*, and the Gram-negative organism *Escherichia coli* (Use of the *Escherichia coli* β-glucuronidase (*gusA*) gene as a reporter gene for

analyzing promoters in lactic acid bacteria. C. Platteeuw, G. Simons, and W. M. de Vos. 1994. *Appl. Environ. Microbiol.* 60:587-593).

2) Construction of *IdhA* overexpression system

5 The *L. bulgaricus* LMG 6901 *IdhA* gene encoding an R-lactate dehydrogenase and published by Bernard et al. (Cloning of the D-lactate dehydrogenase gene from *Lactobacillus delbrueckii* subsp. *bulgaricus* by complementation in *Escherichia coli*. 1991. Bernard, N., T. Ferrain, D. Garmyn, P. Hols, and J. Delcour. *FEBS Lett.* 290:61-64) was generated by PCR using primers with the sequences 5'-

10 GACAATTGATGACTAAAATTTGC-3' and 5'-
GGATTCTCTAGACTGCAGTTAGCCAACCTTAA-3'. The PCR product was cloned as a blunt-*Xba*I fragment into pUC18 digested with *Xba*-*Sma*I and its integrity was confirmed by nucleotide sequence analysis. Subsequently, the *IdhA* gene was cloned as *Rca*-*Xba*I fragment into pJS25 digested with *Nco*I-*Xba*I. The resulting expression vector, pJS26 (Fig. 5), has the *IdhA* gene of *L. bulgaricus* translationally fused to the *B. coagulans* promoter. Subsequently, the complete fragment encompassing the *B. coagulans* promoter and *L. delbrueckii* *IdhA* gene was transferred as a *Pst*I-*Bgl*II fragment (by partial digestion with *Bgl*II) to the thermophilic cloning vector pNW33N digested with *Pst*I-*Bam*HI generating plasmid pJS27 (Fig. 6).

20 3) Overproduction of R-lactate dehydrogenase
Plasmid pJS27 was transformed to *B. coagulans* DSM 1 by electroporation. Specific R-lactate dehydrogenase enzyme activities were determined as decrease of absorbance at 340 nm per min per mg protein at 50°C. The specific activity of *B. coagulans* DSM 1 harbouring pNW33N was 0.45 ΔA min⁻¹ mg protein⁻¹ and that of *B. coagulans* DSM 1 harbouring pJS27 was 2.15 ΔA min⁻¹ mg protein⁻¹ demonstrating that the overproduction of R-lactate dehydrogenase in the modified strains containing the *L. delbrueckii* LMG 6901 *IdhA* gene resulted in a 4.8-times increased activity.

30 **EXAMPLE 5. Genetic modification of *B. coagulans* DSM 1 for R-lactate production**
B. coagulans DSM 1 harbouring pJS27 (EXAMPLE 4) was grown in a batch culture mimicking industrial conditions. *B. coagulans* DSM 1 harbouring pNW33N and *B. coagulans* DSM 1 without plasmids were used as a reference strains. After fermentation the

concentrations of organic acids and the optical purity of the lactic acid were determined (Table 2). Lactate produced by *B. coagulans* DSM 1 and *B. coagulans* DSM 1 harbouring pNW33N was enantiopure in the S-form, while the lactate produced by *B. coagulans* DSM 1 harbouring pJS27 was for a significant part in the R-form. No differences in by-product formation were detected. Concentrations of 2-hydroxy butyric acid, acetic acid, butyric acid, formic acid, pyruvic acid, were below the detection limits (<0.02% for pyruvic acid; <0.01% for others). Concentrations of succinic acid were below 0.1% (v/v). These results demonstrate that R-lactate production by *B. coagulans* can be achieved by introduction of an R-lactate dehydrogenase gene. Construction of a *B. coagulans* strain producing only R-lactate will require the disruption of the gene responsible for S-lactate dehydrogenase activity by random or site-directed mutagenesis.

TABLE 2. Organic acid production from 50 g/L sucrose (duplicate fermentations)

	Glucose (g/L)	Lactic acid (g/L)	Chiral purity of S-lactic acid (S/R+S) * 100%	Chiral purity of R-lactic acid (R/S+R) * 100%
<i>B. coagulans</i> DSM 1	< 0.1	25/28	99.8/99.7	0.2/0.3
<i>B. coagulans</i> DSM 1 + pNW33N	< 0.1	25/22	99.8/99.7	0.2/0.3
<i>B. coagulans</i> DSM 1 + pJS27	< 0.1	26/27	84.3/83.1	15.7/16.9

EXAMPLE 6. Gene replacement in *B. coagulans* DSM 1 for enantiopure R-lactate

15 production

1) Construction of an integration plasmid

A modified *B. coagulans* strain producing R-lactate is constructed by replacing the *B. coagulans* *ldhL* gene coding for the major S-lactate dehydrogenase activity by the *B. bulgaricus* *ldhA* gene coding for R-lactate dehydrogenase. Replacement is achieved by

20 homologous recombination in a two step process using a conditional cloning vector, e.g. a thermosensitive replicon which is functional at 45°C but not at 55°C. We use the pSH71 replicon present in pNZ124 or pMH3, which was discovered to have such a thermosensitive nature in moderately thermophilic *Bacillus* species that are facultative anaerobic and homolactic. The integration vector pRK1 (Fig. 7) contains the pNZ124 replicon, two 1-kb 25 regions flanking *ldhL* in the *B. coagulans* DSM 1 chromosome and now flanking the *B.*

coagulans ATCC 23498 *amy* promoter fused to the *L. bulgaricus* *ldhA* gene and the *cat* gene encoding chloramphenicol resistance. The integration vector is constructed by ligation of the following cassettes: (i) a 1.8-kb *Sall*-*Xba*I fragment, with the *Sall* site made blunt, containing the pNZ124 replicon; (ii) a 1.1-kb *Xba*I-*Bam*HI fragment containing the upstream

5 region of the *ldhL* gene cut from a PCR fragment generated using the primers 5'-
GCGAGATCTAGAGGCCATCTGGGGGGCTTCT-3' and 5'-
CGCGGATCCATGGATAATCTTCCTCCCCATCAAAAGTA-3' and *B. coagulans* DSM 1 as
a template; (iii) a 1.0-kb *Bam*HI-*Pst*I fragment containing the *cat* gene cut from a PCR
fragment generated using the primers 5-CGCGGATCCCCTTCTCAACTAACGGG-3' and
10 5'-GCGCTGCAGTTCGCTACGCTCAAATCC-3' and pMH3 (EXAMPLE 4) as a template; (iv)
a 1.3-kb *Pst*I-*Kpn*I fragment containing the *L. bulgaricus* LMG 6901 *ldhA* gene translationally
fused to the *B. coagulans* ATCC 23498 *amy* promoter cut from pJS27 (EXAMPLE 5); (v) a
1.1-kb *Kpn*I-*Bgl*II fragment, with the *Bgl*II site made blunt containing the downstream region
of the *ldhL* gene cut from a PCR fragment generated using the primers 5'-
15 CGCGGGTACCGGCCGGCTTATGG-3' and 5'-
GCGCAGATCTGTCGAGTAAACGCGGAAAGCATTG-3' and *B. coagulans* DSM 1 as a
template.

2) Exchange of the *B. coagulans* DSM 1 *ldhL* gene with the *L. bulgaricus* LMG 6901 *ldhA*

20 gene

Plasmid pRK1 is transformed to *B. coagulans* DSM 1 by electroporation. Transformants are obtained on BC plates supplemented with 7 mg/L chloramphenicol grown at 45°C. After confirmation of the transformation by plasmid isolation, a single colony is cultured in BC broth at 45°C to mid log-phase after which the temperature is shifted to 55°C and incubation
25 is continued for 1 h. A dilution series is plated on BC plates and incubated overnight at 55°C. The colonies are pooled and plated on BC plates in a second dilution series. After overnight incubation at 55°C colonies are tested for integration by PCR analysis confirming a single crossover event. One colony is selected for continued cultivation by sequential transfer of 1/1000 dilutions (approximately 10 generations) in BC broth with antibiotics at
30 55°C. After approximately 100 generations a dilution series is plated on BC plates with antibiotics. After overnight incubation at 55°C colonies series are tested for the absence of the *ldhL* gene by colony PCR. Alternatively, a double crossover mutant can be screened for among the single crossover mutants of the first event by testing for the absence of the *ldhL*

gene by colony PCR. Chromosomal DNA from a colony negative in these PCR reactions is isolated and further evaluated for the presence of *ldhA* and correct integration is confirmed by Southern blot analysis.

5 **EXAMPLE 7. Enantiopure R-lactate production with a modified *B. coagulans* DSM 1**

1) Construction of modified strain

With the method described in EXAMPLE 6 a *B. coagulans* DSM 1 derivative was constructed with the *ldhL* gene replaced by a cassette containing the *ldhA* gene from an industrial *L. delbrueckii* strain fused to the *ldhL* promoter from an industrial *B. coagulans*

10 strain and the *cat* gene from pMH3. The resulting strain was designated RDSM 1.

2) Fermentation with RDSM 1

The RDSM 1 strain was grown in a batch culture mimicking industrial conditions. After fermentation the concentrations of organic acids and the optical purity of the lactic acid were

15 determined (Table 3). Lactate produced by *B. coagulans* RDSM 1 was for 99.5% in the R-form. No differences in by-product formation were detected compared to *B. coagulans* DSM 1 (Example 5). Concentrations of 2-hydroxy butyric acid, acetic acid, butyric acid, formic acid, pyruvic acid, were below the detection limits (<0.02% for pyruvic acid; <0.01% for others).

20 Concentrations of succinic acid were below 0.1% (v/v). These results demonstrate chromosomal deletion of native *B. coagulans* genes and chromosomal insertion and functional expression of (heterologous) genes is possible and can be applied for production of enantiopure R-lactate by *B. coagulans*.

TABLE 3. Organic acid production from 50 g/L sucrose (duplicate fermentations)

	Glucose (g/L)	Lactic acid (g/L)	Chiral purity of S-lactic acid (S/R+S)	Chiral purity of R-lactic acid (R/S+R) * 100%
<i>B. coagulans</i> DSM 1	< 0.1	25/28	99.8/99.7	0.2/0.3
<i>B. coagulans</i> RDSM 1	< 0.1	27/28	0.5/0.5	99.5/99.5

Claims.

1. Method for modifying moderately thermophilic *Bacillus* species that are facultative anaerobic and homolactic by genetic engineering comprising:

5 introducing a DNA cloned in a thermosensitive plasmid system containing a pSH71 replicon or a homologue thereof into cells of a moderately thermophilic *Bacillus* species that is facultative anaerobic and homolactic;

culturing the cells on a selective medium at a permissive temperature for plasmid replication to select transformed cells capable of growing on said selective medium at said 10 permissive temperature;

culturing said transformed cells on a selective medium at a non-permissive temperature for plasmid replication to select transformed cells capable of growing on said selective medium at said non-permissive temperature.

15 2. Method according to claim 1, wherein the DNA provides a desired functionality.

3. Method according to claim 1 or 2, wherein the culturing at the non-permissive temperature for plasmid replication allows the DNA to be introduced into the *Bacillus* chromosome, preferably by homologous recombination.

20 4. Method according to any one of claims 1-3, wherein an undesired functionality is removed from the *Bacillus* chromosome by homologous recombination.

25 5. Method according to any one of claims 1-3 wherein a desired functionality is introduced in the *Bacillus* chromosome and simultaneously an undesired functionality is removed from the *Bacillus* chromosome by homologous recombination.

30 6. Method according to claim 6, wherein the *Bacillus* species is modified by replacing the gene encoding S-lactate dehydrogenase by a DNA construct comprising a DNA sequence encoding an amino acid sequence according to SEQ ID NO: 1 or an amino acid sequence substantially homologous thereto having R-lactate dehydrogenase activity fused to a promoter functional in the *Bacillus* species.

7. Method according to claim 7, wherein the promoter is of the endogenous S-lactate

dehydrogenase gene that is removed.

8. Method according to any one of the preceding claims wherein the *Bacillus* species are *Bacillus smithii* and/or *Bacillus coagulans*.

5

9. Method according to any one of the preceding claims wherein the *Bacillus* species are sporulation deficient.

10. Genetically engineered moderately thermophilic *Bacillus* strain that is facultative

10 anaerobic and a derivative of a homolactic *Bacillus* strain obtainable by a method according to any one of the preceding claims.

11. Genetically engineered *Bacillus* species that is R-lactic acid- producing.

15 12. Process for the preparation of a compound of interest, preferably lactic acid and/or lactate, wherein the genetically modified moderately thermophilic *Bacillus* species that is facultative anaerobic of claim 11 is used.

13. Process according to claim 12 wherein R-lactic acid and/or R-lactate is produced.

20



Figure 1

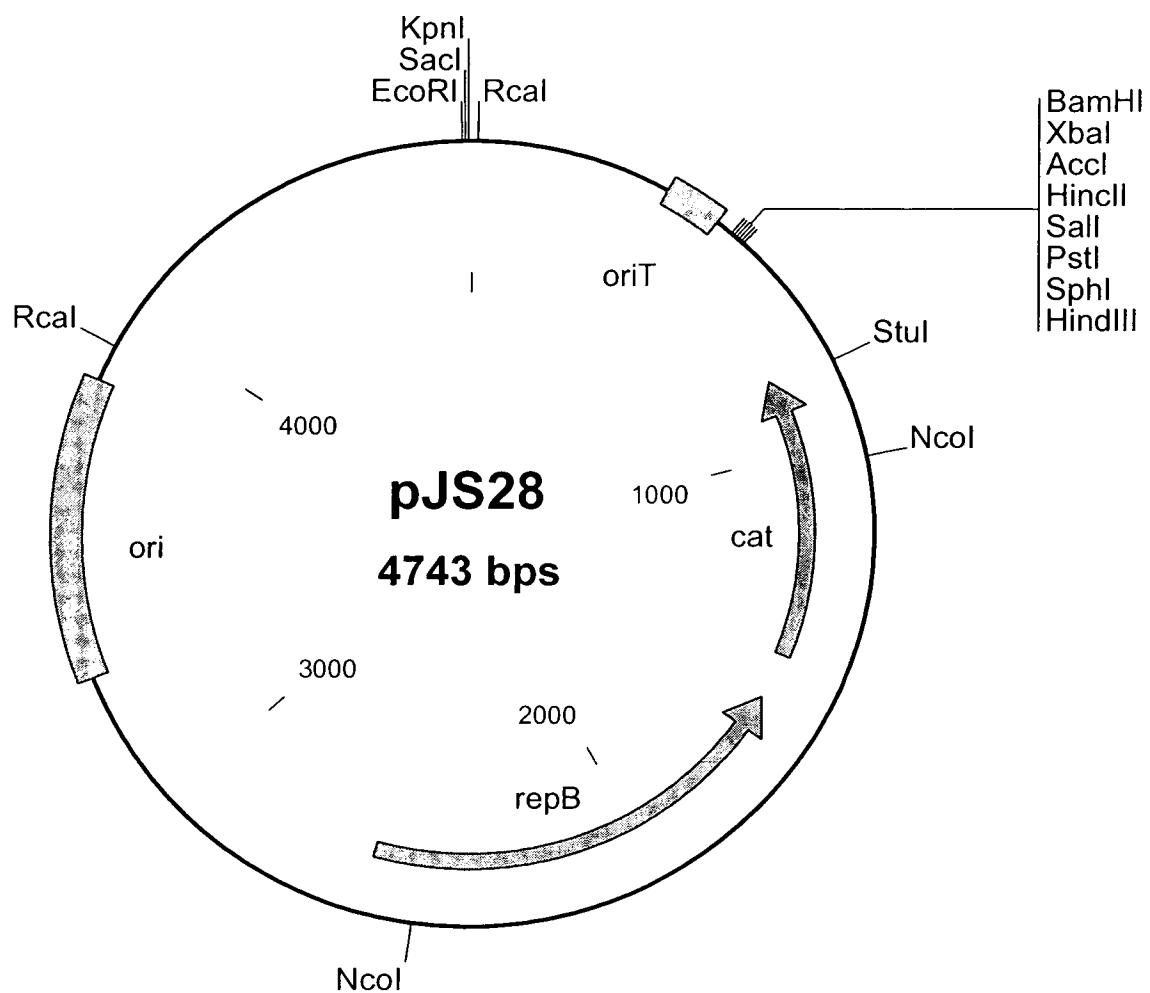


Figure 2

1 AGATCTTGGT TCCCCACCTT TTTTACAGAC TTATCACTAT ATTATTATAG
51 ATAAACCGGC CAAACAACCA AATCGGGGCG CAAAGGAGAG CCGGGGCGTG
101 GATTTAAACC ATTTTGAA AAACAAAAGG AAAACCTGCT TGTAAAAAGA
151 TGTTTCGCG AAACGAAAGC GGGAAATAGTA CCTTTGTTCT CTTCGCCTT
201 TGTCACTGCTT AAAATCATAA TTGATTGAAA ATTTTTCAT GTTCACTTAT
251 ACTAAACGCA TCAACTATTA CTTCTTTGG AAGGGGCAGT TTCCATGGG
301 ATCC

Figure 3

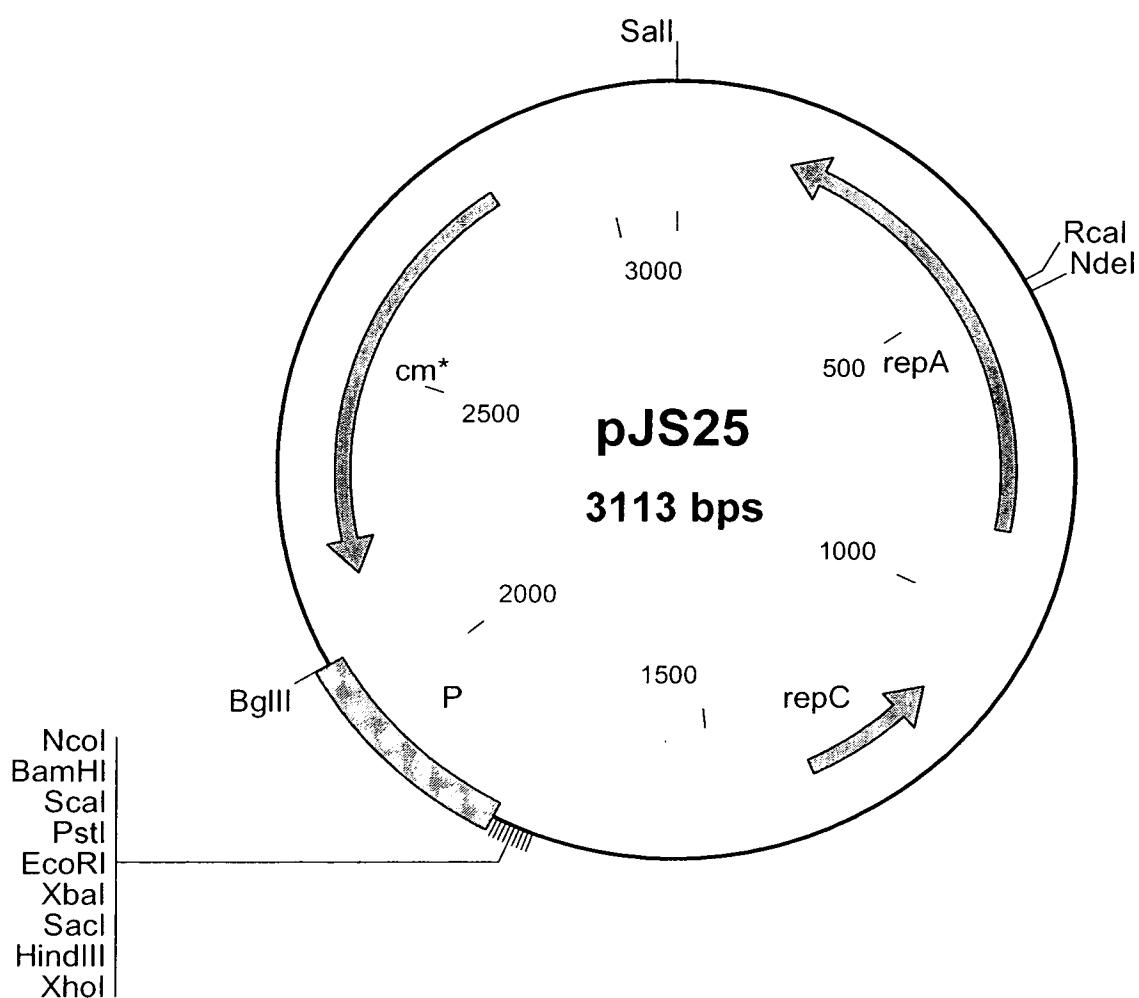


Figure 4

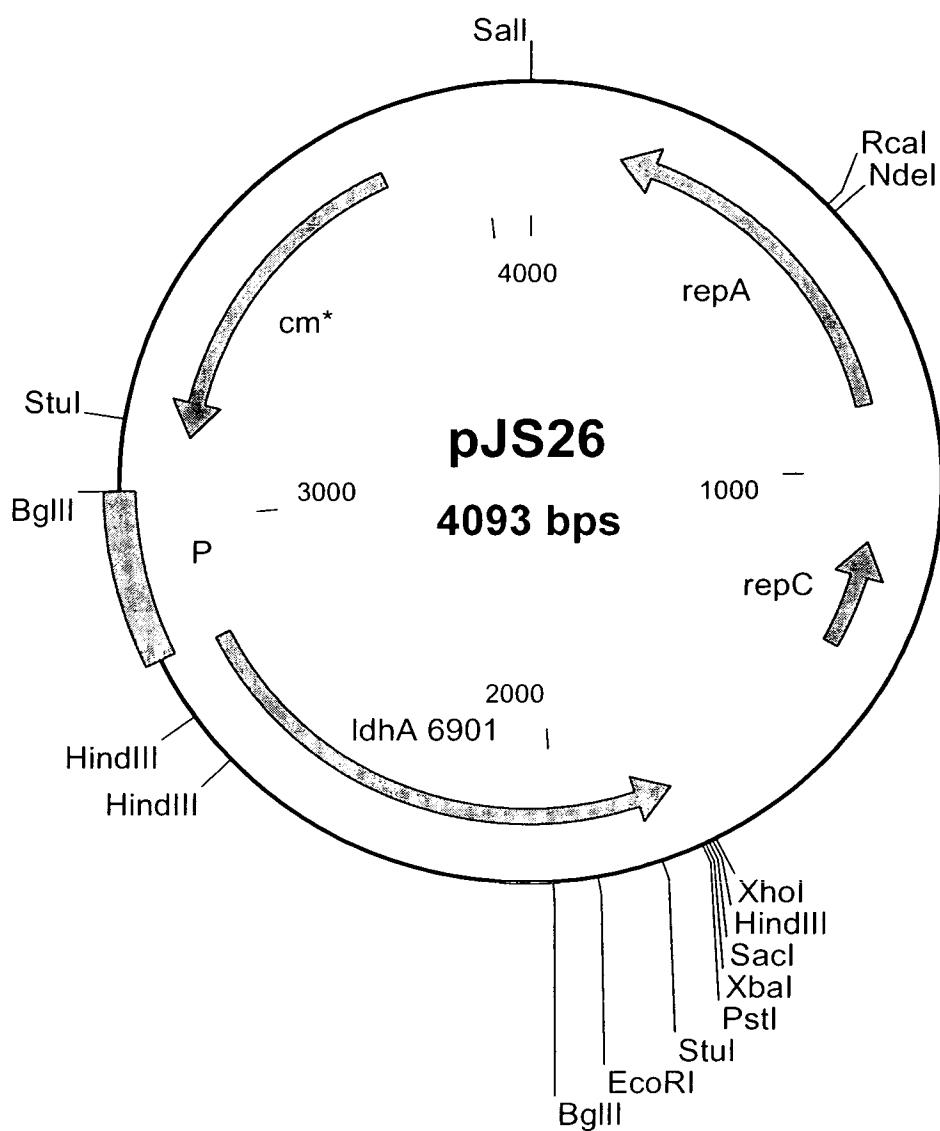


Figure 5

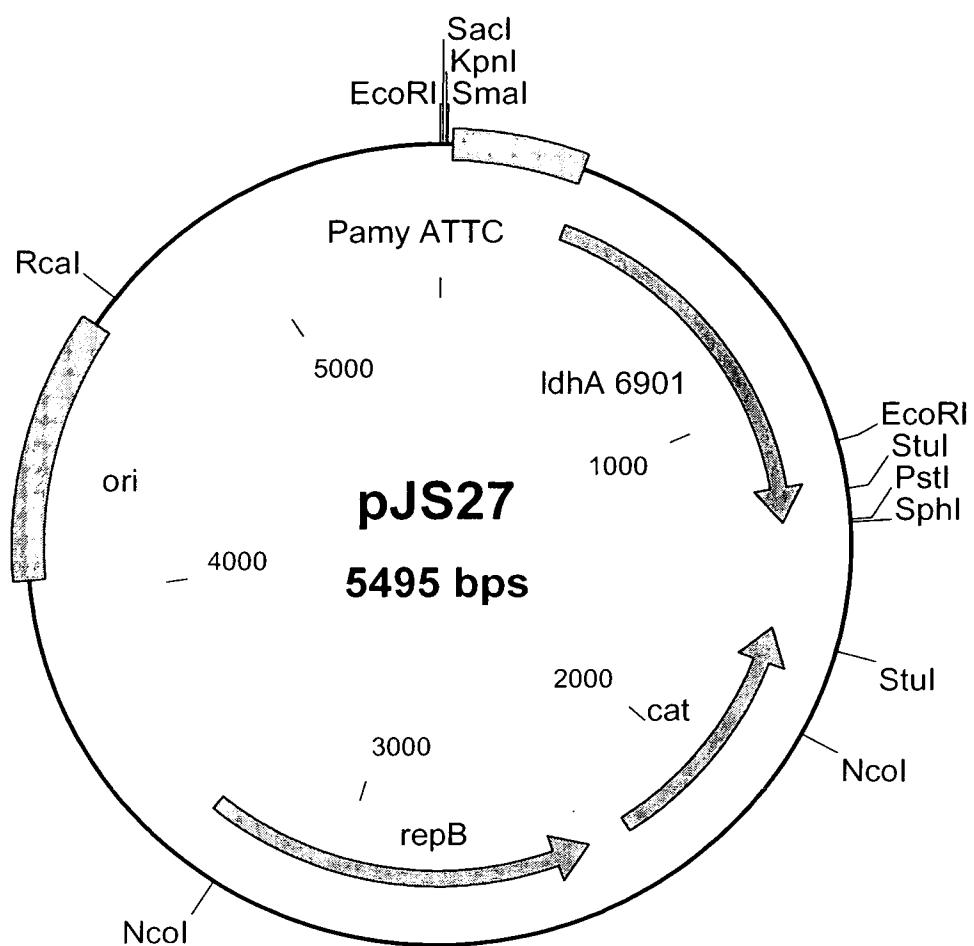


Figure 6

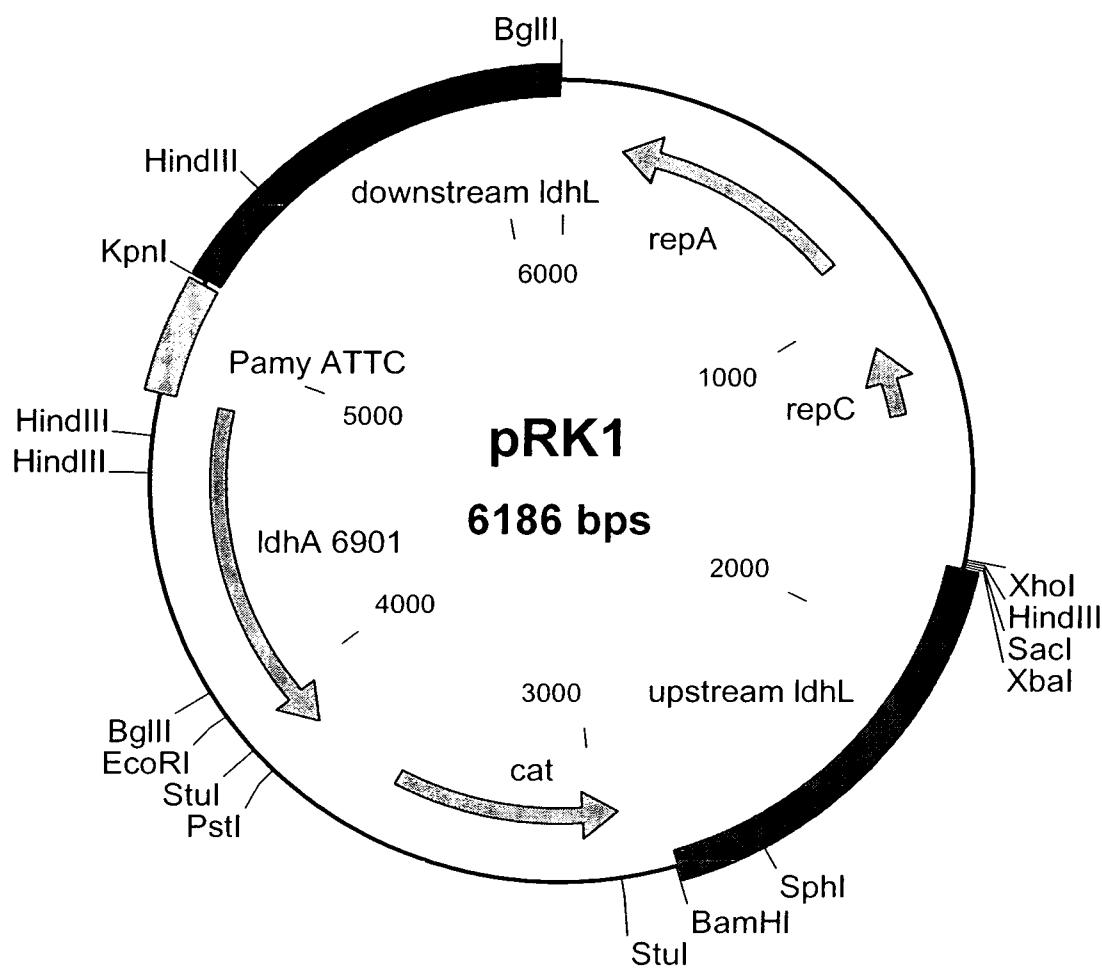


Figure 7