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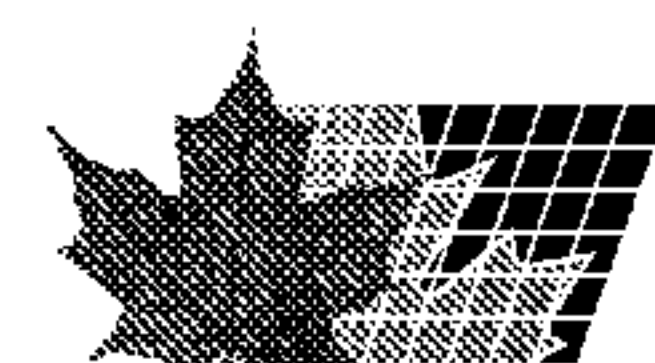
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(57) **Abrégé/Abstract:**

The present invention relates to biocatalysts that are cells, optimally of the Crabtree-negative phenotype, comprising expression vectors encoding genes heterologous to the cell that enable increased production of organic products. More specifically, the invention relates to genetically modified Candida cells, methods for making the Candida cells, and their use in production of organic products, particularly lactic acid.



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(54) Title: METHODS AND MATERIALS FOR THE PRODUCTION OF ORGANIC PRODUCTS IN CELLS OF *S*(CANDIDA) SPECIES

(57) Abstract: The present invention relates to biocatalysts that are cells, optimally of the Crabtree-negative phenotype, comprising expression vectors encoding genes heterologous to the cell that enable increased production of organic products. More specifically, the invention relates to genetically modified *Candida* cells, methods for making the *Candida* cells, and their use in production of organic products, particularly lactic acid.



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METHODS AND MATERIALS FOR THE PRODUCTION OF ORGANIC PRODUCTS IN CELLS OF *CANDIDA* SPECIES

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This application is a continuation-in-part of U.S. Patent Application Serial No. 09/992,430, filed November 23, 2001, which claims priority to U.S. Provisional Application Serial No. 60/252,541, filed November 22, 2000.

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BACKGROUND OF THE INVENTION

The use of microorganisms for synthesizing industrially important organic products is well known. Biosynthetic approaches for producing organic products can be extremely efficient when compared to large-scale chemical synthesis. Advantages a biosynthetic approach may have over a chemical synthetic approach for manufacturing an organic product include more rapid and more efficient product yield, isomeric purity, and reduced cost (*see* Thomas et al., 2002, *Trends Biotechnol.* 20: 238-42).

Lactic acid has wide industrial applicability, including uses in chemical processing and synthesis, cosmetics, pharmaceuticals, plastics, and food production. Lactic acid is a relatively simple organic molecule, and can be produced either by chemical synthesis or by fermentation in microorganisms (biosynthesis). As genetic manipulation of microorganisms has become more advanced, fermentation processes for lactic acid production have become commercially preferred over chemical synthesis. One reason for this preference is that using genetically modified microorganisms enables production of optically pure (*i.e.*, either the L(+) or D(-) isomer) product. Such methods obviate the need for separating racemic product mixtures, thereby reducing cost.

Nevertheless, the use of microorganisms for producing organic products has certain limitations. For example, bacteria can produce large quantities of organic products under fermentation conditions, but the accumulation of organic products within the bacteria itself and in the growth medium can inhibit proliferation of the bacteria, or cause cell death. Even when more robust organisms are engineered and used for production, such as the acidophilic yeast

Saccharomyces cerevisiae, organic products can lead to cell growth suppression, reducing overall yield of organic product. Thus, there remains a need in the art for robust microorganisms that are amenable to genetic manipulation, for use in bioreactors and with other biosynthetic methods for producing industrially
5 important organic products.

SUMMARY OF THE INVENTION

This invention provides methods and reagents, particularly cells and
10 recombinant cells, for producing organic products by biosynthesis. The invention specifically provides recombinant nucleic acid constructs encoding at least one protein useful for the synthesis of an organic product, cells comprising said constructs, particularly Crabtree-negative cells, methods for making such cells, methods for culturing such cells, and methods and reagents for synthesizing
15 numerous organic products *in vivo*.

In one aspect, the invention provides recombinant nucleic acid constructs comprising a sequence encoding at least one protein useful for the synthesis of an organic product. In a preferred embodiment, the recombinant nucleic acid construct encodes lactate dehydrogenase. In one embodiment of this aspect, the
20 recombinant nucleic acid construct comprises a promoter operably linked to the nucleic acid encoding a protein useful for synthesis of an organic product, wherein the promoter is a promoter from a *Candida* species, preferably the *Candida* species that comprises the recombinant nucleic acid construct.

In another aspect, the invention provides a transformed Crabtree-negative
25 cell from the genera *Candida*, comprising the recombinant nucleic acid construct encoding at least one protein useful for the synthesis of an organic product. In a preferred embodiment, the recombinant nucleic acid construct encodes lactate dehydrogenase. In one embodiment of this aspect, the recombinant nucleic acid construct comprises a promoter operably linked to the nucleic acid encoding a
30 protein useful for synthesis of an organic product, wherein the promoter is a promoter from a *Candida* species, preferably the *Candida* species that comprises the recombinant nucleic acid construct. In another aspect, the invention provides a cell of a *Candida* species genetically manipulated so that it has reduced

efficiency in metabolizing pyruvate to ethanol. In preferred embodiments of this aspect of the invention, the cell further comprises a recombinant nucleic acid construct of the invention encoding at least one protein useful for the synthesis of an organic product. In a preferred embodiment, the recombinant nucleic acid
5 construct encodes lactate dehydrogenase. In one embodiment of this aspect, the recombinant nucleic acid construct comprises a promoter operably linked to the nucleic acid encoding a protein useful for synthesis of an organic product, wherein the promoter is a promoter from a *Candida* species, preferably the *Candida* species that comprises the recombinant nucleic acid construct.

10 In another aspect, the invention provides methods for producing organic products comprising fermenting a Crabtree-negative cell from the genera *Candida* comprising a recombinant nucleic acid construct of the invention under conditions that allow for the biosynthesis of said organic products. In preferred embodiments of this aspect of the invention, the organic product is lactic acid. In a preferred
15 embodiment, the recombinant nucleic acid construct encodes lactate dehydrogenase. In one embodiment of this aspect, the recombinant nucleic acid construct comprises a promoter operably linked to the nucleic acid encoding a protein useful for synthesis of an organic product, wherein the promoter is a promoter from a *Candida* species, preferably the *Candida* species that comprises
20 the recombinant nucleic acid construct.

It is an advantage of this invention that the transformed cells provided herein exhibit the "Crabtree negative" phenotype. Crabtree-negative organisms are characterized by the ability to be induced into an increased fermentative state. Both naturally occurring organisms and genetically modified organisms can be
25 characterized as Crabtree-negative. The Crabtree effect is defined as oxygen consumption inhibition in a microorganism when the microorganism is cultured under aerobic conditions in the presence of a high concentration of glucose (*e.g.* >5 mM glucose). Crabtree-positive organisms continue to ferment (rather than respire) irrespective of oxygen availability in the presence of glucose, while
30 Crabtree-negative organisms do not exhibit glucose-mediated inhibition of oxygen consumption. This characteristic is useful for organic product synthesis, since it permits cells to be grown at high substrate concentrations but to retain the beneficial energetic effects of oxidative phosphorylation. Many yeasts and fungi

have the Crabtree-negative phenotype including the non-limiting examples of genera *Kluyveromyces*, *Pichia*, *Hansenula*, *Torulopsis*, *Yamadazyma*, and *Candida*.

Candida species, which are variously characterized as yeasts and
5 dimorphic fungi in the art, can exhibit the Crabtree-negative phenotype (Franzblau & Sinclair, 1983, *Mycopathologia* 82: 185-190). Certain species can ferment glucose, as well as alternative carbon sources, can grow at elevated temperatures (*i.e.*, greater than 37°C), and can tolerate low pH stress. *Candida* species have several of the desirable characteristics of an organism to be used in biosynthetic
10 methods of organic product manufacture: amenability to genetic manipulation, ability to process a variety of carbon sources, Crabtree-negative phenotype, and ability to proliferate under various environmental stresses.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred
15 embodiments and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic diagram of the vector described as pMI260,
20 comprising the G418 resistance-coding gene driven by the *PGK* promoter (*S. cerevisiae*) and linked to the *GAL10* (*S. cerevisiae*) terminator.

FIG. 2 is a schematic diagram of the vector described as pMI268, comprising the G418 resistance-coding gene driven by the *PGK* promoter (*C. sonorensis*) and linked to the *GAL10* (*S. cerevisiae*) terminator.

25 FIG. 3 is a schematic diagram of the vector described as pMI269, comprising the G418 resistance-coding gene driven by the *TDH* promoter (*C. sonorensis*) and linked to the *GAL10* (*S. cerevisiae*) terminator.

FIG. 4 is a schematic diagram of the vector described as pMI270, comprising the hygromycin resistance-coding gene driven by the *PGK* promoter
30 (*C. sonorensis*) and linked to the *GAL10* (*S. cerevisiae*) terminator.

FIG. 5 is a schematic diagram of the vector described as pMI234, comprising the *MEL5* (*S. cerevisiae*) gene driven by the *PGK* promoter (*C. sonorensis*).

FIG. 6 is a schematic diagram of the vector described as pMI238, comprising the *MEL5* (*S. cerevisiae*) gene driven by the *TDH* promoter (*C. sonorensis*).

FIG. 7 is a schematic diagram of the vector described as pMI271,
5 comprising the hygromycin resistance-coding gene driven by the *TDH* promoter (*C. sonorensis*) and linked to the *GAL10* (*S. cerevisiae*) terminator.

FIG. 8 is a schematic diagram of the vector described as pMI246, comprising the *MEL5* (*S. cerevisiae*) and *LDH* (*L. helveticus*) genes each driven by the *PGK* promoter (*C. sonorensis*). The *LDH* gene is linked to the *CYC1*
10 terminator, which is upstream from an S26 rRNA (*C. sonorensis*) region.

FIG. 9 is a schematic diagram of the vector described as pMI247, comprising the *MEL5* (*S. cerevisiae*) gene driven by the *TDH* promoter (*C. sonorensis*) and the *LDH* (*L. helveticus*) gene driven by the *PGK* promoter (*C. sonorensis*). The *LDH* gene is linked to the *CYC1* terminator, which is upstream
15 from an S26 rRNA (*C. sonorensis*) region.

FIG. 10 is a schematic diagram of the vector described as pMI257, comprising the *MEL5* (*S. cerevisiae*) gene driven by the *PGK* promoter (*C. sonorensis*) and the *LDH* (*L. helveticus*) gene driven by the *PGK* promoter (*C. sonorensis*). The *LDH* gene is linked to the *CYC1* terminator. This entire
20 expression cassette is inserted between the *PDC1* promoter and terminator (*C. sonorensis*).

FIG. 11 is a schematic diagram of the vector described as pMI265, comprising the *MEL5* (*S. cerevisiae*) gene driven by the *PGK* promoter (*C. sonorensis*) and the *LDH* (*B. megaterium*; from vector pVR24) gene driven by the
25 *PGK* promoter (*C. sonorensis*). The *LDH* gene is linked to the *PDC1* (*C. sonorensis*) terminator. This entire expression cassette is inserted between the *PDC1* promoter and terminator (*C. sonorensis*).

FIG. 12 is a schematic diagram of the vector described as pMI266, comprising the *MEL5* (*S. cerevisiae*) gene driven by the *PGK* promoter (*C. sonorensis*) and the *LDH* (*R. oryzae*; from vector pVR27) gene driven by the *PGK*
30 promoter (*C. sonorensis*). The *LDH* gene is linked to the *PDC1* (*C. sonorensis*) terminator. This entire expression cassette is inserted between the *PDC1* promoter and terminator (*C. sonorensis*).

FIG. 13 is a schematic diagram of the vector described as pMI267, comprising the *MEL5* (*S. cerevisiae*) gene driven by the *PGK* promoter (*C. sonorensis*). This expression cassette is inserted between the *PDC1* promoter and terminator (*C. sonorensis*).

5 FIG. 14 is a schematic diagram of the vector described as pMI278, comprising the G418 resistance-coding gene driven by the *TDH* promoter (*C. sonorensis*), operatively linked to the *MEL5* terminator, and the *LDH* (*B. megaterium*) gene driven by the *PGK* promoter (*C. sonorensis*). The *LDH* gene is linked to the *GAL10* (*S. cerevisiae*) terminator.

10 FIG. 15 is a schematic diagram of the vector described as pMI286, comprising the G418 resistance-coding gene driven by the *TDH* promoter (*C. sonorensis*), operatively linked to the *MEL5* (*S. cerevisiae*) terminator, and the *LDH* (*B. megaterium*) gene driven by the *PGK* promoter (*C. sonorensis*). The *LDH* gene is linked to the *GAL10* (*S. cerevisiae*) terminator. This entire
15 expression cassette is inserted between the *PDC2* promoter and terminator (*C. sonorensis*).

FIG. 16 is a schematic diagram of the vector described as pMI287, comprising the G418 resistance-coding gene driven by the *TDH* promoter (*C. sonorensis*), operatively linked to the *MEL5* (*S. cerevisiae*) terminator. This
20 expression cassette is inserted between the *PDC2* promoter and terminator (*C. sonorensis*).

FIG. 17 is a schematic diagram of the vector described as pMI288, comprising the G418 resistance-coding gene driven by the *TDH* promoter (*C. sonorensis*), operatively linked to the *MEL5* (*S. cerevisiae*) terminator, and the
25 *LDH* (*L. helveticus*) gene driven by the *PGK* promoter (*C. sonorensis*). The *LDH* gene is linked to the *CYC1* terminator. This entire expression cassette is inserted between the *PDC2* promoter and terminator (*C. sonorensis*).

FIG. 18 is a schematic diagram of the vector described as pMI256, comprising the *MEL5* (*S. cerevisiae*) gene driven by the *PGK* promoter (*C. sonorensis*) and the *LDH* (*L. helveticus*) gene driven by the *PGK* promoter (*C. sonorensis*). The *LDH* gene is linked to the *CYC1* terminator. This entire
30 expression cassette is inserted upstream of the *PDC1* terminator (*C. sonorensis*).

FIG. 19 is a schematic diagram of the vector described as pMI277, comprising the *PDC2* promoter (*C. sonorensis*).

FIG. 20 is a schematic diagram of the vector described as pMI279, comprising the G418 resistance-coding gene driven by the *TDH* promoter (*C. sonorensis*), operatively linked to the *MEL5* (*S. cerevisiae*) terminator, and the *LDH* (*B. megaterium*) gene driven by the PGK promoter (*C. sonorensis*). The *LDH* gene is linked to the *GAL10* terminator (*S. cerevisiae*). This entire expression cassette is inserted downstream of the *PDC2* promoter (*C. sonorensis*).

FIG. 21 is a schematic diagram of the vector described as pVR24.

FIG. 22 is a schematic diagram of the vector described as pVR27.

FIG. 23 A-C are schematic diagrams of the vectors including pMI214, pMI203, pMI205, pMI227, pMI233, and pMI234.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, “organic product” is any compound containing a carbon atom. Non-limiting examples of organic products include carboxylates (*e.g.* lactate, acrylate, citrate, isocitrate, alpha-ketoglutarate, succinate, fumarate, malate, oxaloacetate), carbohydrates (*e.g.* D-xylose), alditols (*e.g.* xylitol, arabitol, ribitol), amino acids (*e.g.* glycine, tryptophan, glutamate), lipids, esters, vitamins (*e.g.*, L-ascorbate), polyols (*e.g.* glycerol, 1,3-propanediol, erythritol), aldehydes, alkenes, alkynes, and lactones. Thus, an organic product can contain one, two, three, four, five, six, seven, eight, nine, ten, or more carbon atoms. In addition, organic products can have a molecular weight that is less than about 1,000 (*e.g.* less than about 900, 800, 700, 600, 500, 400, 300, 200, or 100) daltons. For example, D-xylose ($C_5H_{10}O_5$) is an organic product that has a molecular weight of 150 daltons. Further, organic products can be fermentation products.

The term “fermentation product” as used herein refers to any organic compound that is produced by a fermentation process. Generally, a fermentation process can involve the anaerobic enzymatic conversion of organic compounds (*e.g.* carbohydrates) to compounds such as ethyl alcohol, producing energy in the form of ATP. Cellular fermentation differs from cellular respiration in that organic products rather than molecular oxygen are used as electron acceptors.

Non-limiting examples of fermentation products are acetate, ethanol, butyrate, and lactate.

The organic products can also be derived from pyruvate. A “pyruvate-derived product,” as used herein, refers to any compound that is synthesized from pyruvate within no more than fifteen enzymatic steps. One enzymatic step is considered to be any chemical reaction or series of reactions catalyzed by a polypeptide having enzymatic activity. Such polypeptides are any polypeptide that catalyzes a chemical reaction of other substances without itself being destroyed or altered upon completion of the reaction or reactions. These polypeptides can have any type of enzymatic activity including the non-limiting examples of activities associated with aconitase, isocitrate dehydrogenase, ketoglutarate dehydrogenase, succinate thiokinase, succinate dehydrogenase, fumarase, malate dehydrogenase, citrate synthase, 2,5-dioxovalerate dehydrogenase, 5-dehydro-4-deoxy-D-glucarate dehydrogenase, glucarate dehydratase, aldehyde dehydrogenase, glucuronolactone reductase, L-gulonolactone oxidase, 2-dehydro-3-deoxy-D-pentanoate aldolase, xylonate dehydratase, xylonolactonase, D-xylose dehydrogenase, lactate dehydrogenase, CoA-transferase, lacyl-CoA dehydratase, or acrylyl-CoA hydratase.

The carboxylate products of the invention can be in the free acid or salt form, and can be referred to interchangeably (*e.g.* “lactic acid” or “lactate”). Use of either of the terms is taken to encompass the other, unless specifically noted otherwise. In preferred embodiments, the invention provides the carboxylates in free acid form.

The term “nucleic acid sequence” or “nucleic acid molecule” refers to a DNA or RNA molecule. The term encompasses molecules formed from any of the known base analogs of DNA and RNA such as, but not limited to 4-acetylcytosine, 8-hydroxy-N6-methyladenosine, aziridinyl-cytosine, pseudoisocytosine, 5-(carboxyhydroxymethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, dihydrouracil, inosine, N6-iso-pentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethyl-guanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil,

5-methoxyamino-methyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarbonyl-methyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, oxybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine.

The term "vector" is used to refer to any molecule (*e.g.*, nucleic acid, plasmid, or virus) used to transfer protein-coding information to a host cell.

10 The term "expression vector" refers to a vector that is suitable for transformation of a host cell and contains nucleic acid sequences that direct and/or control expression of inserted heterologous nucleic acid sequences. Expression includes, but is not limited to, processes such as transcription, translation, and RNA splicing, if introns are present.

15 The term "operably linked" is used herein to refer to an arrangement of sequences wherein the sequences are joined together and configured or assembled so as to perform their usual function. Thus, a sequence operably linked to a sequence encoding a protein may flank the coding sequence and be capable of effecting replication and/or transcription of the coding sequence. For example, a coding sequence is operably linked to a promoter when the promoter is capable of directing transcription of that coding sequence. A flanking sequence need not be contiguous with the coding sequence, so long as it functions correctly. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence
20 can still be considered "operably linked" to the coding sequence.

The term "host cell" is used to refer to a cell into which has been introduced or transformed, or is capable of being transformed with a nucleic acid sequence and then of expressing a selected gene of interest. The term includes the progeny of the parent cell, whether or not the progeny is identical in morphology
30 or in genetic make-up to the original parent.

The term "endogenous" as used herein refers to genomic material that is not exogenous, that is, which has not been introduced into the cell. Such endogenous genomic material usually develops within an organism, tissue, or cell

and is not inserted or modified by recombinant technology. Endogenous genomic material encompasses naturally occurring variations.

The term “exogenous” or “heterologous” as used herein refers to genomic material that is not endogenous, that is, material that has been introduced into the
5 cell. Typically such material is inserted or modified by recombinant technology.

As used herein, the term “genetically modified” refers to an organism whose genome has been modified by methods including the non-limiting examples of addition, substitution, or deletion of genetic material. Such methods of genetic manipulation are well known in the art and include, but are not limited
10 to, random mutagenesis, point mutations, including insertions, deletions, and substitutions of one or a plurality of individual nucleotide residues, knock-out technology, and transformation of an organism with a nucleic acid sequence using recombinant technology, including both stable and transient transformants.

The terms “anaerobic” and “anaerobic conditions” are taken to mean that
15 the amount of dissolved oxygen in a solution, typically a culture medium, is not detectable (*i.e.*, about 0%), or alternatively the amount of oxygen in the atmosphere is from about 0% to 2%.

20 Vectors and Host Cells

A nucleic acid molecule encoding the amino acid sequence of a polypeptide useful for synthesis of organic products of interest is inserted into an appropriate cloning or expression vector using standard ligation techniques (see, for example, Sambrook *et al.*, 2001, MOLECULAR CLONING: A LABORATORY
25 MANUAL, 3rd ed., Cold Spring Harbor Laboratory Press, New York). The vector is typically selected to be functional in the particular host cell employed (*i.e.*, the vector is compatible with the host cell machinery such that replication, amplification and/or expression of the gene can occur). A nucleic acid molecule encoding the amino acid sequence of a polypeptide useful for synthesis of organic
30 products of interest can be amplified in any appropriate cell and expressed in any host cell, most preferably a Crabtree-negative host cell.

Preferred Crabtree-negative host cells include those from genera *Candida*, including the non-limiting examples of *C. sonorensis*, *C. methanosorbosa*, *C.*

diddensiae, *C. parapsilosis*, *C. naeodendra*, *C. krusei*, *C. blankii*, and *C. entomophila*.

Flanking sequences (including promoters and terminators) may be homologous (*i.e.*, from the same species and/or strain as the host cell),
5 heterologous (*i.e.*, from a species other than the host cell species or strain), hybrid (*i.e.*, a combination of flanking sequences from more than one source), or synthetic, or the flanking sequences may be native sequences that normally function to regulate expression of the gene of interest. As such, the source of a flanking sequence may be any prokaryotic or eukaryotic organism, any vertebrate
10 or invertebrate organism, or any plant, provided that the flanking sequence is functional in, and can be activated by, the host cell machinery.

Flanking sequences useful in the vectors of this invention may be obtained by any of several methods well known in the art. Typically, flanking sequences useful herein will have been previously identified by mapping and/or by restriction
15 endonuclease digestion and can thus be isolated from a biological source using the appropriate restriction endonucleases. In some cases, the complete nucleotide sequence of a flanking sequence may be known. In such cases, the flanking sequence may be synthesized using methods well known to those of skill in the art, as well as those described herein, for nucleic acid synthesis or cloning.

20 Where all or only a portion of the flanking sequence is known, the full extent of the functional flanking sequence may be obtained using *in vitro* amplification technique such as polymerase chain reaction (PCR) and/or by screening a genomic library with a suitable oligonucleotide and/or flanking sequence fragment from the same or another species. Where the flanking
25 sequence is not known, a fragment of DNA containing a flanking sequence may be isolated from a larger piece of DNA that may contain, for example, a coding sequence or even another gene or genes. Isolation may be accomplished by restriction endonuclease digestion to produce the proper DNA fragment followed by isolation using agarose gel purification, Qiagen® column chromatography
30 (Chatsworth, CA), or other methods known to the skilled artisan. The selection of suitable enzymes to accomplish this purpose will be readily apparent to one of ordinary skill in the art.

A selectable marker gene or element encodes a protein necessary for survival and growth of a host cell grown in a selective culture medium. Useful selection marker genes encode proteins that (a) confer resistance in host cells to antibiotics or other toxins, *e.g.*, ampicillin, tetracycline, or kanamycin; (b) complement auxotrophic deficiencies of the host cell, such as Leu2 deficiency; or
5 (c) supply critical nutrients not available from complex media. Preferred selectable markers include the non-limiting examples of zeocin resistance gene, G418 resistance gene, and the hygromycin resistance gene.

Other selection genes may be used to amplify the gene that will be
10 expressed. Amplification is the process wherein genes that are in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Examples of suitable selectable markers for mammalian cells include dihydrofolate reductase (DHFR) and promoterless thymidine kinase. The mammalian cell transformants
15 are placed under selection pressure wherein only the transformants are uniquely adapted to survive by virtue of the selection gene present in the vector. Selection pressure is imposed by culturing the transformed cells under conditions in which the concentration of selection agent in the medium is progressively increased, thereby leading to the amplification of both the selection gene and DNA that
20 encodes a polypeptide useful for synthesizing an organic product.

Expression and cloning vectors of the present invention will typically contain a promoter that is recognized by the host organism and operably linked to the molecule encoding the polypeptide useful for synthesizing an organic product. Promoters are untranscribed sequences located upstream (*i.e.*, 5') to the translation
25 start codon of a structural gene (generally within about 100 to 1000 bp) and control transcription of the structural gene. Promoters are conventionally grouped into one of two classes: inducible promoters and constitutive promoters. Inducible promoters initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, such as the presence or absence
30 of a nutrient or a change in temperature. Constitutive promoters, on the other hand, initiate continual gene product production; that is, there is little or no regulation of gene expression. A large number of promoters of both promoter types, recognized by a variety of potential host cells, are well known in the art. A

suitable promoter is operably linked to DNA encoding a polypeptide useful for synthesizing an organic product by removing the promoter from the source DNA by restriction enzyme digestion or producing a promoter fragment by *in vitro* amplification and inserting the desired promoter sequence into the vector. Native
5 promoter sequences may be used to direct amplification and/or expression of a nucleic acid molecule that encodes a polypeptide useful for synthesizing an organic product. A heterologous promoter is preferred, however, if it permits greater transcription and higher yields of the expressed protein compared to the native promoter, and if it is compatible with the host cell system that has been
10 selected for use.

Suitable promoters for use with yeast host cells are also well known in the art, and include the non-limiting examples of promoters from yeast genes including phosphoglycerate kinase (PGK), triose dehydrogenase (TDH), pyruvate decarboxylase (PDC), triose phosphate isomerase (TPI), and alcohol
15 dehydrogenase (ADH). Preferred promoters of the invention include PGK and TDH promoters. Yeast enhancers, sequences that increase expression when placed in relative proximity to a promoter are advantageously used with yeast promoters.

Methods of transforming cells are well known in the art, and can include such non-limiting examples as electroporation and calcium chloride or lithium
20 acetate based transformation methods.

Several of the vectors disclosed in the Examples of this invention have been previously constructed and are described in application PCT/US01/44041. Briefly vectors pMI234, pMI238, pMI246, pMI247, and the PDC2 in lambda were
25 constructed as follows.

C. sonorensis gene isolation (PDC2 in lambda): Genomic DNA of *C. sonorensis* (ATCC Accession No. 32109) was isolated from cells grown overnight in YPD using the Easy DNA kit (Invitrogen). DNA was partially digested with *Sau3A* and size fractionated by sucrose gradient centrifugation (Sambrook *et al.*
30 *Id.*). DNA fragments of about 22 kb were ligated to *Bam*HI digested, phosphatase treated lambda DASHTM vector arms (Stratagene) and the ligation mixture was packaged into lambda particles using Gigapack II Gold Packaging Extract (Stratagene). The lambda particles were used to infect *E. coli* MRA P2.

Probes used for isolation of *C. sonorensis* genes from the library were prepared by PCR amplification using the Dynazyme EXT polymerase (Finnzymes, Espoo, Finland), sequence specific primers and genomic DNA of *S. cerevisiae*, *C. albicans* or *C. sonorensis* as a template as follows.

- 5 • Oligonucleotides TGT CAT CAC TGC TCC ATC TT (SEQ ID No.17) and TTA AGC CTT GGC AAC ATA TT (SEQ ID No. 18) corresponding to the *S. cerevisiae* *TDH1* gene were used to amplify a fragment of the TDH gene from genomic *S. cerevisiae* DNA.
- Oligonucleotides GCG ATC TCG AGG TCC TAG AAT ATG TAT ACT AAT
10 TTG C (SEQ ID No. 19) and CGC GAA TTC CCA TGG TTA GTT TTT GTT GGA AAG AGC AAC (SEQ ID No. 20) corresponding to the *C. albicans* *PGK1* gene were used to amplify a fragment of the *PGK1* gene from genomic *C. albicans* DNA.
- Oligonucleotides TGG ACT AGT AAA CCA ACA GGG ATT GCC TTA GT
15 (SEQ ID No. 21) and CTA GTC TAG AGA TCA TTA CGC CAG CAT CCT AGG (SEQ ID No. 22) corresponding to the *C. sonorensis* 26 S rRNA were used to amplify a fragment of the 26S rDNA gene from *C. sonorensis* genomic DNA.
- Oligonucleotides CCG GAA TTC GAT ATC TGG GCW GGK AAT GCC
20 AAY GAR TTR AAT GC (SEQ ID No. 23) and CGC GGA TTC AGG CCT CAG TAN GAR AAW GAA CCN GTR TTR AAR TC (SEQ ID No. 24) were designed based on portions of pyruvate decarboxylase amino acid sequence WAGNANELNA (SEQ ID No. 25) and DFNTGSFSYS (SEQ ID No. 26), that are conserved between *S. cerevisiae* PDC1, *Pichia stipitis* PDC1 and PDC2, and incomplete sequences of *Candida albicans* PDC1 and PDC3. These
25 primers were used were used to amplify a fragment of the PDC gene(s) from *C. sonorensis* genomic DNA. PCR reaction with these primers produced two fragments of different nucleotide sequence termed PDC1 and PDC2.
- Oligonucleotides TCTGTTMCCTACRTAAGA (SEQ ID No. 27) and
30 GTYGGTGGTCACGAAGGTGC (SEQ ID No. 28) were designed based on conserved regions found in fungal alcohol dehydrogenase sequences. These primers were used to amplify a fragment of the ADH gene(s) from *C. sonorensis*

genomic DNA. PCR reaction with these primers produced three fragments of different nucleotide sequences termed ADH1, ADH2, and ADH3.

The library was screened with PCR fragments produced as described above, and products were labeled with ^{32}P α -dCTP using the Random Primed Labeling Kit (Boehringer Mannheim). Hybridization with the radioactive probes was performed by incubation overnight at 42°C in a solution containing 50% formamide, 5x Denhardt's, 5x SSPE, 0.1% SDS, 100 $\mu\text{g/mL}$ herring sperm DNA, 1 $\mu\text{g/mL}$ polyA DNA. For *TDH1*, *PGK1*, and *PDC1* probes, filters were washed after hybridization at room temperature in a solution of 2x SSC for 5 min and repeated, followed by two 30 min washes in a solution of 1x SSC - 0.1% SDS at 68°C. The post hybridization washes for rDNA and PDC2 probes were performed twice for 5 min at room temperature in 2x SSC, followed by two 30 min. washes in 0.1x SSC - 0.1% SDS at 68°C.

Positive plaques were isolated and purified according to manufacturers instructions (Stratagene). Bacteriophage were purified using conventional methods (Sambrook *et al.*, *Id.*), modified by eliminating DNaseI treatment and precipitating phage particles released from lysed host cells using PEG6000. Said phage particles were then dissolved in SM buffer and extracted with chloroform, pelleted by centrifugation at 25,000 rpm in Kontron TST41.14 rotor for 2 h, and again dissolved in SM buffer. Lambda DNA was isolated by digesting the phage particles with proteinase K followed by phenol extraction and ethanol precipitation.

C. sonorensis genomic DNA inserts were partially sequenced using sequence-specific primers. The nucleotide sequences and the amino acid sequences deduced therefrom were compared against sequence databases in order to identify genes encoded in whole or part by the phage insert, using homology to known genes or proteins. The sequences obtained had significant similarity to fungal rDNA, phosphoglycerate kinases, glyceraldehyde-3-phosphate dehydrogenases, or pyruvate decarboxylases depending on the probe used for isolating each clone. The start and end points of the open reading frames encoding sequences of *C. sonorensis* *PGK1*, *PDC1* and *TDH1* were identified thereby.

“Building-block” vectors, pMI203, pMI205 (Zeocin resistance vectors for *C. sonorensis*), pVR24, and pVR27

These plasmids are used in the construction of the vectors described in the Examples and are described in PCT application PCT/US01/44041. Briefly, the
5 construction of these vectors is described.

The plasmid pTEF1/Zeo (Invitrogen) containing the zeocin resistance marker under control of *S. cerevisiae* *TEF1* promoter was modified by adding a *C. sonorensis* rDNA fragment to provide a target for homologous recombination. The following oligonucleotide primers:

10 TGG ACT AGT AAA CCA ACA GGG ATT GCC TTA GT (SEQ ID No. 29)
and

CTA GTC TAG AGA TCA TTA CGC CAG CAT CCT AGG (SEQ ID No. 30),
which correspond to *C. sonorensis* 26 S rRNA (Genbank Accession No. U70185),
were used to amplify *C. sonorensis* genomic DNA to provide a PCR-amplified
15 fragment of the 26S rDNA gene. The resulting PCR product fragment was digested
with restriction enzymes *SpeI* and *XbaI* and ligated with pTEF/Zseo plasmid
digested with *XbaI*. The resulting plasmid was designated pMI203 (FIG 23 B).

The *TEF1* promoter contained in pMI203 was replaced by a promoter of a
gene from another *Candida* species, the *C. albicans* *PGK1* promoter. The
20 following oligonucleotide primers:

GCG ATC TCG AGG TCC TAG AAT ATG TAT ACT AATTTGC (SEQ ID
No. 31)

and

ACT TGG CCA TGG TGA TAG TTA TTC TTC TGC AATTGA (SEQ ID No.
25 32)

were designed based on the available *C. albicans* *PGK1* sequence (Genbank
Accession No. U25180). These primers were used to amplify a 700 bp fragment
from the region upstream of the *C. albicans* *PGK1* open reading frame, using *C.*
albicans genomic DNA as the template. Restriction sites *XbaI* and *SpeI*
30 (underlined above) were added to the primers to facilitate cloning of the fragment.
After amplification, the fragment was isolated and digested with restriction enzymes
XhoI and *NcoI* and then ligated to plasmid pMI203 digested with *XhoI* and *NcoI*.
The resulting plasmid was designated pMI205 (FIG. 23 B).

PVR24 and pVR27: Plasmid pBFY004 (proprietary, NREL) was digested with *NotI* restriction enzyme (Invitrogen), resulting in a 1235bp fragment (SEQ ID No: 33). The fragment was isolated and ligated to a *NotI* digested pGEM5zF(+) (Promega North, Madison, WI). *E. coli* (top10) (Invitrogen) was transformed with
 5 the ligation mixture using standard electroporation protocols (Sambrook, *Id.*). The resultant plasmid was designated pNC002.

B. megaterium DNA encoding the LDH gene was isolated as follows. *B. megaterium* was obtained from the American Type Culture Collection (ATCC Accession #6458) and grown under standard conditions. Genomic DNA was
 10 purified from these cells using an Invitrogen "Easy-DNA" kit according to the manufacturer's protocol. Primers were designed on the basis of the available sequence in Genbank for the L-LDH from *B. megaterium* (Genbank accession # M22305). PCR amplification reactions were performed using standard techniques, with each reaction containing *B. megaterium* genomic DNA (6 ng/μL),
 15 the 4 dNTPs (0.2 mM), and the amplification primers BM1270 and BM179 (1 μM in each). The primers have the sequences:

BM1270 CCTGAGTCCACGTCATTATTC (SEQ ID
 No:34

and

20 BM179 TGAAGCTATTTATTCTTGTTAC (SEQ ID
 No:35)

Reactions were performed according to the following thermocycling conditions: an initial incubation for 10 min at 95°C, followed by 35 cycles consisting of 30 sec at 95°C, 30 sec. at 50°C, 60 sec at 72°C. A strong product fragment of 1100 base
 25 pairs (bp) was gel purified using conventional procedures, cloned, and sequenced. The resulting sequence could be translated into a polypeptide that exhibited excellent homology to known L-LDH-encoding genes.

The coding sequence for the *B. megaterium* LDH-encoding disclosed herein was operatively linked to a promoter from the PGK1 gene and a
 30 transcriptional terminator from the GAL10 gene, both from the yeast *Saccharomyces cerevisiae*. Two oligonucleotide primers, Bmeg5' and Bmeg3',

were designed based on this sequence to introduce restriction sites at the ends of the coding sequence of the gene:

Bmeg5' GCTCTAGATGAAAACACAATTTACACC (SEQ ID No:36) and

5 Bmeg3' ATGGATCCTTACACAAAAGCTCTGTCGC (SEQ ID No:37)

This amplification reaction was performed using dNTP and primer concentrations described above using *Pfu* Turbo polymerase (Stratagene) in a buffer supplied by the manufacturer. Thermocycling was done by initially incubating the reaction mixture for 3 min at 95°C, then by 20 cycles of 30 sec at 95°C, 30 sec at 50°C, 60 sec at 72°C, followed by a final incubation for 9 min at 72°C. The product was digested with restriction enzymes *Xba*I and *Bam*HI and then ligated into the *Xba*I and *Bam*HI sites of plasmid pNC002. This ligation resulted in the PGK promoter and GAL10 terminator becoming operably linked to the *B. megaterium* LDH coding sequence (pVR24; FIG. 21).

Construction of pVR27 (FIG. 22) was performed to create a vector containing *R. oryzae* LDH for its expression under the control of the *S. cerevisiae* PGK1 promoter. LDH was isolated from *Rhizopus oryzae* from genomic DNA purified ("Easy-DNA" kit, Invitrogen) from cells (ATCC Accession #9363) grown under standard conditions. Primers were designed on the basis of the available sequence in Genbank for the LDH from *R. oryzae* (Genbank accession # AF226154). PCR amplification reactions were performed using standard techniques, with each reaction containing *R. oryzae* genomic DNA (6 ng/μL), each of 4 dNTPs (0.2 mM), and each of the amplification primers Ral-5' and Ral-3' (1 μM). The amplification primers had the sequence:

Ral – 5' CTTTATTTTCTTTACAATATAATTC (SEQ ID No:38)and

Ral-3' ACTAGCAGTGCAAAACATG (SEQ ID No:39)

30 Reactions were performed according to the following cycling conditions: an initial incubation for 10 min at 95°C, followed by 35 cycles consisting of 30 sec at 95°C,

30 sec. at 41°C, 60 sec at 72°C. A strong product fragment of 1100 bp was gel purified, cloned in TA vector (Invitrogen, Carlsbad, CA) and sequenced. The resulting sequence could be translated into a polypeptide that exhibited excellent homology to known *Rhizopus oryzae* LDH-encoding gene sequence in Genbank
 5 (Accession # AF226154).

The coding sequence for the *R. oryzae* LDH-encoding gene disclosed herein was operatively linked to a promoter from the PGK1 and a transcriptional terminator from the GAL10 gene, both from the yeast *S. cerevisiae*. In making this construct, the following oligonucleotides were prepared and used to amplify the
 10 coding sequence from the plasmid containing the *Rhizopus* LDH insert. Two oligonucleotide primers, Rapgk5 and Papgk3', were designed based on this sequence to introduce restriction sites at the ends of the coding sequence of the gene.

Rapgk5 GCTCTAGATGGTATTACACTCAAAGGTCG (SEQ ID
 15 No:40) and
 Papgk3 GCTCTAGATCAACAGCTACTTTTAGAAAAG (SEQ ID
 No:41)

This amplification reaction was performed using dNTP and primer concentrations as described above using *Pfu* Turbo polymerase (Stratagene) in a buffer supplied
 20 by the manufacturer. Thermocycling was done by initially incubating the reaction mixture for 3 min at 95°C, then by 20 cycles of 30 sec at 95°C, 30 sec at 53°C, 60 sec at 72°C, followed by a final incubation for 9 min at 72°C. The product was digested with restriction enzymes *Xba*I and then ligated into the *Xba*I site of plasmid pNC002.

25 This ligation resulted in the PGK promoter and GAL10 terminator becoming operably linked to the *R. oryzae* LDH coding sequence (pVR27; FIG. 22)

pMI234 and pMI238: In order to develop a positive selection for *C.*
 30 *sonorensis* transformants, the *S. cerevisiae* *MEL5* gene (Naumov *et al.*, 1990, *MGG* 224: 119-128; Turakainen *et al.*, 1994, *Yeast* 10: 1559-1568; Genbank Accession No. Z37511) was obtained as the 2160 bp *Eco*RI-*Spe*I fragment from

plasmid pMEL5-39 and ligated to pBluescript II KS(-) (Stratagene) digested with *EcoRI* and *SpeI*. The *EcoRI* site in the *MEL5* gene is located 510 bp upstream of the initiator ATG, and the *SpeI* site is located 250 bp downstream of the stop codon of *MEL5*. The resulting plasmid was designated pMI233 (FIG. 23 C).

5 The 1500 bp *PGK1* promoter of *C. sonorensis* was amplified with primers having the sequence: GCG ATC TCG AGA AAG AAA CGA CCC ATC CAA GTG ATG (SEQ ID No. 5) and TGG ACT AGT ACA TGC ATG CGG TGA GAA AGT AGA AAG CAA ACA TTG TAT ATA GTC TTT TCT ATT ATT AG (SEQ ID No. 42) using DNA from the *PGK1* lambda clone isolated above as
10 template. The 3' primer can create a fusion between the *C. sonorensis PGK1* promoter and *S. cerevisiae MEL5*, since it corresponds to nucleotides present in the *PGK1* promoter immediately upstream of the open reading frame and nucleotides corresponding to the 5' end of *MEL5* open reading frame. The resulting amplified fragment was digested with restriction enzymes *SphI* and *XhoI* and ligated to
15 plasmid pMI233 (FIG. 23 C) digested with *SphI* and *XhoI*. The resulting construct in the plasmid contains *C. sonorensis PGK1* promoter upstream of and operatively linked to the *MEL5* open reading frame, and is identified as pMI234 in FIG. 5.

 In a similar fashion, a 650 bp of the *C. sonorensis TDH1* promoter was amplified with primers having the sequence: GCG ATC TCG AGA AAA TGT
20 TAT TAT AAC ACT ACA C (SEQ ID No. 3) and TGG ACT AGT ACA TGC ATG CGG TGA GAA AGT AGA AAG CAA ACA TTT TGT TTG ATT TGT TTG TTT TGT TTT TGT TTG (SEQ ID No. 43) using DNA from the *TDH1* lambda clone isolated above as the template. The 3' primer can create a fusion between *C. sonorensis TDH1* promoter and *S. cerevisiae MEL5*, since it corresponds
25 to nucleotides present in the *TDH1* promoter immediately upstream of the open reading frame and nucleotides corresponding to the 5' end of *MEL5* open reading frame. The amplified fragment was digested with *SphI* and *XhoI* and ligated to plasmid pMI233 (FIG. 23 C) digested with *SphI* and *XhoI*. The resulting plasmid, identified as pMI238 in FIG. 6, contains *C. sonorensis TDH1* promoter upstream of
30 and operatively linked to the *MEL5* open reading frame.

pMI246 and pMI247: Plasmid pMI205 was used to produce a plasmid containing the *MEL5* gene as a selectable marker and the LDH gene for enabling production of lactic acid in *C. sonorensis*. In the resulting plasmid, the zeocin resistance gene in pMI205 was replaced by the *L. helveticus* LDH gene.

- 5 A 1329 bp *NcoI*-*Bam*HI fragment of pVR1 containing the LDH gene and the *CYC1* terminator was ligated to the 3413 bp *NcoI*-*Bam*HI fragment of pMI205 (FIG. 23 B) bringing the *L. helveticus* LDH gene under control of the *C. albicans* *PGK1* promoter; the resulting plasmid was named pMI214. In a second step the *C. albicans* *PGK1* promoter was replaced by the *C. sonorensis* *PGK1* promoter.
- 10 The *C. sonorensis* *PGK1* promoter was isolated by amplification from an isolated lambda clone as described above using primers having the sequence: GCG ATC TCG AGA AAG AAA CGA CCC ATC CAA GTG ATG (SEQ ID No. 5) and ACT TGG CCA TGG TAT ATA GTC TTT TCT ATT ATT AG (SEQ ID No. 44), and the PCR product was digested with *XhoI* and *NcoI* and ligated into pMI214
- 15 digested with *XhoI* and *NcoI*. This plasmid was designated pMI277 and is shown in FIG. 19.

- The LDH expression cassette from pMI227 and *MEL5* marker cassette from pMI234 were combined into the same vector by ligating a 3377 bp *AvrII*-*NheI* fragment of pMI227 (FIG. 23 A) with *SpeI*-digested pMI234 (FIG. 23 C).
- 20 The resulting plasmid was designated pMI246 and is shown in FIG. 8.

- The LDH expression cassette from pMI227 and the *MEL5* marker cassette from pMI238 were combined into the same vector by ligating a 3377 bp *AvrII*-*NheI* fragment of pMI227 with *SpeI*-digested pMI238. The resulting plasmid was designated pMI247 and is shown in FIG. 9.

25

 In one embodiment, the invention provides recombinant nucleic acid constructs comprising a nucleotide sequence that encodes a polypeptide useful for the biosynthesis of an organic product, which is operatively linked to a promoter that is functional in the genera *Candida*.

- 30 In related embodiments the nucleotide sequence encodes a lactate dehydrogenase gene. In preferred embodiments, the lactate dehydrogenase gene is heterologous to the *Candida* yeast cell into which it is introduced. In most preferred embodiments the lactate dehydrogenase gene is from a microorganism

such as, for example, a bacterium or fungus, and the organic product produced according to the methods of the invention is lactic acid (or lactate).

Typically, the methods of the invention for producing lactic acid can yield (based on grams of lactic acid produced / gram of a carbohydrate substrate consumed) about 60% or more, preferably about 70% or more, more preferably about 80% or more, and most preferably about 90% or more, when the carbohydrate substrate is a hexose, for example, glucose.

The methods of the invention for producing lactic acid can result in lactic acid titers of about 75 grams/L or more, preferably about 90 grams/L or more, and most preferably about 100 grams/L or more. The cells of the invention have a specific productivity of lactic acid production (in terms of grams of lactic acid produced / gram of dry cell weight per hour) of about 0.20 or more, preferably about 0.30 or more, and most preferably about 0.50 or more, when a hexose carbohydrate substrate, such as glucose, is used for production.

In one embodiment, the Crabtree-negative cells of the invention can catabolize starch, either naturally or because of a genetic modification. In additional embodiments, the cells are genetically modified to catabolize celluloses through the addition of such molecules as fungal-based cellulases.

In related embodiments, the cells of the invention can metabolize sugars other than glucose or other monosaccharide hexoses, in particular pentoses including the non-limiting examples of xylose and L-arabinose.

The Crabtree-negative cells of the invention are preferably selected from the *Candida* strains *C. sonorensis*, *C. methanosorbosa*, *C. diddensiae*, *C. parapsilosis*, *C. naeodendra*, *C. krusei*, *C. blankii*, and *C. entomophila*. In preferred embodiments the cells *C. sonorensis* and *C. methanosorbosa* cells.

Methods for isolating organic products produced by the cells of the invention are well known in the art. In particular, methods for separating lactic acid from a fermentation mixture, including low pH fermentation mixtures, are disclosed by Eyal *et al.* (International Patent Application, Publication No. WO 99/19290, published April 22, 1999). Such methods for isolating lactic acid include extraction, adsorption, distillation/vaporization, separation *via* a membrane, crystallization, and phase splitting. (*See also*: Vickroy, 1985, Comprehensive Biotechnology, (Moo-Young, ed.), Volume 3, Chapter 38

Pergamon Press, Oxford; Datta *et al.*, 1995, *FEMS Microbiol. Rev.* 16: 221-231; U.S. Patent 4,771,001; U.S. Patent 5,132,456; U.S. Patent 5,510,526; and U.S. Patent 5,420,304).

5 Fermentation Conditions

Various fermentation processes can be used with the various aspects of the instant invention. (See, e.g., Wolf, 1996, Nonconventional Yeasts in Biotechnology, Springer-Verlag Berlin, and Walker, 2000, Yeast Physiology and
10 Biotechnology, John Wiley & Sons, England). Those of skill in the art will recognize that fermentation conditions can be varied to improve various aspects of the fermentation, including product yield, culture productivity, and culture health (among others), depending on the specific host organism and desired product. It is particularly advantageous to use the favorable characteristics of *Candida* in
15 adjusting the fermentation conditions. Thus, the pH can have a range during various stages of processing from about 2.5 to about 9.0. Oxygen levels can vary from about 0% to about 100% (relative to the oxygen content found in air), as measured in the atmosphere above the medium or dissolved in the medium. Oxygen levels can be measured or calculated by any common methods including
20 partial pressure, O₂ electrode, volume/volume, or gas flow rate (VVM). Temperature ranges can span from about ambient temperature (23°C) to about 40°C and above (e.g. to about 45°C).

Preferred fermentation conditions include maintenance of a pH range from about 4 to about 5. It is especially preferred to maintain a pH of about 5 during
25 biomass production and during lactic acid production. Preferably, the pH is maintained throughout the entirety of the fermentation process by automated addition of a base, for example Ca(OH)₂. The temperature during biomass production is preferably maintained at about 35°C. Preferably the biomass is produced under aerobic conditions wherein the culture medium is preferably
30 agitated and supplied an airflow, until an adequate cell mass for lactic acid production is attained. During production of lactic acid, the agitation rate and airflow are preferably slowed, relative to their rate during biomass production.

The following data was generated from three different fermenter cultivations on glucose medium under the preferred conditions detailed above.

Overall yield and productivity

Batch	Strain	CDW (g/l)	pH*	Base	LA g/L	Glu (final)	Yield (%)	Time/h	Productivity	
									g/l/h	g/g-cell/h
602	C40/288-34	5,1	5	Ca(OH) ₂	73	0	81	34	2,15	0,42
802	C40/288-34	5	5/4	Ca(OH) ₂	49	3	70	90	0,54	0,11
902	C40/288-34		4/4	Ca(OH) ₂						

*pH during biomass production/pH during lactate production

5

Yield and productivity in lactate production phase

Batch	Strain	CDW (g/l)	pH	Glu (init.)	Glu (final)	LA (init.)	LA (final)	Time/h	Yield (%)	Productivity	
										g/l/h	g/g-cell/h
602	C40/288-34	5,1	5	63	0	12,5	73	21,5	96	2,81	0,55
802	C40/288-34	5	4	58	3	2	49	77	87	0,61	0,12
902	C40/288-34		4								

10

The following examples serve to illustrate certain embodiments of the invention and do not limit it in scope or spirit.

Examples

15

Example 1: G418 resistance vectors and use of G418 for selection of *C. sonorensis* transformants

Vectors conferring G418 resistance on transformed yeast cells, which permit selection of yeast cell transformants comprising a recombinant nucleic acid construct encoding a protein useful for synthesis of an organic product, were prepared as follows. The G418 resistance marker was cloned to be under the transcriptional control of either the *C. sonorensis* *PGK1* or *TDH1* promoter and the constructs were designated as pMI268 (FIG. 2) and pMI269 (FIG. 3), respectively. The *S. cerevisiae* *GAL10* terminator was used in both cases.

The G418 resistance gene was amplified by polymerase chain reaction (PCR) using the Dynazyme EXT Polymerase (Finnzymes, Espoo, Finland) using a

pair of oligonucleotide primers having the sequence: CTAGTCTAGA ACA ATG AGC CAT ATT CAA CGG GAA ACG (G418 5'; SEQ ID NO:1) and CGC GGATCC GAA TTC TTA GAA AAA CTC ATC GAG CAT CAA ATG (G418 3'; SEQ ID NO:2). The plasmid pPIC9K (obtained from Invitrogen) was used as
 5 template. PCR was performed by initially incubating the reaction mixture for 5 min at 95°C, followed by 29 cycles of 45 sec at 95°C, 45 sec at 55°C, and 2 min at 72°C, with a final incubation for 5 min at 72°C. The PCR product was digested with restriction enzymes *Bam*HI and *Xba*I and an 800 bp fragment was isolated. This fragment was ligated to the 4226 bp *Bam*HI-*Xba*I fragment of pNC101
 10 (obtained from Eric Jarvis at NREL). Plasmid pNC101 was constructed from the phosphoglycerate kinase promoter (pPGK) and the GAL10 terminator sequences from *S. cerevisiae*, using standard cloning techniques (see, e.g., Sambrook et al., *Id.*). This plasmid also harbors an LDH gene from *K. thermotolerans* inserted between *Xba*I and *Eco*RI sites, which, along with a *Bam*HI site, are contained in a
 15 polylinker region found between the yeast promoter and terminator sequences. This plasmid permits expression of various genes or selectable markers, under the control of the yeast promoter and terminator.

The plasmid resulting from these manipulations contains the G418 resistance gene between the *S. cerevisiae* *PGK1* promoter and the *S. cerevisiae*
 20 *GAL10* terminator, and was named pMI260. The structure of this plasmid is shown schematically in FIG. 1.

The 600 bp *TDH1* promoter of *C. sonorensis* was amplified by PCR using the Dynazyme EXT Polymerase with a pair of oligonucleotide primers having the sequence: GCG ATC TCG AGA AAA TGT TAT TAT AAC ACT ACA C (5441;
 25 SEQ ID NO:3) and CTAGTCTAGATT TGT TTG ATT TGT TTG TTT TGT TTT TGT TTG (Cs1; SEQ ID NO:4) using pMI238 as a template (see above "Vectors and Host Cells"; shown in FIG. 6). PCR was performed by initially incubating the reaction mixture for 5 min at 95°C, followed by 29 cycles of 45 sec at 95°C, 45 sec at 55°C, 2 min at 72°C, with a final incubation for 5 min at 72°C.
 30 The PCR product was made blunt ended with Klenow polymerase and each of the 4 dNTPs and then digested with the restriction enzyme *Xba*I. The resulting 600bp fragment was ligated with the 4216 bp *Pst*I (made blunt ended with T4 polymerase)-*Xba*I fragment of pMI260. The resulting plasmid contains the G418

resistance gene operatively linked to the *C. sonorensis* *TDH1* promoter and the *S. cerevisiae* *GAL10* terminator and was named pMI269. The structure of this plasmid is shown schematically in FIG. 3.

The 1500 bp *C. sonorensis* *PGK1* promoter was amplified by PCR using
5 the Dynazyme EXT Polymerase with a pair of oligonucleotide primers having the sequence: GCG ATC TCG AGA AAG AAA CGA CCC ATC CAA GTG ATG (5423; SEQ ID NO:5) and CTA GTC TAG ATG TAT ATA GTC TTT TCT ATT ATT AG (Cs2;SEQ ID NO:6) using pMI234 as the template (*see above* “Vectors and Host Cells”; FIG. 5). PCR was performed by initially incubating the reaction
10 mixture for 5 min at 95°C, followed by 29 cycles of 45 sec at 95°C, 45 sec at 55°C, 2 min at 72°C, with a final incubation for 10 min at 72°C. The 1500 bp PCR product fragment was made blunt ended with Klenow polymerase and each of the 4 dNTPs and then digested with the restriction enzyme *Xba*I. The 1500 bp *PGK1* promoter fragment was ligated with the 4216 bp *Pst*I (made blunt ended
15 with T4 polymerase)-*Xba*I fragment of pMI260. The resulting plasmid contains the G418 resistance gene operatively linked to the *C. sonorensis* *PGK1* promoter and the *S. cerevisiae* *GAL10* terminator, and was named pMI268. The structure of this plasmid is shown schematically in FIG. 2.

The two constructs pMI268 and pMI269 were digested with restriction
20 enzymes *Sal*I and *Not*I and transformed into *C. sonorensis* using the chemical method according to Gietz *et al.* (1992, *Nucleic Acids Res.* 20:1425). This transformation technique was used throughout these Examples, and is described briefly as follows.

Cells from an overnight culture of *C. sonorensis* grown to an OD₆₀₀ of
25 0.8-1.5 were collected by centrifugation, and were washed first with an excess of a solution of 10 mM Tris-HCl, 1 mM EDTA (pH 7.5), followed by washing with an excess of a solution of 100 mM lithium acetate (LiAc), 10 mM Tris-HCl, 1 mM EDTA (pH 7.5), and then resuspended in 2 mL of a solution of 100 mM LiAc, 10 mM Tris-HCl, 1 mM EDTA (pH 7.5). Cells were mixed (about 50 µL of the 2mL
30 suspension) with about 10 µg of transforming DNA and 300 µL of a solution of 40% PEG4000, 100 mM LiAc, 10 mM Tris-HCl, 1 mM EDTA (pH 7.5). The cells were incubated at 30°C for 30 min with slow shaking. Dimethyl sulfoxide

(DMSO; 40 μ L) was added and the cells were incubated in a 42°C water bath for 15 min. The cells were collected by centrifugation, washed with an excess of a solution of 10 mM Tris-HCl, 1 mM EDTA (pH 7.5), resuspended and incubated at 30°C in YPD medium (comprising 10g/L yeast extract, 20g/L peptone and 20 g/L glucose) for 3-7 h. Optionally, the YPD incubation can be continued overnight.

Before applying selection the cells were incubated in liquid YPD for at least 3 h or overnight. The transformants were grown on YPD agar plates (comprising 10g/L yeast extract, 20g/L peptone, 20 g/L glucose and 2% agar) supplemented with G418 antibiotic at a concentration of either 100 μ g/mL or 200 μ g/mL. The plates were incubated at 30°C for 2-5 days and the transformants were then restreaked onto fresh selection plates. Southern analysis of total DNA isolated from the G418 resistant colonies showed that the G418 resistance gene was integrated in the genome of the transformants.

These results showed that the G418 resistance gene can be expressed from the constructs prepared as described herein and is a suitable selection for *C. sonorensis* transformation.

Example 2: Hygromycin resistance (*hph*) vectors and use of hygromycin B for selection of *C. sonorensis* transformants

Vectors conferring hygromycin resistance on transformed yeast cells, which permit selection of yeast cell transformants comprising a recombinant nucleic acid construct encoding a protein useful for synthesis of an organic product, were prepared as follows. The hygromycin resistance marker (*E. coli hph*) was cloned under the transcriptional control of either the *C. sonorensis PGK1* and *TDH1* promoter and the constructs were designated as pMI270 (FIG. 4) and pMI271, respectively. The *S. cerevisiae GAL10* terminator was used in both cases.

The *E. coli hph* gene that confers resistance to hygromycin B was obtained from the plasmid pRLMex30 (Mach *et al.* 1994, *Curr. Genet.* 25, 567-570). pRLMex30 was linearized with the restriction enzyme *NsiI* and made blunt ended with T4 DNA polymerase and then digested with *XbaI*.

The pMI268 plasmid prepared in Example 1 was digested with *EcoRI* and was made blunt ended with Klenow polymerase and each of the 4 dNTPs and then

digested with *Xba*I. The resulting 4900 bp fragment was ligated with the 1035 bp *hph* fragment from pRLMex30. This ligation produced a plasmid that contains the hygromycin resistance gene operatively linked to the *C. sonorensis* *PGK1* promoter and the *S. cerevisiae* *GAL10* terminator, and was named pMI270. The structure of this plasmid is shown schematically in FIG. 4.

The pMI269 plasmid prepared in Example 1 was digested with *Eco*RI and was made blunt ended with Klenow polymerase and each of the 4 dNTPs and then digested with *Xba*I. The resulting 4000 bp fragment was ligated with the 1035 bp *hph* fragment of pRLMex30. This produced a plasmid that contains the hygromycin resistance gene operatively linked to the *C. sonorensis* *TDH1* promoter and the *S. cerevisiae* *GAL10* terminator, and was named pMI271. The structure of this plasmid is shown schematically in FIG. 7.

Yeast cells were transformed using the chemical method according to Gietz *et al.* (1992, *Nucleic Acids Res.* 20: 1425) as described in Example 1 above. The two constructs pMI270 and pMI271 were digested with the restriction enzymes *Xho*I and *Not*I. The transformation mixture was incubated in YPD at 30°C for 3h before plating onto selective plates. The transformants were grown at 30°C for 2-5 days on YPD agar plates supplemented with hygromycin B (Calbiochem) at concentrations of 150-300 µg/mL. Transformants were restreaked onto fresh selection plates. The presence of the transformed DNA in the genome of the hygromycin resistant transformants was verified by PCR using a pair of oligonucleotide primers having the sequence: CCGGACTA GTT GGT ACA GAG AAC TTG TAA ACA ATT CGG (*ScerGal10t*; SEQ ID NO:7) and TAT AAA TAC TTA TCA TTT CCTCC (5436; SEQ ID NO:8). PCR was performed by initially incubating the reaction mixture for 3 min at 94°C, followed by 29 cycles of 45 sec at 94°C, 45 sec at 55°C, 2 min at 72°C, with a final incubation for 10 min at 72°C.

These results show that the *E. coli* *hph* gene can be expressed using the constructs described herein, functions in *C. sonorensis* and that hygromycin B can be used to select *C. sonorensis* transformants.

Example 3: Vectors for expression of the *L. helveticus* LDH and for targeted integration of the transformed DNA into the *PDC1* locus

Vectors comprising a *L. helveticus* LDH gene for targeted integration into the *C. sonorensis* *PDC1* gene locus were prepared as follows. The pMI246 vector contains the *MEL5* expression cassette and the *L. helveticus* LDH expression cassette, shown schematically in FIG. 8 (*see above* “Vectors and Host Cells”). In order to construct a vector that enables targeted integration into the *C. sonorensis* *PDC1* locus and replacement of the *PDC1* protein-coding region, DNA fragments corresponding to sequences immediately upstream and downstream of the *PDC1* protein-coding region were added into pMI246.

The *PDC1* terminator was amplified by PCR using the Dynazyme EXT Polymerase (Finnzymes, Espoo, Finland) with oligonucleotide primers having the sequence: GGG ACT AGT GGA TCC TTG AAG TGA GTC AGC CAT AAG GAC TTA AATTCACC (Cs7; SEQ ID NO:9) and AAGGCCT TGT CGA CGC GGC CGC TTG GTT AGA AAA GGT TGT GCC AAT TTA GCC (Cs8; SEQ ID NO:10), using *C. sonorensis* genomic DNA as a template. PCR was performed by initially incubating the reaction mixture for 5 min at 95°C, followed by 29 cycles of 45 sec at 95°C, 45 sec at 55°C, 2 min at 72°C, with a final incubation for 10 min at 72°C. The 920 bp PCR product fragment was digested with restriction enzymes *Bam*HI and *Not*I and the 920 bp fragment was purified and ligated with the 8900 bp *Bam*HI-*Not*I fragment from pMI246. The resulting plasmid was named pMI256, and is shown schematically in FIG. 18.

The *PDC1* promoter was amplified from *C. sonorensis* with a pair of oligonucleotide primers having the sequence: GGG ACG GGC CCG CGG CCG CTA CAA GTG ATT CAT TCA TTC ACT (Cs5; SEQ ID NO:11) and CCC TGG GCC CCT CGA GGA TGA TTT AGC AAG AAT AAA TTA AAA TGG (Cs6; SEQ ID NO:12) using genomic *C. sonorensis* DNA as a template. PCR was performed by initially incubating the reaction mixture for 5 min at 95°C, followed by 29 cycles of 45 sec at 95°C, 45 sec at 55°C, 2 min at 72°C, with a final incubation for 10 min at 72°C. The PCR product fragment was digested with *Apa*I and the 800 bp fragment was purified and ligated with the 9760 bp *Apa*I linearized pMI256 (*see above* “Vectors and Host Cells”; FIG. 18). The resulting plasmid was named pMI257, and is shown schematically in FIG. 10. pMI257 contains, in order, the *C. sonorensis* *PDC1* promoter, the *C. sonorensis* *PGK1*

promoter operatively linked to the *S. cerevisiae* *MEL5* gene, the *C. sonorensis* *PGK1* promoter operatively linked to the *L. helveticus* *LDH* and the *S. cerevisiae* *CYC1* terminator followed by the *C. sonorensis* *PDC1* terminator.

pMI257 was digested with *NotI* to excise the 7300 bp fragment containing
5 the *MEL5* and *LDH* expression cassettes flanked by the *PDC1* 5' and 3' regions. This 7300 bp fragment was used to transform *C. sonorensis* by the method described in Example 1 above, and the transformants were screened based on expression of the *MEL5* marker. The transformants were grown on YPD agar plates supplemented with the chromogenic substrate of α -galactosidase, 5-bromo-
10 4-chloro-3-indolyl- α -D-galactopyranoside (X- α -gal; ICN Biochemicals) at a concentration of 40 μ g/mL. The plates were incubated at 30°C for 1-3 days and then transferred to 4°C. In the presence of X- α -gal yeast colonies transformed with a functional *MEL5* expression cassette turned blue, whereas the untransformed colonies were white. Blue colonies were purified by restreaking
15 them onto fresh indicator plates. The transformants originating from the transformation of *C. sonorensis* with *NotI* digested pMI257 were designated as 257-1 through 257-15, 257-41, 257-42, and 257-45.

Southern blot analysis of genomic DNA isolated from the pMI257 transformants was carried out with the *C. sonorensis* *PDC1* probe to identify
20 transformants in which the anticipated replacement of the *PDC1* open reading frame by the transformed pMI257 DNA had occurred. The absence of a *PDC1* hybridizing band in transformants 257-3, 257-9, 257-12, 257-15, and 257-41 indicated that *PDC1* gene was deleted. Other pMI257 transformants and *C. sonorensis* gave a positive signal in the *PDC1* hybridization. Hybridization with
25 the *L. helveticus* LDH probe showed that the *LDH* gene was present in one copy in the *pdcl* deletants. Transformants 257-6 257-7, and 257-8 contained two copies of the *L. helveticus* LDH randomly integrated into the genome. Other pMI257 transformants had one copy of LDH randomly integrated in the genome.

These results show that targeted integration of the transformed pMI257
30 DNA into the *PDC1* locus occurred through homologous recombination between *PDC1* promoter and terminator sequences. These results also show that *PDC1* is a single copy gene in *C. sonorensis*. In addition, integration events outside the

PDC1 locus occurred. In some transformants the LDH gene was integrated in more than one copy into the genome.

**Example 4: Vectors for expression of the *B. megaterium* LDH and for
5 targeted integration of the transformed DNA into the *PDC1* locus**

Vectors comprising a *B. megaterium* LDH gene for targeted integration into the *C. sonorensis* *PDC1* gene locus were prepared as follows. In these vectors, the *L. helveticus* LDH in pMI257 was replaced by the *B. megaterium* LDH.

10 pMI257 was linearized with *NcoI* and the 5' overhangs were partially filled in with DNA polymerase I, Klenow fragment, and a mixture of dATP, dCTP, and dTTP, omitting dGTP from the reaction. This was followed by removal of the single stranded extensions by treatment with mung bean nuclease. The DNA was then digested with *BamHI* and the 9200 bp fragment was isolated from a 0.8%
15 agarose gel after electrophoretic separation. Vector pVR24 containing *B. megaterium* LDH was generated from *B. megaterium* genomic DNA, and is shown in FIG. 21. The LDH gene was cloned from the genomic DNA by PCR using primers designed from accession no. M22305, and ligated into a commercially-available vector (see above "Vectors and Host Cells"). The 976 bp
20 fragment containing the *B. megaterium* LDH was excised from pVR24. by *XbaI* digestion followed by fill-in of the 5' overhangs by DNA polymerase I, Klenow fragment and each of the 4 dNTPs, and digestion by *BamHI*. The 9200 bp *NcoI* (blunt)-*BamHI* fragment from pMI257 and the 976 bp *XbaI*(blunt)-*BamHI* fragment from pVR24 were ligated and the resulting plasmid was designated as
25 pMI265, shown in FIG. 11. pMI265 contains, in order, the *C. sonorensis* *PDC1* promoter, the *C. sonorensis* *PGK1* promoter operatively linked to the *S. cerevisiae* *MEL5* gene, the *C. sonorensis* *PGK1* promoter operatively linked to the *B. megaterium* LDH and the *C. sonorensis* *PDC1* terminator. pMI265 was digested with *NotI* to excise the 7300 bp fragment that consisted of the *MEL5* and LDH
30 expression cassettes flanked by the *PDC1* 5' and 3' regions. This 7300 bp fragment was used to transform *C. sonorensis* as described in Example 1 and the transformants were screened on YPD plates supplemented with X- α -gal at a concentration of 40 μ g/mL. The transformants were grown on YPD agar plates

supplemented with X- α -gal (40 μ g/mL). The transformants originating from the transformation of *C. sonorensis* with *NotI*-digested pMI265 were designated as 265-1 through 265-60.

Southern blot analysis of genomic DNA isolated from the pMI265
5 transformants was carried out with the *C. sonorensis* *PDC1* probe to identify transformants in which the anticipated replacement of the *PDC1* open reading frame by the transformed pMI265 DNA had occurred. The absence of a *PDC1* hybridizing band in transformants 265-5, 265-7, 265-15, 265-17, 265-33, 265-34, 265-35, 265-38, 265-39, 265-42, 265-43, 265-47, 265-48, 265-49, 265-51, and
10 265-60 indicated that the *PDC1* gene was deleted. Other pMI265 transformants and untransformed *C. sonorensis* gave a positive signal for *PDC1* hybridization. Hybridization with the *B. megaterium* LDH probe showed that the LDH gene was present in one copy in the *pdcl* deletants. Positively *PDC1*-hybridizing transformants 265-14, 265-22 and 265-23 contained two copies and 265-56
15 contained three copies of the LDH gene randomly integrated into the genome. Other pMI265 transformants had one copy of LDH randomly integrated in the genome.

These results showed that targeted integration of the transformed pMI265 DNA into the *PDC1* locus occurred through homologous recombination between
20 *PDC1* promoter and terminator sequences. These results also confirmed that *PDC1* is a single copy gene in *C. sonorensis*. The transformants deleted of *pdcl* were viable, indicating that *PDC1* is not an essential gene in *C. sonorensis*. In addition to *PDC1*-deleting integrations, integration events outside the *PDC1* locus occurred in certain transformants. In some transformants the LDH gene was
25 integrated in more than one copy into the genome.

Example 5: Vectors for expression of *R. oryzae* LDH and for targeted integration of the transformed DNA into the *PDC1* locus

Vectors comprising a *R. oryzae* LDH gene for targeted integration into the
30 *C. sonorensis* *PDC1* gene locus were prepared as follows. In these vectors, the *L. helveticus* LDH encoding sequences in pMI257 were replaced by *R. oryzae* LDH.

The pMI257 plasmid described in Example 3 above was linearized with *NcoI* and the 5' overhangs were partially filled in with DNA polymerase I, Klenow

fragment, and a mixture of dATP, dCTP, and dTTP, omitting dGTP from the reaction. This was followed by removal of the single stranded extensions by treatment with mung bean nuclease. The DNA was then digested with *Bam*HI and the 9200 bp fragment was isolated from a 0.8% agarose gel after electrophoretic separation. The coding sequence of *R. oryzae* LDH-encoding DNA was operatively linked to the *C. sonorensis* *PGK1* promoter and the transcriptional terminator of the *C. sonorensis* *PDC1* gene. Vector pVR 27 containing *R. oryzae* LDH was generated from *R. oryzae* genomic DNA, and is shown in FIG. 22. The LDH gene was cloned from the genomic DNA by PCR using primers designed from accession no. AF226154, and ligated into a commercially-available vector (see above "Vectors and Host Cells"). The 978 bp fragment containing the *R. oryzae* LDH was excised from pVR27 by *Xba*I digestion followed by fill in of the 5' overhangs by DNA polymerase I, Klenow fragment and each of the 4 dNTPs and digestion by *Bam*HI. The 9200 bp *Nco*I (blunt)-*Bam*HI fragment from pMI257 and the 978 bp *Xba*I(blunt)-*Bam*HI from pVR27 were ligated and the resulting plasmid containing was designated as pMI266, shown schematically in FIG. 12. pMI266 contains, in order, the *C. sonorensis* *PDC1* promoter, the *C. sonorensis* *PGK1* promoter operatively linked to the *S. cerevisiae* *MEL5* gene, the *C. sonorensis* *PGK1* promoter operatively linked to the *R. oryzae* LDH A and the *C. sonorensis* *PDC1* terminator. pMI266 was digested with *Not*I to excise a 7300 bp fragment that consisted of the *MEL5* and LDH expression cassettes flanked by the *PDC1* 5' and 3' regions. This 7300 bp fragment was used to transform *C. sonorensis* by the method described in Example 1 above, and the transformants were screened on YPD plates supplemented with X- α -gal at a concentration of 40 μ g/mL. The transformants originating from the transformation of *C. sonorensis* with *Not*I-digested pMI266 were designated as 266-1 through 266-13.

Southern blot analysis of genomic DNA isolated from the pMI266 transformants was carried out with the *C. sonorensis* *PDC1* probe to identify transformants in which the anticipated replacement of the *PDC1* open reading frame by the transformed pMI266 DNA had occurred. The absence of a *PDC1* hybridizing band in transformants 266-1, 266-3, 266-4, and 266-11 indicated that *PDC1* gene was deleted. In contrast, pMI266 transformants 266-2, 266-7 and 266-8 and untransformed *C. sonorensis* gave a positive signal in the *PDC1*

hybridization. Hybridization with the LDH probe showed that the *R. oryzae* LDH gene was present in one copy in all the transformants.

These results showed that targeted integration of the transformed pMI266 DNA into the *PDC1* locus occurred through homologous recombination between
5 *PDC1* promoter and terminator sequences. In addition, integration events outside the *PDC1* locus occurred.

Example 6: Vector for replacement of *PDC1* without LDH

Vectors were prepared for replacing *PDC1* without introducing exogenous
10 LDH-encoding sequences. The pMI257 plasmid described in Example 3 above was digested with *NcoI* and *BamHI* in order to remove the LDH gene and the *S. cerevisiae* *CYC1* terminator. The 5' overhangs were filled in by DNA polymerase I, Klenow fragment, and each of the 4 dNTPs. The 9200 bp fragment was purified
15 after agarose gel electrophoresis and recircularized by incubation at a concentration of 40 ng/μL in the presence of 400 U of T4 DNA ligase (New England Biolabs) and the appropriate buffer recommended by the manufacturer. The resulting plasmid was named pMI267, and is shown schematically in FIG. 13. pMI267 contains, in order, the *C. sonorensis* *PDC1* promoter, the *C. sonorensis* *PGK1* promoter operatively linked to the *S. cerevisiae* *MEL5* gene, and the *C.*
20 *sonorensis* *PDC1* terminator.

pMI267 was digested with *NotI* to excise the 6300 bp fragment that consisted of the *MEL5* cassette flanked by the *PDC1* 5' and 3' regions. This 6300 bp fragment was used to transform *C. sonorensis* by the method described above in Example 1 and the transformants were screened on YPD plates supplemented
25 with X-α-gal at a concentration of 40 μg/mL. The transformants originating from transformation of *C. sonorensis* with *NotI* digested pMI267 were designated as 267-1 through 267-10.

Southern blot analysis of genomic DNA isolated from the pMI267 transformants was carried out with the *C. sonorensis* *PDC1* probe to identify
30 transformants in which *PDC1* open reading frame was deleted. The absence of a *PDC1* hybridizing band in transformants 267-1 and 267-10 indicated that the *PDC1* gene was deleted.

These results showed that targeted integration of the transformed pMI267 DNA into the *PDC1* locus occurred through homologous recombination between the *PDC1* promoter and terminator sequences. LDH expression was not required to maintain the viability of the *pdcl*-deleted strain. In addition, integration events
5 outside the *PDC1* locus occurred.

Example 7: Construction of a *C. sonorensis* vector containing the *B. megaterium* LDH gene and the G418 marker

A vector comprising the G418 resistance gene and *B. megaterium* LDH
10 gene was prepared as follows. In these vectors, the *B. megaterium* LDH expression cassette from the plasmid pMI265 and the G418 resistance marker cassette from the plasmid pMI269 were combined into the same vector. The pMI269 plasmid described in Example 1 was digested with *EcoRI* and the 5' overhangs were filled in by DNA polymerase I, Klenow fragment, and each of the
15 4 dNTPs, followed by digestion of the DNA with *BamHI*. The 4800 bp *EcoRI*(blunt) -*BamHI* fragment of pMI269 was ligated with 2800 bp *MscI* -*BamHI* fragment from the pMI265 plasmid described in Example 4. The resulting plasmid was named pMI278 and contains, in order, the *C. sonorensis* *TDH1* promoter operatively linked to the G418 resistance gene and the *MEL5* terminator
20 followed by the *C. sonorensis* *PGK1* promoter operatively linked to the *B. megaterium* LDH and the *S. cerevisiae* *GAL10* terminator, and is shown schematically in FIG. 14.

**Example 8: Construction of *C. sonorensis* strains expressing the *R. oryzae*
25 LDH and the *B. megaterium* LDH simultaneously**

The *C. sonorensis* transformant designated 266-3, in which the *R. oryzae* LDH is integrated into the *pdcl* locus, was chosen as host for a second transformation with the *B. megaterium* LDH construct described in Example 4 above. Transformant 266-3 was further transformed with *SaII-NotI* digested
30 pMI278 and the transformants were selected on YPD agar plates supplemented with G418 antibiotic at a concentration of 200 µg/mL. The plates were incubated at 30°C for 2-5 days for selection; transformants were purified by restreaking onto fresh selection plates. The resulting transformants were designated as 278-1

through 278-20. The presence of the *B. megaterium* LDH in the genome of 19 of these transformants was verified by Southern blot analysis of *Hind*III digested yeast DNA using the *B. megaterium* LDH gene as the probe. Some of the transformants had more than one copy of the *B. megaterium* LDH integrated in the
 5 genome. Southern blot analysis was repeated with the *R. oryzae* LDH gene as a probe to verify that the *R. oryzae* LDH was still present.

This experiment showed that *C. sonorensis* could be transformed multiply and independently with different markers. In this way it was demonstrated to be possible to increase the copy number of the gene of interest (LDH) in the host
 10 genome.

Example 9: Vectors for expression of *B. megaterium* LDH and for targeted integration of the transformed DNA into the *PDC2* locus

Vectors comprising a *B. megaterium* LDH gene for targeted integration
 15 into the *C. sonorensis* *PDC2* gene locus were prepared as follows. *C. sonorensis* *PDC2* promoter was amplified by PCR using the Dynazyme EXT polymerase and a pair of oligonucleotide primers having the sequence: GGG ACG GGC CCG CGG CCG CTT ACA GCA GCA AAC AAG TGATGCC (Cs26; SEQ ID NO:13) and CCC TGG GCC CCT CGA GTT TGA TTT ATT TGC TTT GTA
 20 AAGAGAA (Cs27; SEQ ID NO:14). The genomic copy of the *C. sonorensis* *PDC2* cloned in a lambda vector was used as the template (see above). PCR was performed by initially incubating the reaction mixture for 3 min at 94°C, followed by 29 cycles of 45 sec at 94°C, 45 sec at 55°C, 2 min at 72°C, with a final incubation for 10 min at 72°C. The 1000 bp PCR product was cloned into the
 25 TOPO TA vector (Invitrogen) and the resulting plasmid was named pMI277, shown schematically in FIG. 19. The *PDC2* promoter was released by *Eco*RI digestion and made blunt ended with the Klenow polymerase and each of the 4 dNTPs.

The pMI278 plasmid prepared as described in Example 7 was linearized by
 30 *Sal*I and the 5' overhangs were filled in by Klenow polymerase and each of the 4 dNTPs, then ligated to the 1000 bp *Eco*RI (blunt) fragment of the pMI277 plasmid. The plasmid containing the insert in the desired orientation was named pMI279, shown schematically in FIG. 20.

The *PDC2* terminator was amplified by PCR using the Dynazyme EXT polymerase with a pair of oligonucleotide primers having the sequence: TGGACTAGTTAGATAG CAA TTC TTA CTT GAA AAA TTA ATT GAA GCA TTACC (Cs29; SEQ ID NO:15) and GGC CCG CGG CCG CTA AAT
 5 ATA ATT ATC GCT TAG TTA TTA AAA TGG (Cs30; SEQ ID NO:16), using the genomic copy of the *C. sonorensis PDC2* gene cloned in a lambda vector as the template. The *pdv2* terminator fragment includes part of the open reading frame corresponding to the 239 C-terminal amino acids. Translation stop codons were introduced in the PCR oligonucleotide Cs29 in all three frames upstream of
 10 the nucleotides corresponding to the last 239 C-terminal amino acids protein in the terminator fragment. PCR was performed by initially incubating the reaction mixture for 3 min at 94°C, followed by 29 cycles of 45 sec at 94°C, 45 sec at 55°C, 2 min at 72°C, with a final incubation for 10 min at 72°C. The PCR product was made blunt ended with the Klenow polymerase and each of the 4
 15 dNTPs, and purified with a Qiaquick column (Qiagen). The PCR product was phosphorylated with T4 polynucleotide kinase and rATP at a concentration of 1 mM under standard conditions (see Sambrook *et al.*, *Id.*). The 800 bp *PDC2* terminator fragment was purified after agarose gel electrophoresis and ligated with *NcoI* (blunt) digested pMI279 that was dephosphorylated with calf intestinal
 20 phosphatase. The resulting plasmid was named pMI286 and contains, in order, the *C. sonorensis PDC2* promoter, the *C. sonorensis TDH1* promoter operatively linked to the G418 resistance gene and the *S. cerevisiae MEL5* terminator, the *C. sonorensis PGK1* promoter operatively linked to the *B. megaterium* LDH gene, the *S. cerevisiae GAL10* terminator followed by the *C. sonorensis PDC2*
 25 terminator. This construct is shown schematically in FIG. 15.

The pMI286 plasmid was digested with *NotI* to excise the 6400 bp fragment that consisted of the G418 resistance and LDH expression cassettes flanked by the *PDC2* 5' and 3' regions. This 6400 bp fragment was used to transform *C. sonorensis* by the method described in Example 1 above. The
 30 transformants were grown on YPD agar plates supplemented with G418 antibiotic at a concentration of 200 µg /mL. The plates were incubated at 30°C for 2-5 days and the transformants were then restreaked onto fresh selection plates. The transformants were designated as 286-1 through 286-40.

Southern blot analysis of genomic DNA isolated from the pMI286 transformants was carried out with the *C. sonorensis* *PDC2* probe (corresponding to nucleotides in the deleted area) to identify transformants in which *B. megaterium* LDH was integrated into the *PDC2* locus. The absence of a *PDC2* hybridizing band in transformants 286-1, 286-2, 286-4, 286-19, and 286-30 indicated that *PDC2* gene was deleted. Other pMI286 transformants and untransformed *C. sonorensis* gave a positive signal in the *PDC2* hybridization. Hybridization with the *B. megaterium* LDH probe showed the LDH was present in one copy in the *pdv2* deletants. The frequency of targeted integration into the *PDC2* locus was 15%.

These results showed that targeted integration of the transformed pMI286 DNA into the *PDC2* locus occurred through homologous recombination between *PDC2* promoter and *PDC2* terminator sequences. These results also show that the *PDC2* is a single copy gene in *C. sonorensis*. In addition, integration events outside the *PDC2* occurred. In some transformants the LDH gene was integrated in more than one copy into the genome.

Example 10: Construction of *C. sonorensis* strains deleted of *pdv1* and disrupted in *pdv2* and harboring two copies of *B. megaterium* LDH integrated in the genome in the *pdv1* and *pdv2* loci

The *C. sonorensis* transformant 265-15 having *B. megaterium* LDH integrated in the *pdv1* locus was chosen as host for a second transformation with *B. megaterium* LDH. Transformant 265-15 was further transformed with *NotI* digested pMI286 using the methods described in Example 1 above, and the transformants were selected on YPD agar plates supplemented with G418 antibiotic at a concentration of 200 µg/mL. The plates were incubated at 30°C for 2-5 days for selection, and transformants obtained thereby were purified by restreaking them onto fresh selection plates. The transformants were designated as C44/286-1 through C44/286-40.

Disruption of the *pdv2* gene was verified using the *PDC2* probe (corresponding to nucleotides in the deleted area). The absence of *PDC2* hybridizing band in transformants C44/286-10, C44/286-26, C44/286-27, C44/286-28, C44/286-29, C44/286-30, C44/286-31, C44/286-32, and C44/286-33

indicated that the *PDC2* gene was deleted. The presence of *B. megaterium* LDH in the genome in two copies in the *pdcl*, *pdcl* double deletants was verified by Southern analysis of *HindIII* digested yeast DNA using the *B. megaterium* LDH gene as the probe.

5 These results showed that targeted integration of the transformed pMI286 DNA into the *PDC2* locus occurred through homologous recombination between *PDC2* promoter and *PDC2* terminator sequences. These results also confirm that the *PDC2* is a single copy gene in *C. sonorensis*, and that integration events outside the *PDC2* locus can occur. In some transformants the LDH gene was
10 integrated in more than one copy into the genome. The transformants simultaneously deleted of *pdcl* and disrupted in *pdcl* are viable.

 This Example also confirmed that *C. sonorensis* can be transformed multiply and independently when different markers are used. In this way it is also possible to increase copy number of the gene of interest (LDH) in the host
15 genome.

Example 11: Ethanol production by the *pdcl*-*pdcl*- strains

Ethanol production in *Candida* strains bearing deletions or disruptions in the *PDC1* and/or *PDC2* genes was assayed as follows. Transformants designated
20 C44/286-10, C44/286-26, and C44/286-33 and four other strains included as controls were grown in 50 mL of YP + 5% glucose in 250 mL shaker flasks at 250 rpm shaking and at a temperature of 30°C. Samples were withdrawn daily and cells were removed by centrifugation. Culture supernatant samples taken 56 h after inoculation were analyzed for ethanol by the ethanol UV method of
25 Boehringer Mannheim (Table 1). These results showed that ethanol production by the transformants deleted of both *pdcl* and *pdcl* ethanol is reduced more than ten-fold compared to the strains containing an intact *PDC1* or *PDC2* gene.

 These results demonstrated that both *PDC1* and *PDC2* encode functional pyruvate decarboxylases, since a drastic reduction in ethanol production is only
30 observed when both of the genes are simultaneously deleted. The results also indicated that *PDC2* disruption removing approximately 60% of the *PDC2* open reading frame abolished *PDC2* function.

Table 1. Ethanol production by *C. sonorensis* transformants on YP+5% glucose at 56h of cultivation.

strain	Genotype	ethanol g/L
C44/286-10	2 copies of BmLDH, <i>pdcl-pdc2-</i>	0.2
C44/286-26	2 copies of BmLDH, <i>pdcl-pdc2-</i>	0.1
C44/286-33	2 copies of BmLDH, <i>pdcl-pdc2-</i>	0.1
265-15	1 copy of BmLDH, <i>pdcl-</i>	6
286-1	1 copy of BmLDH, <i>pdc2-</i>	6
286-30	1 copy of BmLDH, <i>pdc2-</i>	3
265-23	2 copies of BmLDH, <i>PDC+</i>	4

5 Example 12: Vector for disruption of PDC2 without LDH

Vectors were prepared for replacing *PDC2* without introducing exogenous LDH-encoding sequences. The *B. megaterium* LDH gene was removed from the pMI286 plasmid described in Example 9 as a 1276 bp *SpeI* - *XbaI* fragment. pMI286 was digested with *SpeI* and the linearized molecule partially digested with

10 *XbaI*. The 8100 bp *SpeI-XbaI* fragment was isolated after gel electrophoresis and recircularized. The resulting plasmid termed pMI287 consists, in order, of the *C. sonorensis PDC2* promoter, the *C. sonorensis TDH1* promoter operatively linked to the G418 resistance gene and the *S. cerevisiae MEL5* terminator, the *C. sonorensis PGK1* promoter followed by the *C. sonorensis PDC2* terminator, and

15 is shown schematically in FIG. 16..

pMI287 was digested with *NotI* to excise the 5100 bp fragment that consisted of the G418 expression cassette flanked by the *PDC2* 5' and 3' regions. This 5100 bp fragment was used to transform *C. sonorensis* by the methods described in Example 1 above. Transformants were grown on YPD agar plates

20 supplemented with G418 antibiotic at a concentration of 200 µg/mL. The plates were incubated at 30°C for 2-5 days and the transformants were then restreaked onto fresh selection plates.

Transformants were designated as 287-1 through 287-57. Southern blot analysis of genomic DNA isolated from the pMI287 transformants was performed

25 using a *PDC2* probe that corresponded to nucleotides in the deleted region, in order to identify successful transformants. No *PDC2* hybridizing band was observed in the transformants 287-6 and 287-16, indicating that the *PDC2* gene was deleted.

Example 13: Vectors for expression of *L. helveticus* LDH and for targeted integration of the transformed DNA into the *PDC2* locus

In order to replace sequences encoding *B. megaterium* LDH in pMI286 by
 5 *L. helveticus* LDH-encoding DNA, the pMI286 described in Example 9 was
 digested with the restriction enzyme *SpeI* and made blunt ended with DNA
 polymerase I, Klenow fragment, and each of the four dNTPs and then digested
 with *BspMI*. Plasmid pMI247 shown in FIG. 9 was digested with *BamHI* and
 made blunt ended with DNA polymerase I, Klenow fragment, and each of the four
 10 dNTPs and then digested with *BspMI*. The 6800 bp *SpeI*(blunt)-*BspMI* fragment
 of pMI286 and the 2700 bp *BamHI*(blunt)-*BspMI* fragment of pMI247 were
 ligated. The resulting plasmid termed pMI288 consists, in order, of the *C.*
sonorensis *PDC2* promoter, the *C. sonorensis* *TDH1* promoter operatively linked
 to the G418 resistance gene and the *S. cerevisiae* *MEL5* terminator, the *C.*
 15 *sonorensis* *PGK1* promoter operatively linked to the *L. helveticus* LDH gene and
 the *S. cerevisiae* *CYC1* terminator followed by the *C. sonorensis* *PDC2*
 terminator, and is shown schematically in FIG. 17.

pMI288 was digested with *NotI* to excise the 6400 bp fragment that
 consisted of the G418 resistance and LDH expression cassettes flanked by the
 20 *PDC2* 5' and 3' regions. The *C. sonorensis* transformant designated 257-3 having
 the *L. helveticus* LDH integrated in the *pdcl* locus was chosen as host for a second
 transformation with *L. helveticus* LDH. Transformant 257-3 was further
 transformed with the 6400 bp *NotI* fragment of pMI288 by the methods described
 in Example 1 above. Transformants were selected on YPD agar plates
 25 supplemented with G418 antibiotic at a concentration of 200 µg/mL. The plates
 were incubated at 30°C for 2-5 days, and transformants obtained thereby were
 purified by restreaking them onto fresh selection plates. These transformants were
 designated as C40/288-1 through C40/288-40.

Disruption of the *pdcl* gene was verified using a *PDC2* probe
 30 corresponding to nucleotides in the deleted area of the locus. The absence of a
PDC2 hybridizing band in transformants C40/288-2, C40/288-11, C40/288-29,
 C40/288-34, and C40/288-38, indicated that the *PDC2* gene was deleted. The
 presence of *L. helveticus* LDH in the genome in two copies in the *pdcl*, *pdcl*

double deletants was verified by Southern blot analysis of *Hind*III digested yeast DNA using the *L. helveticus* LDH gene as the probe.

These results demonstrated that targeted integration of exogenous LDH sequences into *C. sonorensis* PDC2 locus was achieved, and provided cells with
5 disrupted PDC2 loci.

Example 14: Production of L-lactic acid in defined or rich glucose medium in aerobic test tube cultures by *C. sonorensis* harboring *L. helveticus* or *B. megaterium* LDH gene integrated into the genome.

10 *C. sonorensis* cells and the transformants disclosed in the Examples above (namely, 246-27, 247-11, 265-03, 265-05, 265-06, 265-07, 265-11, 265-12, 265-14, 265-15, 265-17, 265-18, 265-22, 265-23, 265-29, 265-33, 265-34, 265-35, 265-38, 265-39, 265-42, 265-43, 265-44, 265-45, 265-46, 265-47, 265-48, 265-49, 265-51, 265-52, 265-55, 265-56, 265-57, and 265-60) were cultivated in YPD
15 medium (YP supplemented with 5% glucose and 0.5 M MES pH 5.5) or YD medium (yeast nitrogen base without amino acids supplemented with 2% glucose and 0.5 M MES pH 5.5). Two independent colonies from each transformant were inoculated into a 14 mL disposable plastic tube containing 5 mL of YPD or YD medium and cultivated with 250 rpm shaking at 30°C. Samples were withdrawn
20 during cultivation, OD₆₀₀ measured, and cells removed by centrifugation and the culture supernatant analyzed by HPLC for lactic acid, glucose and ethanol. HPLC analyses were carried out with Waters 510 HPLC pump, Waters 717+ autosampler, and Water System Interfase Module liquid chromatography complex with refractive index detector (Waters 410 Differential refractometer) and UV-
25 detector (Waters 2487 dual λ UV detector). An Aminex HPX-87H Ion Exclusion Column (300 mm x 7.8 mm, Bio-Rad) was used and was equilibrated with 5 mM H₂SO₄ in water at 35°C, and samples were eluted with 5 mM H₂SO₄ in water at a flow rate of 0.6 mL/min. Data acquisition and control were performed using Waters Millennium software. Values are averaged from two independent samples.
30 These results are shown in Table 2 and 3.

After 13 hours of cultivation in defined medium, transformants 246-27 and 247-11 harboring the *L. helveticus* LDH gene produced 0.1 - 0.4 g/L lactic acid; 1.8 - 3.9 g/L lactic acid was produced after 19 hours.

After 13 hours of cultivation in defined medium, transformants 265-03, 265-06, 265-11, 265-12, 265-18, 265-29, 265-44, 265-45, 265-46, 265-52, 265-55 and 265-57 harboring the *B. megaterium* LDH gene integrated in an unknown site in the genome in one copy produced 0.5 - 1.9 g/L lactic acid; 4.0 - 6.3 g/L lactic acid were produced after 19 hours.

After 13 hours of cultivation in defined medium, transformants 265-14, 265-22 and 265-23 harboring two copies of the *B. megaterium* LDH gene integrated in an unknown site in the genome produced 0.5 - 1.2 g/L lactic acid; 3.8 - 6.1 g/L lactic acid were produced after 19 hours .

After 13 hours of cultivation in defined medium, transformant 265-56 harboring three copies of the *B. megaterium* LDH gene produced 0.7 g/L lactic acid; 5.2 g/L lactic acid were produced after 19 hours.

After 13 hours of cultivation in defined medium, transformants 265-05, 265-07, 265-15, 265-17, 265-33, 265-34, 265-35, 265-38, 265-39, 265-42, 265-43, 265-47, 265-48, 265-49, 265-51 and 265-60 harboring the *B. megaterium* LDH gene integrated into the *pdcl* gene locus (*pdcl*- genotype) produced 0.4 - 2.7 g/L lactic acid; 3.4 - 7.5 g/L lactic acid were produced after 19 hours.

After 12 hours cultivation in rich medium, transformants 246-27 and 247-11 harboring the *L. helveticus* LDH gene produced 0.5 - 1.7 g/L lactic acid, and produced 3.7 - 6.1 g/L lactic acid after 17 hours. In comparison, the host strain produced 0.1 g/L lactic acid after 17 hours of cultivation.

After 12 hours cultivation in rich medium, the transformants 265-03, 265-06, 265-11, 265-12, 265-18, 265-29, 265-44, 265-45, 265-46, 265-52, 265-55 and 265-57 harboring the *B. megaterium* LDH gene produced 1.4 - 4.3 g/L lactic acid, and produced 7.2 - 9.8 g/L lactic acid after 17 hours.

After 12 hours of cultivation in rich medium, transformants 265-14, 265-22 and 265-23 harboring two copies of the *B. megaterium* LDH gene produced 2.1 - 1.9 g/L lactic acid, and produced 6.3 - 6.8 g/L lactic acid after 17 hours.

After 12 hours of cultivation in rich medium, transformant 265-56 harboring three copies of the *B. megaterium* LDH gene produced 2.6 g/L lactic acid, and produced 7.5 g/L lactic acid after 17 hours.

After 12 hours of cultivation in rich medium, the transformants 265-05, 265-07, 265-15, 265-17, 265-33, 265-34, 265-35, 265-38, 265-39, 265-42, 265-43,

265-47, 265-48, 265-49, 265-51 and 265-60 harboring the *B. megaterium* LDH gene integrated into the *pdcl* gene locus (*pdcl*- genotype) produced 2.0 - 4.7 g/L lactic acid, and produced 7.1 - 10.7 g/L lactic acid after 17 hours.

These results show that the LDH transformants produced lactic acid when
5 the host strain did not. *B. megaterium* and *L. helveticus* LDHs were shown to be active in *C. sonorensis*. These heterologous LDHs can thus effectively compete for pyruvate in the presence of PDC. The *pdcl* deletion did not seem to have an effect on the overall yield and production of lactate. Residual glucose was higher and ethanol concentration was lower in transformants containing two (265-14,
10 265-22, 265-23) or three (265-56) copies. A higher LDH copy number also resulted in a higher lactic acid yield from glucose, less ethanol production, and a higher ratio of lactic acid to ethanol. The biomass (OD₆₀₀) increased less in strains containing more than one copy of *B. megaterium* LDH.

15

Table 2. OD₆₀₀, residual glucose, lactic acid and ethanol production of *C. sonorensis* and LDH transformants on defined medium.

Strain	OD ₆₀₀ 13 h	OD ₆₀₀ 19 h	Glucose 13 h	Glucose 19h	Lactic acid 13 h	Lactic acid 19 h	Ethanol 13 h	Ethanol 19 h
<i>C. sonorensis</i>	1.87	7.54	16.24	5.53	0.00	0.00	0.83	4.08
246-27	1.04	6.33	18.66	8.06	0.40	3.93	0.06	1.70
247-11	0.43	4.08	19.91	14.26	0.08	1.78	0.00	0.73
265-03	2.45	7.45	15.54	3.17	1.22	6.01	0.60	2.97
265-06	2.93	7.22	15.69	3.18	1.34	6.02	0.52	2.94
265-11	2.66	7.48	15.63	3.24	1.33	6.09	0.48	2.87
265-12	3.58	7.04	17.28	5.60	0.86	4.96	0.27	2.37
265-18	2.03	6.82	18.15	7.66	0.56	4.00	0.24	2.22
265-44	2.52	7.52	17.74	5.65	0.86	4.86	0.22	2.38
265-45	2.04	5.20	18.96	7.85	0.53	4.07	0.11	1.89
265-46	2.96	6.96	16.05	2.65	1.23	6.06	0.55	3.05
265-52	1.94	7.11	18.54	7.32	0.59	4.18	0.23	1.99
265-55	3.22	7.67	15.71	2.86	1.63	6.28	0.64	2.86
265-57	2.03	7.12	18.37	7.58	0.66	3.96	0.28	2.01
265-29	3.27	7.11	14.59	2.78	1.93	6.30	0.71	2.85
265-14	1.65	5.84	19.05	10.42	0.45	3.75	0.05	1.06
265-22	1.94	6.33	18.33	8.10	0.67	5.10	0.11	1.37
265-23	2.52	6.76	16.95	6.01	1.23	6.14	0.23	1.62
265-56	1.86	5.81	19.14	10.26	0.65	5.22	0.00	0.56
265-05	2.19	7.01	16.38	4.95	0.99	4.80	0.46	2.59
265-07	2.54	7.25	16.35	4.35	1.18	5.32	0.43	2.78
265-15	2.82	7.47	15.63	3.09	1.38	5.70	0.62	3.09
265-17	1.79	6.41	18.88	8.66	0.39	3.50	0.06	1.86
265-33	3.85	7.89	13.67	1.74	2.17	6.53	0.96	3.55
265-34	3.96	7.85	12.52	0.15	2.74	7.50	1.19	3.51
265-35	2.71	7.58	16.89	4.82	0.98	4.84	0.36	2.68
265-38	2.66	8.20	17.01	4.56	1.02	5.25	0.36	2.80
265-39	2.38	7.71	17.57	5.76	0.73	4.63	0.28	2.45
265-42	2.64	7.50	17.28	5.34	0.97	4.98	0.35	2.44
265-43	1.81	6.79	18.86	8.94	0.51	3.44	0.11	1.78
265-47	2.71	7.41	17.09	4.30	0.99	5.18	0.38	2.68
265-48	2.90	7.66	16.66	3.86	1.14	5.47	0.45	2.81
265-49	2.73	7.42	16.57	4.07	1.18	5.26	0.41	2.67
265-51	2.71	7.66	16.91	4.06	1.14	5.35	0.37	2.71
265-60	2.41	7.71	17.50	5.54	0.85	4.63	0.35	2.48

Glucose, lactic acid, and ethanol concentrations in g/L

Table 3. OD₆₀₀, residual glucose, lactic acid, and ethanol production of *C. sonorensis* and LDH transformants on rich medium.

Strain	OD ₆₀₀ 12 h	OD ₆₀₀ 17 h	Glucose 12 h	Glucose 17 h	Lactic acid 12 h	Lactic acid 17 h	Ethanol 12 h	Ethanol 17 h
<i>C. sonorensis</i>	3.78	13.28	41.37	22.86	0.00	0.09	1.90	10.05
246-27	3.10	7.33	43.81	32.49	1.68	6.12	0.63	3.27
247-11	2.13	5.60	46.38	38.36	0.52	3.70	0.11	2.18
265-03	3.63	8.53	41.70	28.49	2.07	7.29	0.95	4.53
265-06	4.13	9.25	41.76	26.30	2.75	7.99	1.32	5.28
265-11	4.08	9.15	42.47	28.34	2.27	7.39	0.98	4.97
265-12	4.55	9.98	40.67	25.05	2.81	8.66	1.55	5.40
265-18	4.73	10.38	40.85	23.97	2.61	8.45	1.53	5.72
265-44	5.35	10.30	40.09	23.76	3.60	9.20	1.82	6.00
265-45	4.68	9.90	41.41	26.30	2.55	8.31	1.37	5.88
265-46	4.43	10.05	41.66	27.31	2.33	7.64	1.26	5.13
265-52	4.10	9.38	43.35	29.35	2.48	7.24	1.01	4.53
265-55	4.80	9.30	41.63	29.37	2.92	8.26	1.13	4.67
265-57	6.28	11.25	38.24	21.19	4.30	9.79	2.25	6.83
265-29	5.20	9.80	20.87	23.78	3.67	9.27	1.69	5.90
265-14	3.25	6.70	44.57	34.95	1.57	6.79	0.33	2.25
265-22	3.25	6.75	44.27	34.50	1.49	6.31	0.31	2.38
265-23	3.15	6.73	41.97	33.79	1.89	6.80	0.42	2.27
265-56	4.25	8.18	44.51	33.82	2.57	7.45	0.58	2.57
265-05	4.75	10.85	39.74	23.43	3.02	8.50	1.73	6.21
265-07	4.15	10.05	41.68	26.04	2.26	7.86	1.36	5.46
265-15	5.05	9.98	39.47	22.92	2.77	8.83	1.84	6.56
265-17	4.53	10.10	40.66	24.61	2.75	7.76	1.38	5.87
265-33	4.63	10.28	41.42	24.33	2.42	8.01	1.40	5.94
265-34	5.00	9.93	39.60	24.99	3.09	8.63	1.75	5.44
265-35	5.73	11.33	36.08	19.29	4.74	10.66	2.94	7.24
265-38	4.98	10.45	40.58	25.53	2.64	7.99	1.42	5.65
265-39	4.33	10.45	41.49	27.30	2.41	7.79	1.24	5.08
265-42	6.08	11.15	37.74	21.15	4.37	9.68	2.32	6.79
265-43	4.23	9.60	41.10	29.83	2.02	7.20	0.90	4.61
265-47	4.08	9.43	42.66	28.06	2.32	7.40	1.18	5.12
265-48	4.43	10.23	42.48	27.89	2.28	7.10	1.27	5.00
265-49	5.20	10.20	39.29	25.17	3.18	8.55	1.86	5.94
265-51	4.48	10.15	42.28	26.89	2.34	7.72	1.30	5.60
265-60	4.38	9.45	42.56	28.13	2.41	7.66	1.13	5.11

Glucose, lactic acid, and ethanol concentrations in g/L.

Example 15: Production of L-lactic acid in defined or rich glucose medium in aerobic test tube cultures by *C. sonorensis* harboring *L. helveticus*, *B. megaterium* or *R. oryzae* LDH gene integrated into the genome.

C. sonorensis cells and the transformants disclosed above (namely, 246-27,
 5 247-11, 265-39, 265-5, 265-15, 265-44, 266-1, 266-2, 266-4, 266-6, 266-7, 266-8,
 266-11, 278-2, 278-3, 278-4, 278-6, 278-7, 278-8, 278-9, 278-11, 278-12, 278-13,
 278-14, 278-15, 278-17, 278-18, 278-19, 278-20, 257-3, 257-5, 257-6, 257-8,
 257-8, 257-9, 257-10, 257-11, and 257-12) were cultivated in YPD (YP
 10 nitrogen base without amino acids supplemented with 2% glucose and 0.5 M MES
 pH 5.5). A colony from each transformant was inoculated into a 14 mL
 disposable plastic tube containing 5 mL of YPD or YD medium and cultivated
 with 250 rpm shaking at 30°C. Samples were withdrawn during cultivation at
 time points 12 and 17 hours, OD₆₀₀ measured, and cells harvested by
 15 centrifugation and the culture supernatant analyzed by HPLC as described above
 for lactic acid, glucose and ethanol. HPLC analyses were carried out as detailed
 above in Example 14. These results are shown in Tables 4 and 5.

After 12 hours of cultivation in defined medium, transformants harboring
 the *L. helveticus* LDH gene produced 0.1 - 0.7 g/L lactic acid. In rich medium 0.9
 20 - 2.7 g/L lactic acid was produced by these cells.

After 12 hours of cultivation in defined medium, transformants harboring
 the *B. megaterium* LDH gene produced 0.1 - 0.5 g/L lactic acid. In rich medium
 1.9 - 3.2 g/L lactic acid was produced by these cells.

After 12 hours of cultivation in defined medium, transformants harboring
 25 the *R. oryzae* LDH gene produced 0.2 - 0.6 g/L lactic acid. In rich medium 0.9 -
 2.7 g/L lactic acid was produced by these cells.

After 12 hours of cultivation in defined medium, transformants harboring
 both the *R. oryzae* LDH gene integrated into *pdcl* gene locus and the *B.*
megaterium LDH gene produced 0.1 - 0.9 g/L lactic acid. In rich medium 1.0 - 3.3
 30 g/L lactic acid was produced by these cells.

After 17 hours of cultivation in defined medium, transformants harboring
 the *L. helveticus* LDH gene produced 0.9 - 2.1 g/L lactic acid. In rich medium 6.6
 - 9.9 g/L lactic acid was produced by these cells.

After 17 hours of cultivation in defined medium, transformants harboring the *B. megaterium* LDH gene produced 0.8 - 1.7 g/L lactic acid. In rich medium 8.7 - 11.0 g/L lactic acid was produced by these cells.

After 17 hours of cultivation in defined medium, transformants harboring
5 the *R. oryzae* LDH gene produced 0.7 - 1.3 g/L lactic acid. In rich medium 7.3 - 9.5 g/L lactic acid was produced by these cells.

After 17 hours of cultivation in defined medium, transformants harboring both the *R. oryzae* LDH gene integrated into *pdcl* gene locus and the *B. megaterium* LDH gene produced 0.7 - 3.0 g/L lactic acid. In rich medium 5.0 -
10 10.7 g/L lactic acid was produced by these cells.

These results showed that all three heterologous LDHs were active in *C. sonorensis* and could be used for producing lactic acid. These LDHs can effectively compete for pyruvate in the presence of PDC. Expression of any of these LDH genes reduced glucose utilization, growth and ethanol production,
15 especially in rich medium. The reduction in glucose utilization rate and growth were strongest in strains containing *L. helveticus* LDH and mildest in strains containing *R. oryzae* LDH, while *B. megaterium* LDH transformants showed intermediate behavior. The effects were masked by the presence of the *B. megaterium* LDH in the transformants containing LDHs of two origins.

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Table 4. OD₆₀₀, residual glucose, lactic acid, and ethanol production of *C. sonorensis* and LDH transformants on defined medium.

Strain	OD ₆₀₀ 12h	OD ₆₀₀ 17 h	Glucose 12 h	Glucose 17 h	Lactic acid 12 h	Lactic acid 17 h	Ethanol 12 h	Ethanol 17 h
<i>C. sonorensis</i>	3.78	7.65	15.07	6.86	0.00	0.00	1.16	3.20
246-27	1.56	4.95	19.10	13.41	0.22	0.99	0.00	1.02
247-11	2.16	5.35	17.82	10.40	0.43	1.40	0.22	1.59
265-39	2.30	6.05	17.88	10.08	0.45	1.73	0.28	1.78
265-5	1.04	3.85	19.96	14.50	0.13	0.83	0.00	0.82
265-15	1.68	5.20	18.40	11.45	0.33	1.45	0.11	1.46
265-44	1.62	5.00	18.38	12.55	0.27	1.36	0.09	1.62
266-1	3.22	7.80	15.68	6.68	0.48	1.27	0.87	2.93
266-2	3.52	7.75	15.49	7.42	0.60	1.19	0.82	2.65
266-3	1.80	6.55	18.07	10.53	0.22	0.71	0.28	1.95
266-4	2.58	7.00	17.10	9.00	0.33	1.00	0.48	2.83
266-7	2.84	7.95	16.38	7.50	0.43	1.32	0.67	2.66
266-8	1.96	6.45	17.77	10.30	0.28	1.20	0.35	2.08
266-11	3.00	7.50	15.64	7.14	0.47	1.28	0.82	2.87
278-2	1.78	5.25	18.31	11.97	0.40	2.27	0.10	1.17
278-3	1.62	4.35	18.52	13.85	0.29	1.17	0.00	0.86
278-4	1.72	5.00	18.43	12.63	0.40	1.89	0.00	0.94
278-6	2.24	6.10	17.59	10.39	0.45	1.57	0.25	1.74
278-7	1.98	5.80	17.70	11.00	0.41	1.65	0.21	1.56
278-8	2.76	6.45	15.96	8.65	0.89	2.55	0.46	2.27
278-9	1.78	4.35	18.21	13.43	0.26	0.90	0.00	0.92
278-11	2.80	6.80	16.02	8.83	0.53	2.36	0.54	2.13
278-12	1.96	5.80	17.31	10.71	0.36	1.31	0.18	1.57
278-13	2.30	6.25	17.01	9.22	0.51	1.97	0.29	1.99
278-14	1.84	5.65	17.77	10.69	0.50	2.11	0.12	1.46
278-15	1.46	4.00	18.78	14.66	0.25	1.03	0.00	0.52
278-17	2.38	6.60	16.92	8.60	0.63	2.01	0.37	2.05
278-18	2.26	5.75	17.44	10.27	0.70	3.02	0.16	1.65
278-19	2.62	6.60	16.87	9.06	0.54	1.69	0.35	2.01
278-20	1.34	3.75	19.17	15.66	0.14	0.70	0.00	0.34
257-3	2.20	5.60	17.62	10.57	0.45	1.45	0.24	1.69
257-5	2.44	6.35	17.55	10.38	0.45	1.62	0.25	2.15
257-6	2.10	5.70	17.88	10.58	0.72	2.12	0.12	1.34
257-8	1.58	4.50	18.70	13.21	0.31	1.64	0.00	1.03
257-9	1.34	4.60	19.56	13.93	0.14	0.86	0.00	0.98
257-10	2.88	6.90	16.22	8.18	0.67	1.60	0.52	2.39
257-11	1.24	4.05	19.64	14.99	0.10	0.98	0.00	0.94
257-12	2.16	6.10	17.74	10.53	0.39	1.46	0.23	1.73

Glucose, lactic acid and ethanol concentrations in g/L.

Table 5. OD₆₀₀, residual glucose, lactic acid, and ethanol production of *C. sonorensis* and LDH transformants on rich medium.

Strain	OD ₆₀₀ 12h	OD ₆₀₀ 17 h	Glucose 12 h	Glucose 17 h	Lactic acid 12 h	Lactic acid 17 h	Ethanol 12 h	Ethanol 17 h
<i>C. sonorensis</i>	8.36	18.20	37.48	11.41	0.29	0.34	4.06	14.58
246-27	2.50	7.75	45.77	32.83	1.09	6.94	0.60	3.40
247-11	3.76	8.50	42.59	29.53	2.36	9.39	1.18	4.53
265-39	5.76	11.05	38.25	18.56	3.22	10.97	2.57	7.65
265-5	4.20	10.85	42.29	23.69	2.04	9.03	1.53	6.42
265-15	4.82	10.65	41.50	22.52	2.09	9.67	1.88	7.38
265-44	3.98	9.80	42.74	25.44	1.91	8.73	1.49	6.07
266-1	7.34	15.95	35.67	5.47	2.49	8.13	3.99	14.28
266-2	6.12	15.45	38.62	11.72	2.15	7.79	2.96	11.97
266-3	6.74	16.65	38.31	9.83	2.03	7.38	3.19	12.88
266-4	10.18	16.55	27.26	0.00	4.11	9.53	6.72	16.93
266-7	7.72	16.05	34.83	4.54	2.79	8.49	4.56	14.76
266-8	7.16	16.60	36.67	6.56	2.34	7.93	4.03	14.27
266-11	6.30	16.15	38.99	10.97	1.90	7.34	2.95	13.06
278-2	4.40	9.90	42.30	28.67	2.42	9.24	1.29	5.40
278-3	3.70	8.75	42.95	27.58	1.59	7.45	1.41	5.71
278-4	3.88	9.30	43.39	27.92	2.11	7.60	1.06	4.86
278-6	3.84	10.20	43.69	24.57	1.60	8.22	1.21	6.34
278-7	4.44	11.10	41.90	23.21	2.16	8.64	1.62	7.79
278-8	4.56	10.90	40.59	23.41	2.59	10.06	1.67	6.92
278-9	3.76	8.25	43.22	27.87	1.71	7.31	1.46	5.64
278-11	4.70	12.65	41.29	19.13	2.00	7.60	1.97	8.58
278-12	4.64	10.70	41.99	23.98	1.85	9.43	1.54	7.13
278-13	5.90	10.80	38.64	18.92	3.20	10.74	2.34	8.20
278-14	4.22	8.05	42.35	30.46	3.23	9.77	1.38	3.31
278-15	2.94	5.40	44.64	35.52	1.84	6.61	0.60	2.24
278-17	5.22	10.45	39.23	19.69	3.28	10.51	2.61	7.65
278-18	2.48	5.30	46.55	37.17	1.04	5.01	0.63	2.56
278-19	4.64	11.10	41.03	21.87	2.52	10.17	2.00	7.47
278-20	3.22	7.90	44.53	30.92	1.59	6.55	1.10	3.38
257-3	3.74	7.95	43.93	29.12	1.97	8.75	1.02	4.21
257-5	3.86	8.45	43.67	28.38	2.19	8.96	1.24	4.51
257-6	3.06	6.00	45.26	36.88	1.90	8.03	0.50	1.63
257-8	3.42	6.20	44.21	34.58	2.67	8.18	0.57	1.98
257-9	4.02	8.20	43.16	28.31	2.24	9.16	1.22	4.41
257-10	4.60	10.00	42.66	25.14	2.09	9.69	1.54	5.36
257-11	2.70	8.00	45.80	33.33	0.85	6.57	0.49	3.45
257-12	4.48	9.60	42.09	26.58	2.40	9.91	1.47	4.55

Glucose, lactic acid and ethanol concentrations in g/L.

Example 16: Production of L-lactic acid in defined glucose medium with or without buffering in microaerobic shake flask cultures by *C. sonorensis* harboring *B. megaterium* or *R. oryzae* LDH gene integrated into the genome.

The *C. sonorensis* transformants harboring the *B. megaterium* LDH gene (namely 265-23 and 265-55) or the *R. oryzae* LDH gene (266-8) were cultivated in defined glucose medium. Precultures were grown in YD medium (yeast nitrogen base without amino acids supplemented with 5% glucose and 0.5 M MES pH 5.5), cells collected by centrifugation and resuspended in 50 mL of YD medium (yeast nitrogen base without amino acids supplemented with 10% glucose) to an OD₆₀₀ of 15 for the cultivation experiments. Yeasts were cultivated in 250 mL Erlenmeyer flasks with or without 4 g CaCO₃ with 100 rpm shaking at 30°C. Samples were withdrawn during cultivation, OD₆₀₀ measured from the cultures without CaCO₃, and cells harvested by centrifugation and the culture supernatant analyzed for L-lactic acid (by the L-lactic acid UV method of Boehringer Mannheim, Roche) and glucose (by the glucose/ GOD-Perid method of Boehringer Mannheim, Roche). These results are shown in Table 6.

After 24 hours of cultivation, transformant 265-55 harboring *B. megaterium* LDH gene produced 35.7 g/L lactic acid with CaCO₃ buffering and 6.16 g/L lactic acid without buffering when the pH dropped to 2.75. Transformant 265-23 harboring two copies of *B. megaterium* LDH gene produced 38.2 g/L lactic acid with CaCO₃ buffering and 6.81 g/L lactic acid without buffering when the pH dropped to 2.68 (24 hours of cultivation). Transformant 266-8 harboring *R. oryzae* LDH gene produced 35.4 g/L lactic acid with CaCO₃ buffering and 3.05 g/L lactic acid without buffering when the pH dropped to 2.83 (24 hours of cultivation).

These results demonstrated that in the presence of CaCO₃ at pH 6.5, lactic acid production and glucose utilization were higher than in unbuffered conditions below pH 3. Higher lactic acid titers were reached in the presence of CaCO₃.

Table 6. OD₆₀₀, residual glucose (g/L), and lactic acid (g/L) production of LDH transformants on defined medium.

"C" indicates the presence of CaCO₃ in the cultivation.

Strains	0 h	4 h	8 h	12 h	24 h
265-55, OD ₆₀₀	16.1	15.7	14.1	13.1	12.2
265-23, OD ₆₀₀	15.9	14.6	12.6	10.2	12.3

Strains	0 h	4 h	8 h	12 h	24 h
266-8, OD ₆₀₀	17.6	17.4	14.9	13.0	14.5
265-55 C, OD ₆₀₀	17.4	n.d.	n.d.	n.d.	n.d.
265-23 C, OD ₆₀₀	14.0	n.d.	n.d.	n.d.	n.d.
266-8 C, OD ₆₀₀	17.7	n.d.	n.d.	n.d.	n.d.
265-55, glucose g/L	125.4	105.1	99.08	99.52	85.01
265-23, glucose g/L	109.4	100.8	92.16	77.31	70.93
266-8, glucose g/L	109.8	81.27	87.98	62.02	51.11
265-55C, glucose g/L	106.6	91.94	93.03	78.08	49.83
265-23 C, glucose g/L	86.66	110.6	73.13	75.00	46.56
266-8 C, glucose g/L	105.1	101.0	73.13	75.66	41.48
265-55, lactic acid g/L	0.67	3.35	4.33	5.58	6.16
265-23, lactic acid g/L	0.69	3.52	4.53	5.76	6.81
266-8, lactic acid g/L	0.67	3.43	4.58	4.22	3.05
265-55 C, lactic acid g/L	0.57	6.92	12.21	18.73	35.73
265-23 C, lactic acid g/L	0.64	7.26	13.28	17.90	38.18
266-8 C, lactic acid g/L	0.35	5.01	10.36	15.76	35.36

Example 17: Intracellular lactic acid in CaCO₃-buffered and unbuffered cultivation

Cell pellets from *C. sonorensis* transformants harboring the *B. megaterium* LDH gene (namely 265-23 and 265-55) or the *R. oryzae* LDH gene (266-8) cultivated in defined glucose medium, as described above in Example 16, were analyzed to determine intracellular lactic acid concentration. Samples (2 mL) were withdrawn during cultivation at 8h and 24h, OD₆₀₀ measured and cells harvested by centrifugation. The supernatant was discarded and each of the pellets was washed with 1 mL of ice-cold 10 mM K₂HPO₄/KH₂PO₄, pH 7.5, supplemented with 2 mM EDTA. Washed cell pellets were resuspended in 0.5 mL of the same buffer and stored at -70°C. Samples were thawed and washed (1 mL) once in 1 M Tris-HCl, pH 9.0, and centrifuged at 13,000 rpm for 1 min. The pellet was suspended into 1 mL ice cold 5% trichloroacetic acid (TCA) and vortexed 1 min. After vortexing, the sample was kept on ice for about 30 min. After incubation on ice, the sample was vortexed for 1 min and centrifuged at 13,000 rpm for 30 min at 4°C. Lactic acid levels were measured in the collected supernatant. Lactic acid concentration was analyzed from the sample by using an enzymatic method (L-lactic acid UV method, Boehringer Mannheim, Roche) or by HPLC (as in Example 14). Intracellular concentration of lactic acid was calculated as follows:

1. The intracellular volume of the cells (in the sample):

Dry weight of the culture (g/L) * volume of the sample (L) * 2 mL/g cell = cell volume (mL).

5

Cell volume is converted into liters by multiplying by 0.001. One gram of cell (dry weight) corresponds to 2 mL cell volume (Gancedo & Serrano, 1989, "Energy Yielding Metabolism," in The yeasts. (Rose & Harrison, eds.), Vol 3. Academic Press: London).

10

2. The lactic acid amounts in the cells:

Measured lactic acid concentration (g/L) * volume of used 5% TCA (L) = lactic acid amount (g) in the sample. To calculate lactic acid concentration in the cell: divide lactic acid amount in the sample (g) by cell volume (L).

15

After 24 hours of cultivation transformant 265-55 harboring the *B. megaterium* LDH gene had an intracellular concentration of 28.2 g/L lactic acid with CaCO₃ buffering and 7.2 g/L of lactic acid without buffering. Transformant 265-23 harboring two copies of the *B. megaterium* LDH gene had an intracellular concentration of 46.1 g/L lactic acid with CaCO₃ buffering and 8.2 g/L of lactic acid without buffering, after 24 hours of cultivation. Transformant 266-8 harboring *R. oryzae* LDH gene had an intracellular concentration of 45.4 g/L of lactic acid with CaCO₃ buffering and 4.9 g/L of lactic acid without buffering (24 hours cultivation). These results are shown in Table 7.

25 These results showed that after 8h of cultivation intracellular lactic acid levels were twice as high as extracellular levels in transformants 265-55 and 265-23 when grown in unbuffered culture. At 8h of cultivation for the other transformants, the difference between intra- and extracellular levels was small, about 10%. When CaCO₃ was included in the cultures, the intracellular and
30 extracellular lactic acid levels in all strains were higher than cultures without CaCO₃. The intra- and extracellular lactic acid concentrations in all strains increased from 8 to 24h in the CaCO₃-buffered culture. The intracellular lactic acid concentrations in the unbuffered cultures are similar at 8h and at 24h. The

intracellular lactic acid levels of strain 266-8 are lower than the levels of the other strains.

- 5 **Table 7.** Intracellular lactic acid concentration (g/L). "C" indicates the presence of CaCO₃ in the cultivation.

Transformant	8 h	24 h
265-55	10.42	7.16
265-23	10.18	8.16
266-8	4.77	4.87
265-55 C	11.38	28.15
265-23 C	11.85	46.11
266-8 C	8.53	45.40

- 10 **Example 18: Enzyme activities of lactate dehydrogenase and pyruvate decarboxylase in *C. sonorensis* harboring *L. helveticus* or *B. megaterium* LDH gene integrated into the genome.**

The *C. sonorensis* transformants (namely, 246-27, 247-11, 257-3, 257-12, 257-6, 247-9, 246-27, 247-11, 265-39, 265-15, 265-44, 265-55, 265-23, 265-22, 15 265-56, 278-14, 278-17, 286-4, 286-30, and 286-1) were cultivated in 50 mL of YD -medium (yeast nitrogen base without amino acids supplemented with 5% glucose and 0.5 M MES pH 5.5), in 250 mL Erlenmeyer flasks with 250 rpm shaking to an OD₆₀₀ of 10 at 30°C. Cells were harvested by centrifugation and the culture supernatant was analyzed by HPLC. Cell samples to be used for enzyme 20 activity measurements (2 mL) were collected by centrifugation and washed with 1 mL of ice-cold 10 mM K₂HPO₄/ KH₂PO₄, pH 7.5 supplemented with 2 mM EDTA. Washed cell pellets were resuspended in 0.5 mL of the same buffer and stored at -70°C. Samples were thawed at room temperature and washed (1 mL) once in sonication buffer (10 0 mM KH₂PO₄/ K₂HPO₄, pH 7.5 supplemented with 25 2 mM MgCl₂ and 10 mM DTT). Washed samples were resuspended in 0.5 mL of sonication buffer and homogenized with 0.5 mL of glass beads with a Bead Beater homogenizer for 1 minute. After homogenization samples were centrifuged at 14,000 rpm for 30 min at 4°C. Supernatant samples were collected and lactate dehydrogenase activity was determined spectrophotometrically (A₃₄₀) with Cobas 30 MIRA automated analyzer at 30°C in sodium acetate buffer (50 mM Na-acetate

pH 5.2) (*Lactobacillus helveticus* LDH) or in imidazole buffer (40 mM imidazole-HCl, pH 6.5) (*Bacillus megaterium* LDH) containing 0.4 mM NADH, 5 mM fructose-1,6-diphosphate, 1 mM glyoxylic acid and 2 mM pyruvate. The protein concentrations were determined by the Lowry method (Lowry *et al.*, 1951, *J. Biol. Chem.* 193: 265-275). Bovine serum albumin (Sigma) was used as a protein standard. Pyruvate decarboxylase activity was determined spectrophotometrically (A_{340}) with Cobas MIRA automated analyzer at 30°C in imidazole buffer (40 mM imidazole-HCl pH 6.5) containing 0.2 mM NADH, 50 mM $MgCl_2$, 0.2 mM thiamin pyrophosphate (cocarboxylase), 90 units of ADH and 50 mM pyruvate. 1 U of enzyme activity was defined as the amount of activity converting 1 μ mol of NADH to NAD^+ per min. These results are shown in Table 8.

This Example demonstrated that intracellular LDH activity correlated with the copy number of the LDH genes in the genome. The calculated LDH activity in strains harboring one copy of the *L. helveticus* LDH was 8 U/mg total cellular protein, and the activity in strains harboring two copies was 15 or 35 U/mg total cellular protein. Lactic acid titers and yields from glucose were greater in the strains containing multiple copies of the LDH gene, however the ethanol titers were lower than in strains containing only one copy of the LDH gene. Calculated LDH activity in strains harboring one copy of the *B. megaterium* LDH was 2-3 U/mg total cellular protein, the activity in strains harboring 2 copies was 10 U/mg, and the activity in strains harboring 3 copies was 40 U/mg.

Pyruvate decarboxylase activity was typically 2-4 U/mg total cellular protein in strains containing an intact *PDC2* gene. When *pdc2* was disrupted, PDC activity dropped below 0.4 U/ mg total cellular protein. If both *pdc1* and *pdc2* were deleted or disrupted (strain C44/286-10) PDC activity decreased to 0.07 U/mg total cellular protein.

Table 8. LDH and PDC enzyme activities; glucose, lactic acid, and ethanol concentrations; lactic acid yield in the culture supernatant measured from cultures grown on YD -medium. n.d.: not determined. 1x, 2x, and 3x indicate the LDH gene copy number.

Strain	Genotype	LDH U/mg total cellular protein	PDC U/mg total cellular protein	Glucose g/L	Lactic acid g/L	Ethanol g/L	lactic acid yield (%)
246-27	1xLhLDH, <i>PDC+</i>	8.01	3.82	31.8	4.73	2.63	26.00
247-11	1xLhLDH, <i>PDC+</i>	7.00	3.58	30.7	5.10	2.90	26.44
257-3	1xLhLDH, <i>pdcl-</i>	8.70	3.81	29.6	5.76	2.96	28.25
257-12	1xLhLDH, <i>pdcl-</i>	8.00	2.85	34.1	3.60	2.19	22.64
257-6	2x LhLDH, <i>PDC+</i>	15.0	3.73	34.1	5.79	1.31	36.51
247-9	2x LhLDH, <i>PDC+</i>	35.2	9.69	33.1	7.87	0.64	46.57
278-14	1xRo+ 2x BmLDH, <i>pdcl-</i>	5.99	3.59	28.6	5.50	3.37	25.71
278-17	1xRo + 1xBmLDH, <i>pdcl-</i>	0.48	2.46	33.84	4.11	0.61	25.43
265-39	1xBmLDH, <i>pdcl-</i>	2.73	2.89	31.99	7.03	1.82	39.03
265-15	1xBmLDH, <i>pdcl-</i>	2.00	1.86	33	8.28	0.92	48.71
265-44	1xBmLDH, <i>PDC+</i>	3.48	3.42	33.33	7.89	1.02	47.33
265-55	1xBmLDH, <i>PDC+</i>	1.81	1.15	28.75	3.56	4.5	16.75
265-23	2x BmLDH, <i>PDC+</i>	8.56	0.95	36.95	5.51	3.36	42.22
265-22	2x BmLDH, <i>PDC+</i>	11.1	2.37	28.97	7.13	2.45	33.90
265-56	3x BmLDH, <i>PDC+</i>	40.7	5.01	29.38	7.02	2.24	34.04
286-4	1xBmLDH, <i>pdcl2-</i>	0.43	0.22	34.6	3.13	0.59	20.32
286-30	1xBmLDH, <i>pdcl2-</i>	2.51	0.13	28.63	3.25	0.44	15.21
286-1	1xBmLDH, <i>pdcl2-</i>	3.28	0.35	28.71	3.5	0.48	16.44
C44/ 286-10	2x BmLDH, <i>pdcl-, pdcl2-</i>	9.30	0.07	n.d.	n.d.	n.d.	n.d.

Example 19: Production of L-lactic acid in defined glucose medium by *C. sonorensis* harboring the *L. helveticus*, *B. megaterium* or *R. oryzae* LDH encoding gene or both *B. megaterium* and *R. oryzae* LDH genes integrated into the genome.

C. sonorensis cells and the transformants (namely 266-7, 266-8, 246-27, 247-11, 257-3, 257-12, 257-6, 247-9, 265-39, 265-15, 265-44, 265-55, 265-23, 265-22, 265-56, 266-3, 278-14, 278-17, 286-4, 286-30, 286-1) were cultivated in YD medium (yeast nitrogen base without amino acids, pH 5.5, supplemented with 5% glucose and 0.5 M MES), and collected by centrifugation. The cells were resuspended in 50 mL of YD (yeast nitrogen base without amino acids supplemented with 10 % glucose) to an OD₆₀₀ of 15 for the cultivation

experiments. The cells were cultivated in 250 mL Erlenmeyer flasks containing 4 g CaCO₃ with 100 rpm shaking at 30°C. Samples were withdrawn during cultivation, the cells were harvested by centrifugation, and the growth medium was analyzed for lactic acid, glucose, and ethanol, by HPLC as described above
 5 (Example 14). These results are shown in Tables 9-13.

The maximal lactic acid titers in the culture supernatants were typically reached at 72 h or later in the cultivation after all glucose had been consumed. The maximal lactic acid titers and yields reached as classified on the basis of the different genetic backgrounds were as follows:

- 10 - 1 copy of *R. oryzae* LDH (strain 266-7): 81 g/L and 79% yield at 96 h
- 1 copy of *B. megaterium* LDH (strain 265-55): 85 g/L and 82 % yield at 96 h
- 1 copy of *L. helveticus* LDH (strain 257-3): 85 g/L and 84% yield at 96 h
- 2 copies of *B. megaterium* LDH (strain 265-22): 87 g/L and 84 % yield at 72 h
- 3 copies of *B. megaterium* LDH (strain 265-56): 83 g/L and 80 % yield at 72 h
- 15 - 2 copies of *L. helveticus* LDH (strain 247-9): 90 g/L and 89% yield at 72 h
- 1 copy of *R. oryzae* LDH and 1 copy of *B. megaterium* LDH (strain 278-17): 79 g/L and 76% yield at 72 h
- 1 copy of *R. oryzae* LDH and 2 copies of *B. megaterium* LDH (strain 278-14): 89 g/L and 86 % yield at 96 h

20 After all glucose was consumed a calcium lactate precipitate was formed in the following cultures: strains 246-27, 247-11, 265-39, 265-15, 265-44, 265-23, 265-22, 278-14, 278-17, 286-4, 286-30, and 286-1. The precipitate formation also indicated that very high lactic acid titers were obtained.

These results demonstrated that *C. sonorensis* overexpressing *L.*
 25 *helveticus*, *R. oryzae* or *B. megaterium* LDH reached high final lactic acid titers (>80 g/L) and yields (>80%) from glucose in CaCO₃ buffered defined medium at pH 6.5. *L. helveticus* and *B. megaterium* LDH transformants performed essentially equally well, and better than *R. oryzae* LDH transformants that gave slightly lower lactic acid titers and yields. LDH copy number especially affected
 30 byproduct formation: a higher LDH copy number and LDH activity resulted in less ethanol and acetate production. Both *L. helveticus* and *B. megaterium* LDH transformants produced less ethanol and acetate than *R. oryzae* LDH transformants. Other measured byproducts, including glycerol and pyruvate were

present in negligible amounts, and did not significantly differ between the *PDC*⁺, *pdc1*- or *pdc2*- genotypes.

Table 9. Maximal lactic acid titers and yields and ethanol and acetate production by *C. sonorensis* LDH transformants in CaCO₃ buffered cultivation on defined medium. 1x, 2x, and 3x indicate the LDH gene copy number.

LDH genotype	Highest LA yield g/g used glucose	Highest LA titer g/L	EtOH g/L	acetate g/L	hrs	Ca-lactate ppt.	strain	PDC
1xRoLDH	0.76	79	<0.02	2	120	no	266-3	<i>pdcl</i> -
1xRoLDH	0.77	78	0.9	5	96	no	266-11	<i>pdcl</i> -
1xRoLDH	0.78	81	0.4	4	96	no	266-7	<i>PDC</i> +
1xRoLDH	0.77	80	0.7	5	96	no	266-8	<i>PDC</i> +
1xBmLDH	0.82	85	0.0	<0.01	72	yes	265-39	<i>pdcl</i> -
1xBmLDH	0.82	85	<0.02	<0.01	72	yes	265-15	<i>pdcl</i> -
1xBmLDH	0.80	83	<0.02	<0.01	72	yes	265-44	<i>PDC</i> +
1xBmLDH	0.82	85	<0.02	<0.01	96	yes	265-55	<i>PDC</i> +
1xBmLDH	0.77	80	<0.02	<0.01	72	yes	286-4	<i>pdcl</i> -
1xBmLDH	0.77	80	<0.02	<0.01	72	yes	286-30	<i>pdcl</i> -
1xBmLDH	0.73	76	0.2	<0.01	48	yes	286-1	<i>pdcl</i> -
1xLhLDH	0.79	80	<0.02	<0.01	120	no	246-27	<i>PDC</i> +
1xLhLDH	0.79	80	<0.02	<0.01	144	no	247-11	<i>PDC</i> +
1xLhLDH	0.84	85	<0.02	<0.01	96	no	257-3	<i>pdcl</i> -
1xLhLDH	0.81	82	<0.02	<0.01	144	no	257-12	<i>pdcl</i> -
1xLhLDH	0.81	84	<0.02	<0.01	72	yes	246-27	<i>PDC</i> +
1xLhLDH	0.82	85	<0.02	<0.01	72	yes	247-11	<i>PDC</i> +
2xBmLDH	0.80	83	<0.02	<0.01	72	yes	265-23	<i>PDC</i> +
2xBmLDH	0.84	87	<0.02	<0.01	72	yes	265-22	<i>PDC</i> +
3xBmLDH	0.80	83	<0.02	<0.01	72	no	265-56	<i>PDC</i> +
2xLhLDH	0.77	78	<0.02	<0.01	120	no	257-6	<i>PDC</i> +
2xLhLDH	0.89	90	<0.02	<0.01	72	no	247-9	<i>PDC</i> +
1xRoLDH+ 1xBmLDH	0.76	79	<0.02	<0.01	72	yes	278-17	<i>pdcl</i> -
1xRoLDH+ 2xBmLDH	0.86	89	<0.02	<0.01	96	yes	278-14	<i>pdcl</i> -

5

Table 10. Glucose g/L at different time points. n.d.= not determined; *= calcium lactate precipitate.

Strain	0h	4h	8h	12h	24h	48h	72h	96	120h	144h
<i>C. sonorensis</i>	100.04	81.49	73.03	66.6	59.77	41.03	31.81	31.19	16.21	0.41
<i>C. sonorensis</i>	99.33	83.46	75.43	69.44	57.14	46.84	38.55	41.97	29.81	20.78
266-3	99.79	83.58	79.35	73.54	57.52	43.14	33.63	36.48	29	17.12
266-11	101.71	79.68	74.38	65.46	39.54	2.29	0.00	0.00	0.00	0.00
266-7	98.72	82.13	74.22	64.9	44.61	17.3	1.29	0.00	0.00	0.00
266-8	100.01	82.07	71.6	62.67	42.13	15.63	0.87	0.00	0.00	0.00
246-27	100.32	85	77.22	74.09	58.28	33.65	23.01	13.99	0.00	0.00
247-11	101.89	84.23	76.25	n.d.	58.09	33.9	22.68	8.28	0.00	0.00
257-3	104.51	84.1	73.31	n.d.	53.18	27.2	16.14	0.00	0.00	0.00

Strain	0h	4h	8h	12h	24h	48h	72h	96	120h	144h
257-12	99.69	81.74	77.63	n.d.	52.95	29.22	15.69	2.55	0.00	0.00
257-6	101.32	84.78	78.34	72.66	60.85	40.53	34.2	17.44	0.00	0.00
247-9	100.78	85.97	77.99	68.14	50.65	9.7	0.00	0.00	0.00	0.00
246-27	69.69	82.04	64.32	57.84	34.01	0.00	0.00	*		
247-11	98.29	83.26	79.02	62.51	45.27	10.76	0.00	0.00	*	
<i>C. sonorensis</i>	95.67	80.28	72.24	56.24	39.83	16.38	0.26	0.00	0.00	0.00
<i>C. sonorensis</i>	91.63	81.42	69.91	61.29	47.36	30.73	19.69	7.91	0.00	0.00
265-39	96.51	83.44	79.68	40.33	44.55	13.96	0.00	*		
265-15	94.7	89.05	74.6	65.28	37.52	6.58	0.00	*		
265-44	95.32	86.01	72.89	65.3	49.24	12.38	0.00	0.00	*	
265-55	91.56	90.02	70.48	68.18	49.91	24.81	4.06	0.00	0.00	0.00
265-23	93.34	84.3	73.42	64.48	45.73	9.2	0.00	*		
265-22	92.13	85.92	70.78	65.54	46.82	8.19	0.00	*		
265-56	91.85	89.76	73.76	69.46	54.69	24.35	0.00	0.00	0.00	0.00
266-3	90.68	86.01	70.34	63.32	35.98	0.00	0.00	0.00	0.00	0.00
278-14	92.44	89.84	74.84	67.31	50.96	20.31	0.00	0.00	*	
278-17	97.57	88.18	71.68	61.92	43.86	6.90	0.00	*		
286-4	96.11	85.80	71.01	63.90	45.67	6.08	0.00	*		
286-30	92.22	83.59	68.22	61.48	40.07	0.42	0.00	*		
286-1	96.42	84.21	68.06	60.66	46.22	0.00	*			

Table 11. Lactic acid g/L; n.d.= not determined; *= calcium lactate precipitate.

Strain	0h	4h	8h	12h	24h	48h	72h	96h	120h	144h
<i>C. sonorensis</i>	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.18	0.00	0.00
<i>C. sonorensis</i>	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.48	0.00	0.00
266-3	0.34	3.15	6.27	9.41	20.34	29.26	39.2	44.51	45.82	50.54
266-11	0.46	5.5	12.27	18.65	37.6	65.04	77.75	77.71	76.81	77.69
266-7	0.46	5.7	11.88	17.64	35.15	57.11	74.23	80.88	76.81	78.78
266-8	0.48	5.87	12.04	18.21	36.34	58.92	75.07	80.45	74.72	77.76
246-27	0.48	5.37	10.16	15.16	28.75	40.7	58.15	70.84	80.06	77.72
247-11	0.49	5.38	10.27	n.d.	29.24	45.05	58.11	76.73	79.92	80.27
257-3	0.57	6.64	12.34	n.d.	34.72	52.53	71.92	85.71	81.50	80.98
257-12	0.51	5.76	11.46	n.d.	32.55	51.57	56.12	81.15	80.02	82.48
257-6	0.55	5.82	10.09	14.02	24.78	38.11	53.13	66.47	78.03	77.82
247-9	0.63	5.44	11.46	16.50	35.7	57.83	89.75	89.66	88.58	88.47
246-27	0.26	8.74	15.51	23.23	43.69	76.83	84.09	*		
247-11	0.32	6.71	14.17	17.98	37.32	73.23	85.56	84.08	*	
<i>C. sonorensis</i>	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>C. sonorensis</i>	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.49
265-39	0.18	6.85	14.67	12.27	40.22	69.97	85.02	*		
265-15	0.23	7.43	13.78	19.78	40.3	76.86	85.17	*		
265-44	0.21	6.91	11.63	17.11	33.47	(37.27)	83.02	70.25	*	
265-55	0.24	7.05	10.97	17.46	32.41	59.35	79.00	85.32	78.81	87.49
265-23	0.2	7.49	13.28	19.27	37.36	69.74	83.44	*		

Strain	0h	4h	8h	12h	24h	48h	72h	96h	120h	144h
265-22	0.22	7.26	12.65	19.67	38.60	74.97	87.36	*		
265-56	0.18	6.58	10.50	15.82	30.69	55.16	83.20	76.95	80.08	78.47
266-3	0.14	5.77	11.34	17.43	39.25	75.38	75.31	74.41	79.04	72.06
278-14	0.16	6.77	11.60	17.36	34.13	66.85	87.54	89.33	*	
278-17	0.25	7.51	12.62	18.58	38.91	78.41	79.16	*		
286-4	0.23	6.02	10.91	17.13	37.77	75.62	79.59	*		
286-30	0.28	7.95	13.52	20.08	39.68	80.22	81.63	*		
286-1	0.39	8.33	14.44	21.51	36.82	75.68	*			

Table 12. Ethanol g/L. Values below the detection limit (0.02 g/L) are not shown. n.d.= not determined.

Strain	0h	4h	8h	12h	24h	48h	72h	96h	120h	144h
<i>C. sonorensis</i>	0.48	2.84	5.5	7.71	11.29	14.86	13.33	15.91	17.29	20.75
<i>C. sonorensis</i>	0.4	2.52	4.88	6.77	10.37	0.82	11.34	11.8	10.47	11.4
266-3	0.29	0.81	1.43	2.05	3.14	0.27	1.5	0.47		
266-11	0.36	0.93	1.53	2.28	3.85	3.46	2.21	0.94		
266-7	0.33	0.89	1.34	1.92	2.92	2.38	1.65	0.35		
266-8	0.4	1.05	1.72	2.43	3.38	2.29	2.06	0.72		
246-27		0.21	0.26	0.21	0.12					
247-11		0.2	0.17	n.d.						
257-3	0.12	0.12	0.17	n.d.						
257-12	0.09	0.11	0.14	n.d.						
257-6										
247-9										
246-27	0.05	0.18	0.21							
247-11	0.06	0.14	0.12							
<i>C. sonorensis</i>	0.56	4.1	7.38	6.41	10.67	15.91	18.11	16.19	13.53	20.9
<i>C. sonorensis</i>	0.54	3.66	6.58	5.21	8.05	9.48	11.51	12.05	10.88	13.13
265-39	0.09	0.21	0.15				0.03			
265-15	0	0.23	0.26	0.02	0.15					
265-44	0	0.16								
265-55	0	0.13								
265-23										
265-22		0.14								
265-56										
266-3	0.27	1.38	1.18	1.65	2.15	2.13	1.52	0.47		
278-14										
278-17		0.31	0.1	0.13	0.09	0.03				
286-4		0.22	0.15	0.21	0.29	0.37				
286-30		0.24	0.1	0.17	0.21	0.23				
286-1		0.23	0.09	0.14		0.16				

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Table 13. Lactic acid (g/ g glucose consumed). n.d.= not determined; *= calcium lactate precipitate.

	0h	4h	8h	12h	24h	48h	72h	96h	120h	144h
<i>C. sonorensis</i>	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>C. sonorensis</i>	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00
266-3	0.28	0.18	0.29	0.34	0.47	0.51	0.58	0.69	0.64	0.60
266-11	0.00	0.26	0.46	0.52	0.61	0.66	0.77	0.77	0.76	0.77
266-7	0.20	0.30	0.44	0.49	0.62	0.68	0.74	0.80	0.76	0.78
266-8	0.48	0.31	0.41	0.48	0.62	0.69	0.75	0.80	0.74	0.77
246-27	0.71	0.34	0.43	0.56	0.67	0.60	0.75	0.81	0.79	0.77
247-11	0.00	0.32	0.41	n.d.	0.68	0.67	0.74	0.83	0.79	0.79
257-3	0.00	0.39	0.45	n.d.	0.73	0.71	0.85	0.85	0.81	0.80
257-12	0.39	0.30	0.49	n.d.	0.68	0.72	0.66	0.82	0.79	0.82
257-6	0.00	0.36	0.45	0.49	0.62	0.63	0.80	0.80	0.77	0.77
247-9	2.86	0.36	0.50	0.50	0.71	0.63	0.89	0.89	0.88	0.88
246-27	0.01	0.40	0.39	0.50	0.65	0.74	0.81	*		
247-11	0.06	0.32	0.57	0.43	0.64	0.79	0.82	0.81	*	
<i>C. sonorensis</i>	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>C. sonorensis</i>	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01
265-39	0.02	0.33	0.60	0.19	0.68	0.78	0.82	*		
265-15	0.02	0.50	0.47	0.51	0.61	0.79	0.82	*		
265-44	0.02	0.38	0.37	0.44	0.61	(0.41)	0.80	0.68	*	
265-55	0.02	0.50	0.33	0.49	0.60	0.75	0.79	0.82	0.76	0.84
265-23	0.02	0.38	0.43	0.49	0.64	0.74	0.80	*		
265-22	0.02	0.40	0.38	0.51	0.68	0.78	0.84	*		
265-56	0.01	0.46	0.35	0.46	0.62	0.69	0.80	0.72	0.77	0.75
266-3	0.01	0.32	0.34	0.43	0.58	0.72	0.72	0.74	0.76	0.69
278-14	0.01	0.48	0.40	0.47	0.64	0.80	0.84	0.86	*	
278-17	0.04	0.47	0.39	0.44	0.65	0.81	0.76	*		
286-4	0.03	0.33	0.33	0.43	0.65	0.77	0.77	*		
286-30	0.02	0.39	0.38	0.47	0.62	0.77	0.78	*		
286-1	0.05	0.42	0.40	0.50	0.64	0.73	*			

Example 20: Production of L-lactic acid in defined glucose medium in nitrogen sparged tubes by *C. sonorensis* harboring *L. helveticus* or *R. oryzae*

5 LDH encoding gene integrated into the genome.

Production of L-lactic acid in transformed *C. sonorensis* cells was demonstrated as follows. *C. sonorensis* cells and the transformants harboring the *L. helveticus* LDH gene (namely, 246-14, 246-14, 246-18, 246-23, 246-27, 247-7, 247-8, 247-11, and 257-3) or the *R. oryzae* LDH gene (266-3 and 266-4) were

10 cultivated in YD medium (yeast nitrogen base without amino acids supplemented with 12% glucose and 0.4 M MES pH 5.5). Precultures were grown in 50 mL of YD medium (yeast nitrogen base without amino acids supplemented with 6.5%

glucose and 0.4 M MES, pH 5.5) in 250 mL Erlenmeyer flasks with 250 rpm shaking at 30°C. Cells were collected by centrifugation and washed once with 0.9% NaCl, then resuspended in 50 mL of YD medium to an OD₆₀₀ of 11 for the cultivation experiments. Yeasts were cultivated in 50 mL disposable plastic tubes sparged with nitrogen with 250 rpm shaking at 30°C ((nearly) anaerobic conditions). Samples were withdrawn during cultivation, and after that the tubes were sparged with nitrogen. OD₆₀₀ was measured, and cells harvested by centrifugation and the culture supernatant analyzed by HPLC as described above for lactic acid, glucose and ethanol. These results are shown in Tables 14-20.

After 94 hours of cultivation the transformants harboring *L. helveticus* LDH gene produced 6.9 - 7.2 g/L lactic acid (equivalent to 66 - 84% yield) and 1-1.4 g/L ethanol, whereas the host strain produced 0.1 g/L lactic acid and 40 g/L ethanol. The transformants harboring *R. oryzae* LDH gene produced 7.2-8.8 g/L lactic acid (equivalent to 13-18% yield) and 17-28 g/L ethanol after 94 hours of cultivation. Glucose consumption and ethanol production by the *R. oryzae* LDH transformants were faster than those of the *L. helveticus* transformants.

These results showed that *C. sonorensis* transformed with *L. helveticus* LDH or *R. oryzae* LDH produced lactic acid from glucose in nitrogen sparged tube cultures.

Table 14. Lactic acid g / g glucose consumed

Strains	0 h	27 h	46 h	70 h	94 h
247-7	0	0.93	1.04	0.90	0.75
247-8	0	1.09	0.98	0.90	0.66
247-11	0	0.84	0.91	0.88	0.80
246-14	0	1.02	1.02	0.91	0.78
246-18	0	1.08	0.91	0.77	0.71
246-23	0	0.89	0.94	0.87	0.84
246-27	0	0.83	0.95	0.88	0.83
<i>C. sonorensis</i>	0	0.00	0.00	0.00	0.00
266-3	0	0.37	0.22	0.17	0.13
266-4	0	0.32	0.26	0.22	0.18
257-3	0	1.00	0.88	0.93	0.76

Example 21: Production of L-lactic acid in rich glucose medium without buffering in microaerobic shake flask cultures by *C. sonorensis* harboring *L. helveticus* or *B. megaterium* LDH gene integrated into the genome.

Production of L-lactic acid in transformed *C. sonorensis* cells was demonstrated as follows. The *C. sonorensis* transformants harboring the *B. megaterium* LDH gene (namely, 265-23 and 286-1) and *L. helveticus* LDH gene (246-27 and 247-11) disclosed above were cultivated in 50 mL of YD medium (yeast nitrogen base without amino acids supplemented with 5% glucose and 0.5 M MES, pH 5.5) in 250 mL Erlenmeyer flasks with 250 rpm shaking at 30°C. Cells were collected by centrifugation and then resuspended in 50 mL of YP supplemented with 5% glucose to an OD₆₀₀ of 15 for the cultivation experiments. Cells were cultivated in 250 mL Erlenmeyer flasks with 100 rpm shaking at 30°C. Samples were withdrawn during cultivation, OD₆₀₀ measured, and cells were harvested by centrifugation. The culture supernatant analyzed for L-lactic acid (by the L-lactic acid UV method of Boehringer Mannheim, Roche), for glucose (by the glucose/ GOD-Perid method of Boehringer Mannheim, Roche), for acetate (by the acetic acid UV method of Boehringer Mannheim, Roche), and for ethanol (by the ethanol UV method of Boehringer Mannheim, Roche). These results are shown in Tables 15-20.

Transformants 246-27 and 247-11 harboring *L. helveticus* LDH gene integrated randomly into the yeast genome (*PDC+* genotype) produced 7.8-9.0 g/L lactic acid (equivalent to 24-29% yield) after 24 hours of cultivation. The transformant 286-1 harboring *B. megaterium* LDH gene integrated into the *pdc2* gene locus (*pdc2-* genotype) produced 8.9 g/L lactic acid (equivalent to 31% yield) after 24 hours of cultivation. Transformant 265-23 harboring two copies of *B. megaterium* LDH gene integrated randomly into genome (*PDC+* genotype) produced 9.1 g/L lactic acid (equivalent to 30% yield) after 24 hours of cultivation. After 24 hours of cultivation the transformants harboring *B. megaterium* LDH gene produced 8.9 - 9.1 g/L lactic acid, equivalent to 30-31% yield from glucose. Transformants harboring the *L. helveticus* LDH gene produced 7.8-9.0 g/L lactic acid, which is equivalent to 24-29% yield from glucose. Although some glucose was unconsumed at 24 h, all glucose was eventually consumed (at 120 h). No further increase in lactic acid concentration

occurred after 24 h, however. Glucose consumption by all strains was very similar. The pH of the culture medium was between 3.4 - 3.8 during this experiment. The transformant 265-23 containing two copies of *B. megaterium* LDH produced less ethanol and acetate early in the cultivation whereas the *pdh2*-
 5 transformant 286-1 produced less ethanol and acetate towards the end of the cultivation than the other strains.

These results demonstrated that *C. sonorensis* transformed with *L. helveticus* LDH or *B. megaterium* LDH was capable of producing lactic acid from glucose under microaerobic conditions at low pH up to 9 g/L.

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Table 15. Absorbance OD₆₀₀

Strain	0 h	4 h	8 h	12 h	24 h	48 h	72 h	96 h	120 h
265-23	17.40	16.1	15.2	15.30	18.40	15.4	13.6	14.8	17.8
286-1	15.80	16.4	15.6	15.30	20.40	16.2	16.5	20.1	25.0
246-27	16.20	15.9	15.8	16.90	18.20	15.1	14.1	17.0	23.7
247-11	15.80	15.4	16.3	15.40	18.60	15.8	13.4	16.1	23.3

Table 16. Glucose g/L in different time points

Strain	0 h	4 h	8 h	12 h	24 h	48 h	72 h	96 h	120 h
265-23	n.d.	n.d.	n.d.	27.1	19.51	10.2	8.4	6.7	2.8
286-1	n.d.	n.d.	n.d.	25.5	21.6	13.2	8.1	0.9	0.0
246-27	n.d.	n.d.	n.d.	25.3	18.0	9.9	6.8	2.3	0.0
247-11	n.d.	n.d.	n.d.	24.6	18.9	11.2	7.3	2.4	0.0

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Table 17. Lactic acid g/L

Strain	0 h	4 h	8 h	12 h	24 h	48 h	72 h	96 h	120 h
265-23	0.46	4.41	6.31	7.57	9.10	6.49	6.04	5.78	4.35
286-1	0.46	4.62	5.57	7.57	8.85	7.43	5.55	5.68	5.01
246-27	0.49	4.38	5.23	7.75	7.75	6.96	5.97	7.12	5.86
247-11	0.49	4.35	5.18	7.40	8.97	8.18	6.08	7.39	5.06

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Table 18. Ethanol g/L

Strain	0 h	4 h	8 h	12 h	24 h	48 h	72 h	96 h	120 h
265-23	0.28	0.55	0.82	1.50	3.62	6.29	7.47	8.12	6.45
286-1	0.25	0.77	1.14	1.57	3.84	5.43	6.23	6.96	4.50
246-27	0.31	0.72	1.22	1.97	4.14	6.47	7.33	7.43	5.48
247-11	0.33	0.70	1.16	1.64	4.04	6.44	7.50	7.76	6.06

Table 19. pH

Strain	0 h	4 h	8 h	12 h	24 h	48 h	72 h	96 h	120 h
265-23	5.11	3.79	3.66	3.55	3.41	3.58	3.69	3.7	3.66
286-1	4.98	3.73	3.62	3.58	3.38	3.54	3.57	3.51	3.66
246-27	5.09	3.74	3.66	3.57	3.41	3.53	3.68	3.63	3.62
247-11	5.05	3.73	3.57	3.53	3.43	3.49	3.54	3.6	3.61

Table 20. Acetate g/L

Strain	0 h	4 h	8 h	12 h	24 h	48 h	72 h	96 h	120 h
265-23	0.1	0.4	0.9	1.1	2.8	5.5	6.1	4.7	1.2
286-1	0.1	0.5	1.1	1.3	2.8	3.8	2.1	1.3	0.1
246-27	0.1	0.5	1.1	1.4	3.0	5.0	4.6	1.4	0.0
247-11	0.1	0.5	1.0	1.2	2.7	4.4	4.1	1.4	0.0

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Example 22: Production of L-lactic acid in a bioreactor in rich glucose medium by *C. sonorensis* harboring *B. megaterium* LDH gene integrated into the genome.

C. sonorensis transformants designated 265-55, 286-30 and 265-15, described above, were cultivated in aerobic bioreactors. Batch cultivation was performed at 35°C in a laboratory bioreactor (Biostat CT-DCU3, Braun, Germany) with a working volume of 2 L. During the production phase the pH was maintained at 5.0 ±0.1 or increased to 6.0 ± 0.1 after 48 hours of cultivation by automated addition of 5 M potassium hydroxide (KOH). Biomass was produced with YP medium supplemented with 150 g/L glucose. The biomass production phase was inoculated with 20 mL of culture stored in 23% (w/v) glycerol at -80°C to an initial OD₆₀₀ of 0.7-1. The bioreactor was flushed with 100% air at a flow rate of 1.0 L/min and stirred at 800 rpm during this phase. After 23.5 hours of cultivation 10-21 g/L cell mass was produced (dry weight) (equivalent to 0.2-0.3 g dry weight per used gram of glucose). After the 24 hour biomass production, the bioreactor was emptied and cells were collected by centrifugation (4000 rpm, 20°C, 10 min). The medium for lactate production (YP supplemented with 100 g/L glucose) was pumped into the bioreactor and was inoculated with the cells collected from the biomass production phase, to a density corresponding to 5 g/L dry weight. The bioreactor was flushed with 10% air - 90% nitrogen gas at a flow rate of 1.0 L min⁻¹ and stirred at 500 rpm.

Samples were withdrawn during cultivation. For each sample, dry cell weight was determined, the OD₆₀₀ was measured, and the cells were harvested by centrifugation. Culture supernatants were analyzed by HPLC as described above for lactic acid, glucose, ethanol, and acetate. These results are shown in Tables 21 and 22.

The transformant harboring the *B. megaterium* LDH gene integrated randomly into the genome (265-55, *PDC+* genotype) produced 28 g/L lactic acid (equivalent to 67% yield) at pH 5.0, after 52 hours of cultivation in the lactate production phase. The same transformant produced 28 g/L lactic acid (equivalent to 60% yield) at pH 6.0 after 72 hours of cultivation in the production phase.

The transformant harboring the *B. megaterium* LDH gene integrated into the *pdcl* gene locus (265-15, *pdcl-* genotype) produced 23 g/L lactic acid (equivalent to 66% yield) at pH 5.0 after 51 hours of cultivation in the lactate production phase.

The transformant harboring the *B. megaterium* LDH gene integrated into the *pdc2* gene locus (286-30, *pdc2-* genotype) produced 27 g/L lactic acid (equivalent to 54% yield) at pH 5.0 after 46 hours of cultivation in the lactate production phase.

After 46 to 52 hours of lactic acid production phase, the transformants produced 23 - 28 g/L lactic acid (equivalent to a 54 - 67% yield).

These results demonstrated that *C. sonorensis* overexpressing a heterologous lactate dehydrogenase encoding gene produced lactic acid from glucose in batch fermentation under microaerobic condition (e.g. 0%-2% O₂ in the atmosphere).

Table 21: Biomass production in bioreactor cultivations in YPD medium with 150 g/L glucose

<i>C. sonorensis</i> transformants	Cultivation time (h)	Initial OD ₆₀₀	Final OD ₆₀₀	Glucose consumed (g/L)	Final biomass (cell dry weight g/L)
265-55	23.5	0.68	90	96	20.8
265-55	21.5	0.69	98	73	20.5
286-30	23.5	1.01	42	65	21.3
265-15	23.5	0.85	41	46	10.0

Table 22: Lactate production with *C. sonorensis* in bioreactor cultivations in YPD medium with 100 g/L glucose. Glucose consumption, lactate concentration, and lactate yield at the time when the peak lactate concentration was achieved. *= pH was increased to 6.0 at 48h.

<i>C. sonorensis</i> transformants	Initial biomass (cell dry weight g/L)	Ferm. time at peak LA	Glucose consumed (g/L)	L.A. (g/L)	LA yield (g LA/g glucose used)
265-55	4.5	52h	42	28	67%
265-55*	6.4	72h	47	28	60%
286-30	4.3	46h	50	27	54%
265-15	5.3	51h	35	23	66%

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Table 23: Byproduct production with *C. sonorensis* in bioreactor cultivations in YPD medium with 100 g/L glucose. Byproduct concentrations at the time when the peak lactate concentration was achieved.

*= pH was increased to 6.0 at 48h.

<i>C. sonorensis</i> transformants	Initial biomass (dry wt. g/L)	Ferm. time at peak LA	Ethanol (g/L)	Acetate (g/L)	Glycerol (g/L)	Pyruvate (g/L)
265-55	4.5	52h	1.9	4.5	0	0.71
265-55*	6.4	72h	0.6	10.6	0	0.79
286-30	4.3	46h	2.3	5.0	0	0.39
265-15	5.3	51h	1.4	5.0	0	0.06

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Intracellular lactic acid and pyruvate

Intracellular lactic acid and pyruvate concentrations were determined as described above in Example 17, except that the sample volume was 1 mL and the cell pellet was washed (1 mL) in 1 M Tris-HCl, pH 9.0, centrifuged at 13,000 rpm for 1 min., and stored at -70°C . After thawing, the pellet was directly suspended into 1 mL of ice-cold 5% TCA. Intracellular pyruvate concentration was analyzed from the sample enzymatically (pyruvate kit, Sigma Diagnostics). These results are shown in Tables 24-27.

15

The transformant harboring the *B. megaterium* LDH gene integrated randomly into the genome (265-55, *PDC+* genotype) produced 60.9 g/L of lactic acid in the cells at 52 hours of cultivation in the lactate production phase, at pH 5.0. The same transformant produced 38.7 g/L of lactic acid, at pH 6.0, at 72 hours of cultivation in the lactate production phase.

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The transformant harboring the *B. megaterium* LDH gene integrated into the *pdcl* locus (265-15, *pdcl*- genotype) produced 13.4 g/L of lactic acid in the cells at 51 hours of cultivation in the lactate production phase.

5 The transformant harboring the *B. megaterium* LDH gene integrated into the *pdcl* locus (286-30, *pdcl*- genotype) produced 14.3 g/L lactic acid at 49 hours of cultivation in the lactate production phase.

The transformant harboring the *B. megaterium* LDH gene integrated randomly into the genome (265-55, *PDC*+ genotype) produced 0.1 g/L of pyruvate in the cells during the cultivation at pH 5.0 and at pH 6.0.

10 The transformant harboring the *B. megaterium* LDH gene integrated into the *pdcl* locus (265-15, *pdcl*- genotype) or into the *pdcl* locus (286-30, *pdcl*- genotype) produced 0.3 g/L pyruvate in the cells during the cultivations.

15 These results showed that deletion of *pdcl* and disruption of *pdcl* caused an increase in intracellular pyruvate levels. In the *PDC*+ strain intracellular lactic acid levels increased towards the end of the cultivations, although this trend was not as clear in the *pdcl*- and in the *pdcl*- strains.

Table 24. Intracellular lactate and pyruvate concentration (g/L) in the strain 265-55 (*PDC*+, pH 5.0)

Time (h)	lactic acid (g/L)	pyruvate (g/L)
0	15.9	0.1
3.1	17.0	0.1
6.1	13.9	0.1
9.0	17.9	0.0
20.9	20.9	0.1
24.2	40.6	0.1
27.9	25.2	0.1
44.8	28.4	0.0
49.1	40.3	0.1
51.6	60.9	0.2

20

Table 25. Intracellular lactate and pyruvate concentration (g/L) in the strain 265-55 (*PDC*+, pH 6.0)

Time (h)	Lactic acid (g/L)	Pyruvate (g/L)
0	10.67	0.2
4.7	11.15	0.1
21.1	12.97	0.2
19.4	95.83	0.2

Time (h)	Lactic acid (g/L)	Pyruvate (g/L)
22.4	23.08	0.1
24.5	24.89	0.1
48.3	39.67	0.1
71.7	38.7	0.1
91.6	42.0	0.1
96.1	53.4	0.1
97.9	55.5	0.1

Table 26. Intracellular lactate and pyruvate concentration (g/L) in the strain 286-30 (*pdc2*-, pH 5.0)

Time (h)	Lactic acid (g/L)	Pyruvate (g/L)
1.7	14.3	0.4
3.1	15.0	0.1
22.2	24.2	0.2
24.5	26.0	0.2
39.1	15.1	0.3
48.7	14.3	0.3
66.7	45.8	0.4
70.5	43.7	0.5

5

Table 27. Intracellular lactate and pyruvate concentration (g/L) in the strain 265-15 (*pdcl*-, pH 5.0)

Time (h)	Lactic acid (g/L)	Pyruvate (g/L)
1.3	11.8	0.2
16.6	11.1	0.3
19.7	14.6	0.2
22.4	17.7	0.2
50.6	13.4	0.3
76.1	13.4	0.3
89.0	41.5	0.2
94.8	23.5	0.1

10

Lactate dehydrogenase and pyruvate decarboxylase activities

Lactate dehydrogenase and pyruvate decarboxylase activities were determined as follows. Samples for enzyme activity measurements (2 mL) were collected by centrifugation and the cell pellets were washed with 1 mL of ice-cold 10 mM K₂HPO₄/ KH₂PO₄, pH 7.5 supplemented with 2 mM EDTA. Washed pellets were resuspended in 0.5 mL of the same buffer and stored at -70°C. Samples were thawed at room temperature and washed (1 mL) once in homogenization buffer (100 mM KH₂PO₄/ K₂HPO₄, pH 7.5, supplemented with 2

mM MgCl₂ and 10 mM DTT). Washed samples were resuspended in 0.5 mL of homogenization buffer and homogenized with 0.5 mL of glass beads with a Bead Beater homogenizer for 1 minute. After homogenization samples were centrifuged 14,000 rpm for 30 min at 4°C. Supernatant samples were collected
 5 and lactate dehydrogenase and pyruvate decarboxylase activities were determined spectrophotometrically (A₃₄₀) as described above in example 18, except that glyoxylic acid was not used. These results are shown in Tables 28-31.

The transformant harboring *B. megaterium* LDH gene integrated randomly into the genome (265-55, *PDC+* genotype) produced lactate dehydrogenase
 10 activity of 1.4 U/mg total cellular protein and pyruvate decarboxylase activity of 0.8 U/mg total cellular protein at 52 hours of cultivation in the lactate production phase at pH 5.0. The same transformant produced lactate dehydrogenase activity of 1.2 U/mg total cellular protein and pyruvate decarboxylase activity of 0.4 U/mg total cellular protein, at pH 6.0, at 72 hours of cultivation in the lactate production
 15 phase.

The transformant harboring the *B. megaterium* LDH gene integrated into the *pdcl* locus (265-15, *pdcl-* genotype) produced lactate dehydrogenase activity of 1.5 U/mg total cellular protein and pyruvate decarboxylase activity of 0.5 U/mg total cellular protein at 51 hours of cultivation in the lactate production phase.

20 The transformant harboring the *B. megaterium* LDH gene integrated into the *pdc2* locus (286-30, *pdc2-* genotype) produced lactate dehydrogenase activity of 0.7 U/mg total cellular protein and pyruvate decarboxylase activity of 0.1 U/mg total cellular protein, at 49 hours of cultivation in the lactate production phase.

These results demonstrated that LDH activity is similar in all strains that
 25 contain one copy of the *B. megaterium* LDH integrated in the genome. LDH activity was higher than PDC activity (U/mg total cellular protein) and thus LDH could compete efficiently with PDC for pyruvate. The *pdc2-* strain 286-30 has clearly reduced PDC activity compared with the wild type. The observed effect of the *pdcl* deletion on PDC activity in the *pdcl-* strain 265-15 was a more gradual
 30 decrease in activity over time.

Table 28. Lactate dehydrogenase and pyruvate decarboxylase activities (265-55 (*PDC+*, pH 5.0))

hours	LDH (U/ mg total cellular protein)	PDC (U/ mg total cellular protein)
0	1.9	0.41
3.1	1.83	0.58
6.08	1.78	0.79
8.95	1.74	0.82
20.92	1.6	0.95
24.17	1.92	0.98
27.93	2.13	0.92
44.83	n.d.	1
49.13	1.88	0.97
51.62	1.35	0.77

Table 29. Lactate dehydrogenase and pyruvate decarboxylase activities (265-55 (*PDC+*, pH 6.0))

Hour	LDH (U/ mg total cellular protein)	PDC (U/ mg total cellular protein)
0	1.13	0.28
2.08	1.11	0.3
4.73	0.75	0.31
19.35	1.19	0.5
22.35	1.14	0.51
24.47	1.8	0.55
48.33	1.09	0.46
71.73	1.21	0.35
91.63	0.69	0.27
96.08	n.d.	0.22
97.88	1.72	0.23

5

Table 30. Lactate dehydrogenase and pyruvate decarboxylase activities (265-15 (*pdcl-*, pH 5.0))

hours	LDH (U/ mg total cellular protein)	PDC (U/ mg total cellular protein)
16.63	0.91	0.52
19.65	1.09	0.57
22.38	0.78	0.52
50.58	1.54	0.46
76.08	4.38	0.34
88.95	2.25	0.16
94.83	1.41	0.21

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Table 31. Lactate dehydrogenase and pyruvate decarboxylase activities (286-30 (*pdcl-*, pH 5.0))

hours	LDH (U/ mg total cellular protein)	PDC (U/ mg total cellular protein)
1.65	0.82	0.19
3.08	1.09	0.23
22.22	0.78	0.14
24.52	1.56	0.26
39.05	0.85	0.15
48.73	0.72	0.12
66.67	0.17	0.06
70.5	0.26	0.08

Example 23: Anaerobic production of L-lactic acid in a bioreactor in rich glucose medium by *C. sonorensis* harboring the *B. megaterium* LDH gene integrated into the genome.

The *C. sonorensis* transformant designated 265-55 described above was cultivated in a bioreactor. Batch cultivation was carried out at 35°C in a laboratory bioreactor (Biostat CT-DCU3, Braun, Germany) with a working volume of 2 L. Biomass was produced aerobically on YP medium supplemented with 150 g/L glucose. The biomass production phase was inoculated with 20 mL of culture stored in 23% (w/v) glycerol at -80°C. The bioreactor was flushed with 100 % air at a flow rate of 1.0 L/min, and stirred at 800 rpm. The dissolved-oxygen concentration was continuously monitored with an oxygen electrode (Mettler Toledo). After 22.5 hours of biomass production the bioreactor was emptied and cells were collected by centrifugation (4000 rpm, 20°C, 10 min). Medium for lactic acid production (YP supplemented with 100 g/L glucose) was pumped into the bioreactor, and was inoculated with the centrifuged biomass to a density equivalent of 4.5 g/L cell dry weight. The bioreactor was flushed with 100% nitrogen at a flow rate of 1.0 L/min and stirred at 500 rpm. The pH was maintained at 5.0 ±0.1 by automated addition of 5 M potassium hydroxide (KOH).

Samples were withdrawn during cultivation. Cell dry weight was determined, OD₆₀₀ was measured, and cells were harvested by centrifugation. The culture supernatants were analyzed for L-lactic acid (by the L-lactic acid UV method of Boehringer Mannheim) and glucose content (by the glucose/ GOD-Perid method of Boehringer Mannheim). These results are shown in Tables 32 and 33.

After 120 h of cultivation 4.7 g/L lactic acid was produced from glucose (equivalent to a 52% yield).

This example demonstrated that *C. sonorensis* overexpressing a heterologous lactate dehydrogenase was capable of producing lactic acid from glucose under anaerobic batch fermentation.

Table 32: Aerobic biomass production in a bioreactor cultivation in YPD medium with 150 g/L glucose.

<i>C. sonorensis</i> transformant	Cultivation time (h)	Initial OD ₆₀₀	Final OD ₆₀₀	Final biomass (cell dry weight g/L)
265-55	22.5	0.77	74	20.1

Table 33: Lactic acid production with *C. sonorensis* overexpressing *B. megaterium* LDH in anaerobic bioreactor cultivation in YPD medium with 100 g/L glucose: glucose consumption, lactic acid concentration and lactic acid yield.

<i>C. sonorensis</i> transformant	Initial biomass (dry weight g/L)	Ferm. time	Glucose consumed (g/L)	Lactic acid (g/L)	L.A. yield (gLA/ g glucose consumed)
265-55	4.5	120h	9	4.7	0.52

Example 24: Production of L-lactic acid in a bioreactor in Ca(OH)₂ - buffered rich glucose medium by *C. sonorensis* harboring *B. megaterium* LDH gene integrated into the genome.

The *C. sonorensis* transformant designated 265-55 described above was cultivated by batch cultivation in a bioreactor (Biostat CT-DCU3, Braun, Germany) at 35°C as described in Example 23. After 18.5 hours of biomass production the bioreactor was emptied and cells were collected by centrifugation (4000 rpm, 20°C, 10 min). The medium for lactic acid production (YP supplemented with 100 g/L glucose) was pumped into the bioreactor and inoculated with the centrifuged biomass to a density equivalent of 6.7 g/L cell dry weight. The bioreactor was flushed with 90% nitrogen and 10% air at a flow rate of 1.0 L/min and stirred at 500 rpm. The pH was maintained at 5.0 ±0.1 by automated addition of 2.5 M calcium hydroxide (Ca(OH)₂).

Samples were withdrawn during cultivation. Cell dry weight was determined, OD₆₀₀ was measured and cells were harvested by centrifugation. The

culture supernatants were analyzed for L-lactic acid (by the L-lactic acid UV method, Boehringer Mannheim) and glucose content (by the glucose/GOD-Perid method, Boehringer Mannheim). These results are shown in Tables 34 and 35.

After 53 hours of cultivation, 26 g/L lactic acid was produced from glucose
5 (equivalent to a 67% yield).

These results demonstrated that *C. sonorensis* overexpressing *B. megaterium* lactate dehydrogenase was capable of producing lactic acid from glucose in a microaerobic batch fermentation, (2% O₂) with calcium hydroxide buffering.

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Table 34: Aerobic biomass production in a bioreactor cultivation in YPD medium with 150 g/L glucose.

<i>C. sonorensis</i> transformant	Cultivation time (h)	Initial OD ₆₀₀	Final OD ₆₀₀	Final biomass (cell dry weight g/L)
265-55	18.5	2.19	46	16.5

15 **Table 35:** Lactic acid production with *C. sonorensis* overexpressing *B. megaterium* LDH in a bioreactor cultivation in YPD medium with 100 g/L glucose: glucose consumption, lactic acid concentration and lactic acid yield.

<i>C. sonorensis</i> transformant	Initial biomass (dry weight g/L)	Ferm. time	Glucose consumed (g/L)	Lactic acid (g/L)	L.A. yield (g L.A./g glucose consumed)
265-55	6.7	53h	39	26	0.67

20 **Example 25: Production of L-lactic acid in a bioreactor in rich glucose medium by *C. sonorensis* harboring *L. helveticus* LDH gene integrated into the genome.**

The *C. sonorensis* transformants designated 247-5 and 247-11 described above were cultivated by batch cultivation in a laboratory bioreactor (Biostat CT-
25 DCU3, Braun, Germany) at 30°C (strain 247-11) or 35°C (strain 247-5) with a working volume of 2 L. The cultivation medium was YP supplemented with 40 g/L glucose. Precultures were grown on YPD medium to an OD₆₀₀ of 11-16, cells were collected by centrifugation and the bioreactor was inoculated to an OD₆₀₀ of 1. Cultivation continued until all glucose was consumed. The pH was maintained
30 at 5.0 ±0.1 by automated addition of 5 M potassium hydroxide (KOH). The bioreactor was flushed with 5% air and 95 % nitrogen gas at a flow rate of 0.5

L/min and stirred at 500 rpm. The dissolved-oxygen concentration was continuously monitored with an oxygen electrode (Mettler Toledo).

Samples were withdrawn during cultivation. Cell dry weight was determined, OD₆₀₀ was measured, and the cells were harvested by centrifugation.

5 The culture supernatants were analyzed by HPLC as described above for lactic acid, glucose, ethanol and acetate. These results are shown in Tables 36 and 37.

After 52 to 69 hours of cultivation, the transformants produced 26-29 g/L of lactic acid (equivalent to a 67 -72 % yield) from glucose.

10 These results demonstrated that *C. sonorensis* overexpressing the *L. helveticus* lactate dehydrogenase gene was capable of producing lactic acid from glucose in a microaerobic batch fermentation.

15 **Table 36:** Lactate production with *C. sonorensis* overexpressing *L. helveticus* LDH in two bioreactor cultivations in YPD medium with 40 g/L glucose: glucose consumption, lactate concentration and lactate yield at the end of fermentation (all glucose used).

<i>C. sonorensis</i> transformants	Initial biomass (dry weight g/L)	Ferm. time	Glucose used (g/L)	L.A. (g/L)	L.A. yield (g L.A./g glucose consumed)
247-5	0.25	52h	40.0	28.9	0.72
247-11	0.26	69h	39.5	26.6	0.67

20 **Table 37:** Lactate production with *C. sonorensis* overexpressing *L. helveticus* LDH in two bioreactor cultivations in YPD medium with 40 g/L glucose: byproduct concentrations.

<i>C. sonorensis</i> transformants	Initial biomass (dry weight g/L)	Ferm. time	Final biomass (dry wt. g/L)	Ethanol (g/L)	Acetate (g/L)	Glycerol (g/L)
247-5	0.25	52h	1.5	0.76	0.21	0
247-11	0.26	69h	1.8	1.91	0.30	0

25 **Example 26: Production of L-lactic acid in defined xylose medium by *C. sonorensis* cells harboring *L. helveticus* LDH gene integrated into the genome.**

C. sonorensis cells and the transformants disclosed above (specifically, 246-1, 246-10, 247-2, and 247-5) were cultivated in YX -medium (yeast nitrogen base without ammonium sulfate and amino acids supplemented with 0.3% urea and 5% xylose). Precultures were grown in YPD-medium, cells were collected by

centrifugation, washed once with YX -medium and then resuspended in 50 mL of YX medium to an OD₆₀₀ of 14-22 for the cultivation experiments. Yeast cells were cultivated in 100 mL Erlenmeyer flasks with 100 rpm shaking at 30°C. When the pH reached approximately 3.5, 0.2% solid calcium carbonate was added. Samples were withdrawn during cultivation, OD₆₀₀ measured, cells were harvested by centrifugation, and the culture supernatant was analyzed by HPLC, as described above. These results are shown in Table 38.

After 71 hours of cultivation, the transformants harboring *L. helveticus* LDH produced 3.6 - 5.0 g/L lactic acid, equivalent to 18 - 34 % yield from xylose, whereas *C. sonorensis* host did not produce detectable lactic acid. The biomass increased less than 10% during the 167 hour experiment. The transformants utilized 10-30 g/L xylose and produced 4-9 g/L of lactic acid. One third of used xylose was converted into lactic acid by the transformants 246-10 and 247-5.

These results demonstrated that *C. sonorensis* overexpressing a heterologous lactate dehydrogenase gene was capable of producing lactic acid from xylose.

Table 38. OD₆₀₀, residual xylose and lactic acid production by *C. sonorensis* and the *L. helveticus* LDH transformants on defined xylose medium.

Strain	1 h	3 h	24 h	48 h	71 h	167 h
<i>C. sonorensis</i> , OD ₆₀₀	18.0	19.1	18.4	18.8	19.1	23.7
246-1, OD ₆₀₀	22.4	25.7	24.6	23.9	23.4	25.3
247-2, OD ₆₀₀	20.5	21.2	21.3	21.7	18.8	23.2
246-10, OD ₆₀₀	19.5	19.3	17.3	18.8	18.4	16.2
247-5, OD ₆₀₀	14.8	14.9	15.4	14.9	13.9	14.9
<i>C. sonorensis</i> , xylose g/L	45.00	43.99	40.32	34.95	26.79	7.22
246-1, xylose g/L	42.67	42.56	40.53	35.43	29.79	13.15
247-2, xylose g/L	44.61	44.45	41.81	33.63	23.66	11.18
246-10, xylose g/L	45.21	44.90	44.01	38.8	35.44	33.73
247-5, xylose g/L	45.31	44.74	42.94	37.81	30.34	17.75
<i>C. sonorensis</i> , lactic acid g/L	0	0	0	0	0	0
246-1, lactic acid g/L	0.45	0.55	1.18	2.44	4.12	5.84
247-2, lactic acid g/L	0.00	0.10	1.16	3.01	4.09	6.42
246-10, lactic acid g/L	0.30	0.40	0.95	2.18	3.55	4.12
247-5, lactic acid g/L	0.26	0.37	1.09	3.08	4.97	9.02
<i>C. sonorensis</i> , lactic acid g/ g DW	0.00	0.00	0.00	0.00	0.00	0.00
246-1, lactic acid g/ g DW	0.07	0.07	0.16	0.34	0.59	0.77
247-2, lactic acid g/ g DW	0.00	0.02	0.18	0.46	0.72	0.92
246-10, lactic acid g/ g DW	0.05	0.07	0.18	0.39	0.64	0.85
247-5, lactic acid g/ g DW	0.06	0.08	0.24	0.69	1.19	2.02

Strain	1 h	3 h	24 h	48 h	71 h	167 h
<i>C. sonorensis</i> , lactic acid g/ used xylose g	0.00	0.00	0.00	0.00	0.00	0.00
246-1, lactic acid g/ used xylose g	0.14	0.16	0.21	0.23	0.25	0.18
247-2, lactic acid g/ used xylose g	0.00	0.06	0.28	0.24	0.18	0.18
246-10, lactic acid g/ used xylose g	0.38	0.36	0.48	0.30	0.34	0.34
247-5, lactic acid g/ used xylose g	0.38	0.29	0.36	0.38	0.32	0.32

Example 27: Production of L-lactic acid in defined L-arabinose medium by *C. sonorensis* harboring *L. helveticus* LDH gene integrated into the genome.

5 *C. sonorensis* cells and the transformants described above (specifically 246-1, 246-10, 247-2, and 247-5) were cultivated in YA-medium (yeast nitrogen base without ammonium sulfate and amino acids supplemented with 0.3% urea and 2% L- arabinose). Precultures were grown in YPD-medium, cells were collected by centrifugation, washed once with YA-medium and resuspended on
10 the 50 mL of YA-medium to an OD₆₀₀ of 14-20 for the cultivation experiments. Yeast cells were cultivated in 100 mL Erlenmeyer flasks with 100 rpm shaking (microaerobic conditions) at 30°C. When the pH reached approximately 3.5, 0.2% solid calcium carbonate was added. Samples were withdrawn during cultivation, OD₆₀₀ measured, the cells were harvested by centrifugation, and the
15 culture supernatant analyzed for lactic acid and xylose by HPLC as described above. These results are shown in Table 39.

After 71 hours of cultivation the transformants harboring the *L. helveticus* LDH produced 2.3 - 3.2 g/L lactic acid equivalent of 14 – 34% yield from arabinose, whereas the control strain did not produce detectable lactic acid. The
20 biomass increased 20-60% during the 167h experiment. The transformants used almost all the 20 g/L arabinose initially provided and produced 3.3-4.5 g/L of lactic acid. About 20% of used arabinose was converted into lactic acid by transformants 246-10 and 247-5.

This example showed that *C. sonorensis* expressing a heterologous LDH
25 gene produced lactic acid from arabinose.

Table 39. OD₆₀₀, residual xylose and lactic acid production by *C. sonorensis* and the *L. helveticus* LDH transformants on defined arabinose medium (OD₆₀₀).

Strain	1 h	3 h	24 h	48 h	71 h	167 h
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Strain	1 h	3 h	24 h	48 h	71 h	167 h
<i>C. sonorensis</i> , OD ₆₀₀	18.2	19.0	19.0	20.7	22.1	29.2
246-1, OD ₆₀₀	19.7	22.2	22.6	29.5	23.2	31.2
247-2, OD ₆₀₀	18.4	19.1	19.2	22.1	23	27.5
246-10, OD ₆₀₀	17.2	21.7	17.7	20.5	18.5	24.4
247-5, OD ₆₀₀	14	14.5	13.9	14.2	14	18.2
<i>C. sonorensis</i> , arabinose g/L	19.01	18.11	16.74	12.79	9.24	0
246-1, arabinose g/L	17.77	17.17	15.72	12.90	9.68	0
247-2, arabinose g/L	19.20	19.11	16.40	11.72	5.78	0.58
246-10, arabinose g/L	18.09	18.24	17.05	13.72	10.73	0.32
247-5, arabinose g/L	18.89	18.52	17.11	14.67	2.82	0
<i>C. sonorensis</i> , lactic acid g/L	0.00	0.00	0.00	0.00	0.00	0.01
246-1, lactic acid g/L	0.44	0.52	0.78	1.54	2.50	3.54
247-2, lactic acid g/L	0.00	0.00	0.43	1.64	2.31	3.28
246-10, lactic acid g/L	0.25	0.30	0.64	1.75	3.16	4.41
247-5, lactic acid g/L	0.28	0.32	0.76	1.51	2.37	3.40
<i>C. sonorensis</i> , lactic acid g/g DW	0.00	0.00	0.00	0.00	0.00	0.00
246-1, lactic acid g/g DW	0.07	0.08	0.11	0.17	0.36	0.38
247-2, lactic acid g/g DW	0.00	0.00	0.07	0.25	0.34	0.40
246-10, lactic acid g/g DW	0.05	0.05	0.12	0.28	0.57	0.60
247-5, lactic acid g/g DW	0.07	0.07	0.18	0.36	0.57	0.62
<i>C. sonorensis</i> , lactic acid g/ used arabinose g	0.00	0.00	0.00	0.00	0.00	0.00
246-1, lactic acid g/ used arabinose g	0.20	0.18	0.18	0.22	0.24	0.18
247-2, lactic acid g/ used arabinose g	0.00	0.00	0.12	0.20	0.16	0.17
246-10, lactic acid g/ used arabinose g	0.13	0.17	0.22	0.28	0.34	0.22
247-5, lactic acid g/ used arabinose g	0.25	0.21	0.26	0.28	0.14	0.17

Example 28: Transformation of *C. methanosorbosa* and production of lactic acid by strains harboring *B. megaterium* LDH integrated in the genome

5 *C. methanosorbosa* was transformed with the *C. sonorensis* vector pMI278 described above for lactic acid production. pMI278 was digested with *Sa*I and *Not*I. Lithium acetate transformation according to a modification of the method of Gietz *et al.* (1992, *Nucleic Acids Res.* 20: 1425) described above in Example 1.

10 Cells from an overnight culture of *C. methanosorbosa* grown to OD₆₀₀ = 0.9-1.1 were collected by centrifugation, washed first with an excess of a solution of 10 mM Tris-HCl, 1 mM EDTA (pH 7.5), and then with an excess of a solution of 100

mM LiAc / 10 mM Tris-HCl, 1 mM EDTA (pH 7.5), and resuspended in 2 mL
100 mM LiAc / 10 mM Tris-HCl, 1 mM EDTA (pH 7.5). 50 μ L of cells was
mixed with 10 μ g of transforming DNA and 50 μ g of heat-denatured herring
sperm DNA. To the cells was added 300 μ L of a 40% PEG-4000 solution in 100
5 mM LiAc / 10 mM Tris-HCl, 1 mM EDTA (pH 7.5) and the cells were then
incubated at 30°C for 30 min with slow shaking. DMSO was then added (40 μ L)
and the cells were incubated in a 42°C water bath for 15 min. Cells were collected
by centrifugation, washed with an excess of a solution of 10 mM Tris-HCl, 1 mM
EDTA (pH 7.5), resuspended in YPD and incubated at 30°C overnight. Cells
10 were spread onto solid YPD medium containing 200 μ g/mL G418 and incubated
at 30°C for three to five days. Transformants were streaked onto fresh selection
plates twice. The transformants were designated as Cm/278-1 through Cm/278-
74.

Transformants were tested for their ability to produce L-lactic acid as
15 follows. 5 mL of YPD in a 10 mL plastic tube was inoculated with a colony
grown on G418 plates and incubated with shaking at 250 rpm at 30°C overnight.
The cells were removed by centrifugation and the supernatant was analyzed for L-
lactic acid using the L-lactic acid UV method of Boehringer Mannheim. L-lactic
acid was produced at 2.3-4.3 g/L. The presence of a single copy of *B. megaterium*
20 LDH gene in the genome was verified by Southern blot analysis of *Hind*III
digested yeast DNA using the *B. megaterium* LDH gene as the probe.

These results showed that *B. megaterium* LDH was able to function in *C.*
methanosorbosa and produced lactic acid from glucose. The *B. megaterium* LDH
is operatively linked to *C. sonorensis* *PGK1* promoter that is able to drive
25 expression of a heterologous gene in *C. methanosorbosa*. Furthermore, the *C.*
sonorensis *TDH1* promoter that is operatively linked to the G418 resistance gene
is also able to function in *C. methanosorbosa*.

Example 29: Production of L-lactic acid in rich glucose medium without
30 **buffering by *C. methanosorbosa* harboring the *B. megaterium* LDH gene**
integrated into the genome.

One of the *C. methanosorbosa* transformants disclosed above (Cm/278-1) was cultivated in YD-medium (yeast nitrogen base without amino acids supplemented with 5% glucose and 0.5 M MES pH 5.5). Cells were then collected by centrifugation and resuspended in 50 mL of YP supplemented with 5% glucose to an OD₆₀₀ of 16. Yeast cells were cultivated in 250 mL Erlenmeyer flasks with 100 rpm shaking at 30°C. Samples were withdrawn during cultivation, OD₆₀₀ was measured, the cells were harvested by centrifugation, and the culture supernatant was analyzed for L-lactic acid (by the L-lactic acid UV method of Boehringer Mannheim, Roche), glucose (by the glucose/ GOD-Perid method of Boehringer Mannheim, Roche), for acetate (by the acetic acid UV method of Boehringer Mannheim, Roche), and ethanol (by the ethanol UV method of Boehringer Mannheim, Roche). These results are shown in Table 40.

After 24 hours of cultivation the transformant produced 8.1 g/L lactic acid (equivalent to 19 % yield) from glucose and the pH dropped to 3.5.

These results demonstrated that *C. methanosorbosa* overexpressing a heterologous LDH produced lactic acid from glucose in rich medium at low pH.

Table 40. OD₆₀₀, residual glucose, lactic acid, ethanol and acetate production and pH of the culture supernatant of the strain Cm/278-1.

	0 h	4 h	8 h	12 h	24 h	48 h	72 h	96 h	120 h	144 h
OD ₆₀₀	16.0	17.9	18.5	18.7	22.3	17.3	17.3	16.8	26.4	17.0
Glucose	41.2	33.2	21.6	20.4	8.7	n.d.	0.6	0.0	0.0	0.0
Lactic acid	0.2	2.9	4.5	6.7	8.1	5.8	5.6	6.9	6.4	5.6
Ethanol	0.3	2.2	5.6	7.4	10.1	12.2	11.9	11.0	9.4	8.3
pH	5.5	3.9	3.7	3.6	3.5	3.6	3.7	3.6	3.5	3.6
Acetate	0.1	0.2	0.7	0.9	2.3	3.5	4.1	4.2	3.9	3.8

Glucose, lactic acid, ethanol, and acetate all in g/L.

Example 30: Production of L-lactic acid in CaCO₃-buffered defined glucose medium by *C. methanosorbosa* harboring the *B. megaterium* LDH gene integrated into the genome.

The transformed *C. methanosorbosa* cells disclosed above (specifically, transformants designated Cm/278-1 and Cm/278-14) and the untransformed host strain (Cm) were cultivated in YD-medium (yeast nitrogen base without amino acids supplemented with 5% glucose and 0.5 M MES, pH 5.5). The cells were

then collected by centrifugation and resuspended in 50 mL of YD medium (yeast nitrogen base without amino acids supplemented with 10 % glucose) to an OD₆₀₀ of 15 for the cultivation experiments. Yeast cells were cultivated in 250 mL Erlenmeyer flasks containing 4 g CaCO₃ with 100 rpm shaking at 30°C. The pH of the culture medium throughout the cultivation was 6.5. Samples were withdrawn during cultivation, cells harvested by centrifugation and the culture supernatant analyzed by HPLC for lactic acid, glucose and ethanol, as described above. These results are shown in Tables 41-44.

The transformants had consumed glucose at 96 hours of cultivation and had produced 63-65 g/L of lactic acid (equivalent to 63-64% yield) and 6.5-6.9 g/L of ethanol. The host strain (Cm) had used all glucose by 120 hours of cultivation and it had produced 23 g/L of ethanol and no lactic acid.

These results demonstrated that *C. methanosorbosa* cells overexpressing a heterologous LDH gene produced lactic acid from glucose in defined medium at neutral pH. High lactic acid titers, 63-65 g/L, and yields 63-64 % were achieved.

Table 41. Glucose g/L

Strain	0h	4h	8h	12h	24h	48h	72h	96h	120h
278-1	99.81	83.83	80.72	75.18	63.28	33.17	16.08	0.0	0.0
278-14	102.07	85.14	81.51	75.01	63.01	33.28	16.36	0.0	0.0
Cm	89.18	87.29	79.69	72.78	60.28	36.40	22.56	2.11	0.0

Table 42. Lactic acid g/L

Strain	0h	4h	8h	12h	24h	48h	72h	96h	120h
278-1	0.25	1.77	4.19	6.86	13.24	33.34	53.18	63.22	59.54
278-14	0.25	2.31	4.59	7.00	13.41	35.49	54.38	64.98	64.60
Cm	0.00	0.00	0.00	0.00	0.00	0.00	0.68	0.40	0.00

Table 43. Ethanol g/L

Strain	0h	4h	8h	12h	24h	48h	72h	96h	120h
278-1		0.62	1.45	2.07	3.13	4.53	6.17	6.51	4.18
278-14		1.11	1.68	2.12	2.36	4.58	6.16	6.91	5.71
Cm	0.3	1.75	3.66	5.47	9.80	14.83	21.7	27.08	23.51

Table 44. Lactic acid g / used glucose g.

	0h	4h	8h	12h	24h	48h	72h	96h	120h
278-1	0.21	0.10	0.21	0.27	0.35	0.49	0.63	0.63	0.59

	0h	4h	8h	12h	24h	48h	72h	96h	120h
278-14	0.00	0.15	0.24	0.27	0.35	0.52	0.64	0.64	0.64
Cm	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00

Example 31: Enzyme activities of lactate dehydrogenase and pyruvate decarboxylase and production of L-lactic acid in defined glucose medium by
5 *C. methanosorbosa* harboring the *B megaterium* LDH gene integrated into the genome.

The *C. methanosorbosa* transformants disclosed above (Cm/278-1 and Cm/278-14) were cultivated in 50 mL of YD-medium (yeast nitrogen base without amino acids supplemented with 5% glucose and 0.5 M MES, pH 5.5). Yeast cells
10 were cultivated in 250 mL Erlenmeyer flasks with 250 rpm shaking to an OD₆₀₀ of 10 at 30°C. Samples were collected (2 mL) and cells were harvested by centrifugation. The culture supernatant was analyzed by HPLC.

For enzyme activity measurements the cell pellet was washed with 1 mL of ice-cold 10 mM K₂HPO₄/KH₂PO₄, 2 mM EDTA (pH 7.5). Washed pellets were
15 resuspended with 0.5 mL of the same buffer and stored at -70°C. Samples were thawed at room temperature and washed once with 1 mL of sonication buffer (100 mM KH₂PO₄/K₂HPO₄, 2 mM MgCl₂, 10 mM DTT, pH 7.5). Washed samples were resuspended to 0.5 mL of sonication buffer and homogenized with 0.5 mL of glass beads in a Bead Beater homogenizer for 1 min. After homogenization, the
20 samples were centrifuged at 14,000 rpm for 30 min at 4°C. Supernatant samples were collected and lactate dehydrogenase and pyruvate decarboxylase activities were determined spectrophotometrically (A₃₄₀) as described above in example 18. These results are shown in Table 45.

At 20 h of cultivation, transformants 278-1 and 278-14 produced 0.69 and
25 0.33 g/L lactic acid (equivalent to 7 and 4 % yield from glucose), respectively. At that time point, lactate dehydrogenase activity was 0.05 and 0.16 U/mg total cellular protein, and pyruvate decarboxylase activity was 0.71 and 0.53 U/mg total cellular protein in the transformant 278-1 and 278-14, respectively.

These results demonstrated that lactate dehydrogenase activity is detected
30 in *C. methanosorbosa* cells overexpressing a heterologous LDH gene and confirmed that the cells were capable of producing lactic acid from glucose. The

lower activity could be attributed to a lower starting OD₆₀₀ and higher aeration (250 rpm), resulting in predominantly cell growth and small amount of lactate production.

- 5 **Table 45.** Intracellular LDH and PDC enzyme activities (U/mg total cellular protein) and residual glucose, lactic acid and ethanol (g/L) in the culture supernatant of *C. methanosorbosa* expressing the *B. megaterium* LDH.

Strain		LDH U/mg	PDC U/mg	Glucose g/L	Lactic acid g/L	Ethanol g/L
Cm/278-1	Cm/BmLDH	0.05	0.71	40.8	0.69	1.31
Cm/278-14	Cm/BmLDH	0.16	0.53	41.9	0.33	0.92

- 10 **Example 32. Production of lactic acid in defined xylose medium by *C. sonorensis* harboring the *L. helveticus* or the *B. megaterium* LDH encoding gene and by *C. methanosorbosa* harboring the *B. megaterium* LDH encoding gene integrated in the genome.**

Lactic acid was produced from xylose in CaCO₃ buffered cultures of *C. sonorensis* and *C. methanosorbosa* cultivated on defined medium. The cell biomass was generated either on glucose or on xylose in two stages before transfer into the xylose-containing production medium.

A) Biomass generation on glucose and lactate production on xylose

- 20 5 mL of YP+5% glucose medium was inoculated with a yeast colony (strain C40/288-34) grown on YPD plates. The culture was incubated overnight with 250 rpm shaking at 30°C. 50 mL of YD -medium (yeast nitrogen base, no amino acids supplemented, 5% glucose, and 0.5 M MES, pH 5.5) in 250 mL Erlenmeyer flasks was inoculated into an initial OD₆₀₀ of 0.1 and incubated with
- 25 250 rpm shaking overnight at 30°C until an OD₆₀₀ of 10 was reached. The cells were resuspended in 50 mL of YX- medium (yeast nitrogen base without amino acids supplemented with 5 % xylose) to an OD₆₀₀ of 11-13. The cells were cultivated in 250 mL Erlenmeyer flasks containing 2 g CaCO₃ with 100 rpm shaking at 30°C. Samples were withdrawn during cultivation. The cells were
- 30 removed by centrifugation, and the culture supernatant was analyzed for lactic

acid and xylose by HPLC as described above (Example 14). Two independent experiments were carried out and the results are shown in Table 46.

The *C. sonorensis* transformant C40/288-34 consumed 50 g/L of xylose in 7-8 days and produced 13-16 g/L of lactic acid, corresponding to 28-32% lactic acid yield from xylose.

Table 46. Lactic acid production from xylose in CaCO₃ buffered cultivation on defined medium by glucose-grown cells. The yields were calculated at peak lactate concentration (168 or 198 h). The data are from two independent experiments.

Strain	Genotype	Time h	Residual xylose g/L	L.A. g/L	L.A. yield from xylose g/g
C40/288-34	2xLhLDH <i>pdc1-pdc2-</i>	168	0	13	0.28
C40/288-34	2xLhLDH <i>pdc1-pdc2-</i>	198	2.2	16	0.32

10

B) Biomass generation on xylose and lactate production on xylose

Transformants 265-55 and 265-44 (*C. sonorensis*) harboring the *B. megaterium* LDH, transformants C40/288-34, C40/288-36, 257-3, and 246-27 (*C. sonorensis*) harboring the *L. helveticus* LDH, and transformants Cm/278-1 and Cm/278-42 (*C. methanosorbosa*) harboring the *B. megaterium* LDH were used.

50 mL of YP+5% xylose medium in a 250 mL shake flask were inoculated with a yeast colony grown on YP+2% xylose plates. The culture was incubated overnight with 250 rpm shaking at 30°C until an OD₆₀₀ of 10 was reached, then 50 mL of YX -medium (yeast nitrogen base, no amino acids supplements, 5% xylose, and 0.5 M MES, pH 5.5) in a 250 mL Erlenmeyer flasks was inoculated to an initial OD₆₀₀ of 0.2. The cells were incubated with 250 rpm shaking overnight at 30°C until an OD₆₀₀ of 7-10 was reached. The cells were resuspended in 50 mL of YX- medium (yeast nitrogen base, no amino acids supplements, and 5 % xylose) to an OD₆₀₀ of 11-12. The cells were cultivated in 250 mL Erlenmeyer flasks containing 2 g CaCO₃ with 100 rpm shaking at 30°C. Samples were withdrawn during cultivation. The cells were removed by centrifugation, and the culture supernatant was analyzed for lactic acid and xylose by HPLC as described above (Example 14). The results are shown in Table 47.

C. sonorensis LDH transformants consumed 50 g/L of xylose, typically in 5-6 days. Maximal lactic acid titers were measured 4-5 days after transfer into CaCO₃-buffered xylose medium, when at least 95% of the xylose was consumed. The amount of lactic acid produced was 30-37 g/L, corresponding to a 63-76 %
5 lactic acid yield from xylose.

C. methanosorbosa LDH transformants consumed 50 g/L of xylose, typically in 5-6 days. Maximal lactic acid titers were measured 4-5 days after transfer into CaCO₃-buffered xylose medium, when at least 95% of the xylose was consumed. Transformants produced 8-14 g/L of lactic acid, corresponding to a
10 16-28% lactic acid yield from xylose.

Table 47. Lactic acid production from xylose in CaCO₃-buffered defined medium by cells grown on xylose. The yields were calculated at peak lactic acid concentration.

Strain	Genotype	Time h	Residual xylose g/L	L.A. g/L	L.A. yield from xylose g/g
265-55	1xBmLDH <i>PDC+</i>	96	1.5	36	0.74
265-44	1xBmLDH <i>PDC+</i>	96	0.9	37	0.76
C40/288-34	2xLhLDH <i>pdcl-pdc2-</i>	96	3.0	33	0.71
C40/288-36	2xLhLDH <i>pdcl-PDC2+</i>	96	1.9	36	0.76
257-3	1xLhLDH <i>pdcl-PDC2+</i>	120	2.2	30	0.63
246-27	1xLhLDH <i>PDC+</i>	120	1.2	32	0.66
<i>C. sonorensis</i>	<i>PDC+</i>	96	17.3	0	0.00
Cm/278-1	1xBmLDH <i>PDC+</i>	120	1.2	14	0.28
Cm/278-42	1xBmLDH <i>PDC+</i>	96	1.8	8	0.16
<i>C. methano-sorbosa</i>	<i>PDC+</i>	96	1.6	0	0.00

15

This Example demonstrated that the culture conditions and the history of the inoculum have a major effect on lactic acid production from xylose. When the biomass was generated on glucose, the *C. sonorensis* LDH transformant C40/288-

34 converted xylose into lactic acid, after transfer into xylose-containing medium, at approximately 30% yield. In comparison, when the biomass was generated on xylose, the same transformant converted xylose into lactic acid with a much higher yield (63-76%), after transfer into xylose-containing medium. The xylose-grown biomass also consumed xylose faster than the glucose-grown biomass under lactic acid production conditions. The data suggests that increased lactic acid yields can be obtained when the cells are "adapted" to sugars other than glucose, for example xylose, by growth on xylose-containing medium, prior to their transfer to the xylose-containing lactic acid production medium.

10

Example 33. Production of L-lactic acid in defined glucose medium by *C. sonorensis* comprising a deleted *pdcl* gene and a disrupted *pdcl* gene and harboring the *L. helveticus* LDH encoding gene integrated into the genome.

The *C. sonorensis* transformants designated 257-3, C40/288-2, C40/288-34 and C40/288-11 (described above in Example 13) were cultivated and assayed as described in Example 19, with the exception that the cells were suspended to an $OD_{600} = 18$ for the lactate production phase. The culture supernatant was analyzed for lactic acid, glucose, and ethanol as described above. These results are shown in Table 48.

The *pdcl*- strain 257-3 (where *pdcl* is deleted) produced 89 g/L of lactic acid in 48 h, corresponding to a 93% yield from glucose (g/g). The *pdcl*- (deleted) *pdcl*- (where *pdcl* is disrupted) strains C40/288-2, C40/288-34 and C40/288-11 produced 86-87 g/L of lactic acid in 72 h, corresponding to 89-90% yield from glucose (g/g). No ethanol was detected at these time points.

25

Table 48. Lactic acid titers and yields obtained on $CaCO_3$ -buffered defined medium containing glucose, upon consumption of all glucose (initially 96 g/L).

Strain	Genotype	Time h	L.A. g/L	L.A. yield from glucose g/g
257-3	2xLhLDH <i>pdcl</i> - <i>PDC2</i> +	48	89	0.93
C40/288-2	2xLhLDH <i>pdcl</i> - <i>pdcl</i> -	72	87	0.90
C40/288-34	2xLhLDH <i>pdcl</i> - <i>pdcl</i> -	72	86	0.90

Strain	Genotype	Time h	L.A. g/L	L.A. yield from glucose g/g
C40/288-11	2xLhLDH <i>pdcl</i> - <i>pdcl</i> 2-	72	86	0.89

It is to be understood that while the invention is described in conjunction with the foregoing detailed description and examples, they are intended to
5 illustrate and not limit the scope or the spirit of the invention, which is defined by the appended claims. Other aspects, advantages, and modifications are within the scope of the claims.

SEQUENCE LISTING

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 Ruohonen, Laura
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<120> METHODS AND MATERIALS FOR THE PRODUCTION OF ORGANIC PRODUCTS IN CANDIDA

<130> MBHB00-1237-D

<160> 50

<170> PatentIn version 3.1

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<220>
 <223> Primer for G418 resistance gene

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<220>
 <223> Primer for G418 resistance gene (3')

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 <223> Primer for TDH1 promoter (C. sonorensis)

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<210> 7
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<220>
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<210> 8
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<220>
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<400> 8
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<210> 9

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<220>
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<210> 12
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 <212> DNA
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<220>
 <223> Primer for PDC1 promoter (C. sonorensis)

<400> 12
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<210> 13
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 <212> DNA
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<220>
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<400> 13
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<210> 14
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<220>
 <223> Primer for PDC2 promoter (C. sonorensis)

<400> 14
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<210> 15
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 <212> DNA
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<220>
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<400> 15
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<210> 16
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<220>
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<400> 16
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<210> 17
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<220>
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<400> 17
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<210> 18
 <211> 20
 <212> DNA
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<220>
 <223> Primer for TDH (S. cerevisiae)

<400> 18
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<210> 19
 <211> 37
 <212> DNA
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<220>
 <223> Primer for PGK1 (C. albicans)

<400> 19
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<210> 20
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 <212> DNA
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<220>
 <223> Primer for PGK1 (C. albicans)

<400> 20
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<210> 21
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 <212> DNA
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<220>
 <223> Primer for 26 S rRNA (C. sonorensis)

<400> 21
 tggactagta aaccaacagg gattgcctta gt 32

<210> 22
 <211> 33
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Primer for 26 S rRNA (C. sonorensis)

<400> 22
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<210> 23
 <211> 44
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<223> Primers for isolating PDC

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44

<210> 24

<211> 44

<212> DNA

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<223> Primer for isolating PDC

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<222> (21)..(21)

<223> n stands for any nucleotide

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<222> (33)..(33)

<223> n stands for any nucleotide

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44

<210> 25

<211> 10

<212> PRT

<213> Artificial Sequence

<220>

<223> Conserved PDC region in yeasts

<400> 25

Trp Ala Gly Asn Ala Asn Glu Leu Asn Ala

1 5 10

<210> 26

<211> 10

<212> PRT

<213> Artificial Sequence

<220>

<223> Conserved PDC region in yeasts

<400> 26

Asp Phe Asn Thr Gly Ser Phe Ser Tyr Ser

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<223>	Primer for 26 S rRNA (C. sonorensis)		
<400>	29		
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<212>	DNA		
<213>	Artificial Sequence		
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<223>	Primer for 26 S rRNA (C. sonorensis)		
<400>	30		
ctagtctaga	gatcattacg ccagcatcct agg		33
<210>	31		
<211>	37		
<212>	DNA		
<213>	Artificial Sequence		
<220>			

<223> Primer for PGK1 sequence (C. albicans)

<400> 31
gcgatctcga ggtcctagaa tatgtatact aatttgc 37

<210> 32
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for PGK1 sequence (C. albicans)

<400> 32
acttggccat ggtgatagtt attcttctgc aattga 36

<210> 33
<211> 1235
<212> DNA
<213> Artificial Sequence

<220>
<223> 1,235 kbp NotI digested fragment comprising the S. cerevisiae PGK promoter, multiple cloning site, and the Gal10 terminator.

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ctgcatttaa agatgccgat ttgggcgcga atcctttatt ttggcttcac cctcatacta 240
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ggttctggaa tggcgggaaa gggtttagta ccacatgcta tgatgccac tgtgatctcc 540
agagcaaagt tcgttcgatc gtactgttac tctctctct tcaaacagaa ttgtccgaat 600
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atgcaagaaa tacatatattg gtcttttcta attcgtagtt tttcaagttc ttagatgctt 780
tctttttctc ttttttacag atcatcaagg aagtaattat ctacttttta caacaaatct 840
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aaccttgaaa aacggtgaaa cttacggggtc caagaccctc tacagatttt cctgatttgc    960
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atagatttgc tgtataacga attttatgct attttttaaa tttggagttc agtgataaaa    1080
gtgtcacagc gaatttcctc acatgtagga ccgaattggt tacaagttct ctgtaccacc    1140
atggagacat caaagattga aaatctatgg aaagatatgg acggtagcaa caagaatata    1200
gcacgagccg cggatttatt tcgttacgca tgcgc                                1235

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<210> 34
<211> 21
<212> DNA
<213> Artificial Sequence

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<220>
<223> Primer for LDH isolation (B. megaterium)

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<400> 34
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<210> 35
<211> 22
<212> DNA
<213> Artificial Sequence

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<220>
<223> Primer for LDH isolation (B. megaterium)

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<400> 35
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<210> 36
<211> 27
<212> DNA
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<220>
<223> Primer to introduce restriction sites

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<400> 36
gctctagatg aaaacacaat ttacacc                            27

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<220>
<223> Primer to introduce restriction sites

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<400> 37
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<210> 38
 <211> 26
 <212> DNA
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<220>
 <223> Primer for LDH isolation (R. oryzae)

<400> 38
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<210> 39
 <211> 19
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Primer for LDH isolation (R. oryzae)

<400> 39
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<210> 40
 <211> 29
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Primer for introduction of restriction sites

<400> 40
 gctctagatg gtattacact caaaggctcg 29

<210> 41
 <211> 30
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Primer for introduction of restriction sites

<400> 41
 gctctagatc aacagctact tttagaaaag 30

<210> 42
 <211> 68
 <212> DNA
 <213> Artificial Sequence

<220>

<223> Primer for PGK1 promoter (C. sonorensis)

<400> 42

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attattag 68

<210> 43

<211> 75

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer for TDH1 promoter (C. sonorensis)

<400> 43

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ttttgttttt gtttg 75

<210> 44

<211> 35

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer for PGK1 promoter (C. sonorensis)

<400> 44

acttgccat ggtatatagt cttttctatt attag 35

<210> 45

<211> 957

<212> DNA

<213> Bacillus megaterium

<220>

<221> CDS

<222> (1)..(957)

<223> B. megaterium LDH protein

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Met Lys Thr Gln Phe Thr Pro Lys Thr Arg Lys Val Ala Val Ile Gly
1 5 10 15

act ggt ttt gtt ggc tca agc tac gct ttt tca atg gtg aat caa ggt 96

Thr Gly Phe Val Gly Ser Ser Tyr Ala Phe Ser Met Val Asn Gln Gly
20 25 30

att gcc aat gaa tta gtg tta atc gat atg aac aaa gaa aaa gca gaa 144

Ile	Ala	Asn	Glu	Leu	Val	Leu	Ile	Asp	Met	Asn	Lys	Glu	Lys	Ala	Glu	
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Gly	Glu	Ala	Arg	Asp	Ile	Asn	His	Gly	Met	Pro	Phe	Ala	Thr	Pro	Met	
	50					55					60					
aaa	atc	tgg	gct	gga	gat	tat	aaa	gac	tgt	gct	gac	gct	gat	tta	gca	240
Lys	Ile	Trp	Ala	Gly	Asp	Tyr	Lys	Asp	Cys	Ala	Asp	Ala	Asp	Leu	Ala	
65					70					75					80	
gtt	att	aca	gcg	ggc	gct	aat	caa	gct	cca	ggg	gaa	aca	cgc	tta	gat	288
Val	Ile	Thr	Ala	Gly	Ala	Asn	Gln	Ala	Pro	Gly	Glu	Thr	Arg	Leu	Asp	
				85					90					95		
cta	gtt	gaa	aaa	aac	gtt	aaa	att	ttc	gaa	tgc	att	gta	aaa	gat	att	336
Leu	Val	Glu	Lys	Asn	Val	Lys	Ile	Phe	Glu	Cys	Ile	Val	Lys	Asp	Ile	
			100						105				110			
atg	aac	agc	gga	ttt	gac	ggc	atc	att	tta	gtg	gca	aca	aat	cca	gtt	384
Met	Asn	Ser	Gly	Phe	Asp	Gly	Ile	Ile	Leu	Val	Ala	Thr	Asn	Pro	Val	
		115					120					125				
gat	att	ctc	gca	cac	gtt	aca	caa	aaa	gta	tca	gga	tta	cca	aac	gga	432
Asp	Ile	Leu	Ala	His	Val	Thr	Gln	Lys	Val	Ser	Gly	Leu	Pro	Asn	Gly	
	130					135					140					
cgg	gta	att	ggt	tca	gga	acg	att	ctt	gac	aca	gct	cgc	ttc	cgc	tac	480
Arg	Val	Ile	Gly	Ser	Gly	Thr	Ile	Leu	Asp	Thr	Ala	Arg	Phe	Arg	Tyr	
145					150					155					160	
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Leu	Leu	Ser	Asp	Tyr	Phe	Glu	Val	Asp	Ser	Arg	Asn	Val	His	Ala	Tyr	
				165					170					175		
att	atg	ggg	gaa	cat	gga	gat	acg	gaa	ttt	cct	gtt	tgg	agc	cac	gcg	576
Ile	Met	Gly	Glu	His	Gly	Asp	Thr	Glu	Phe	Pro	Val	Trp	Ser	His	Ala	
			180					185					190			
caa	att	ggc	ggt	gtg	aag	ctc	gaa	cat	ttt	atc	aat	act	gcc	gct	att	624
Gln	Ile	Gly	Gly	Val	Lys	Leu	Glu	His	Phe	Ile	Asn	Thr	Ala	Ala	Ile	
		195					200					205				
gaa	aaa	gaa	ccg	gat	atg	cag	cat	cta	ttc	gaa	caa	acc	cgc	gat	gcg	672
Glu	Lys	Glu	Pro	Asp	Met	Gln	His	Leu	Phe	Glu	Gln	Thr	Arg	Asp	Ala	
	210					215					220					
gct	tac	cat	att	att	aat	cga	aaa	gga	gcg	act	tat	tac	gga	att	gca	720
Ala	Tyr	His	Ile	Ile	Asn	Arg	Lys	Gly	Ala	Thr	Tyr	Tyr	Gly	Ile	Ala	
225					230					235					240	
atg	ggg	ctt	gta	cgc	att	acc	aag	gct	att	tta	gat	gat	gaa	aat	tct	768
Met	Gly	Leu	Val	Arg	Ile	Thr	Lys	Ala	Ile	Leu	Asp	Asp	Glu	Asn	Ser	
				245					250					255		
att	tta	aca	gta	tct	gct	tta	tta	gaa	gga	caa	tac	ggg	att	tct	gat	816
Ile	Leu	Thr	Val	Ser	Ala	Leu	Leu	Glu	Gly	Gln	Tyr	Gly	Ile	Ser	Asp	

260	265	270	
gtg tat atc ggc gta cca gct atc att aat aaa aac ggc gtg cgt caa			864
Val Tyr Ile Gly Val Pro Ala Ile Ile Asn Lys Asn Gly Val Arg Gln			
275	280	285	
att att gaa ttg aat tta act cct cac gaa cag cag cag ctc gag cac			912
Ile Ile Glu Leu Asn Leu Thr Pro His Glu Gln Gln Gln Leu Glu His			
290	295	300	
tct gct agc att ctt aag caa act cgc gac aga gct ttt gtg taa			957
Ser Ala Ser Ile Leu Lys Gln Thr Arg Asp Arg Ala Phe Val			
305	310	315	
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Ile Ala Asn Glu Leu Val Leu Ile Asp Met Asn Lys Glu Lys Ala Glu			
	35	40	45
Gly Glu Ala Arg Asp Ile Asn His Gly Met Pro Phe Ala Thr Pro Met			
	50	55	60
Lys Ile Trp Ala Gly Asp Tyr Lys Asp Cys Ala Asp Ala Asp Leu Ala			
65	70	75	80
Val Ile Thr Ala Gly Ala Asn Gln Ala Pro Gly Glu Thr Arg Leu Asp			
	85	90	95
Leu Val Glu Lys Asn Val Lys Ile Phe Glu Cys Ile Val Lys Asp Ile			
	100	105	110
Met Asn Ser Gly Phe Asp Gly Ile Ile Leu Val Ala Thr Asn Pro Val			
	115	120	125
Asp Ile Leu Ala His Val Thr Gln Lys Val Ser Gly Leu Pro Asn Gly			
	130	135	140

Arg Val Ile Gly Ser Gly Thr Ile Leu Asp Thr Ala Arg Phe Arg Tyr
 145 150 155 160

Leu Leu Ser Asp Tyr Phe Glu Val Asp Ser Arg Asn Val His Ala Tyr
 165 170 175

Ile Met Gly Glu His Gly Asp Thr Glu Phe Pro Val Trp Ser His Ala
 180 185 190

Gln Ile Gly Gly Val Lys Leu Glu His Phe Ile Asn Thr Ala Ala Ile
 195 200 205

Glu Lys Glu Pro Asp Met Gln His Leu Phe Glu Gln Thr Arg Asp Ala
 210 215 220

Ala Tyr His Ile Ile Asn Arg Lys Gly Ala Thr Tyr Tyr Gly Ile Ala
 225 230 235 240

Met Gly Leu Val Arg Ile Thr Lys Ala Ile Leu Asp Asp Glu Asn Ser
 245 250 255

Ile Leu Thr Val Ser Ala Leu Leu Glu Gly Gln Tyr Gly Ile Ser Asp
 260 265 270

Val Tyr Ile Gly Val Pro Ala Ile Ile Asn Lys Asn Gly Val Arg Gln
 275 280 285

Ile Ile Glu Leu Asn Leu Thr Pro His Glu Gln Gln Gln Leu Glu His
 290 295 300

Ser Ala Ser Ile Leu Lys Gln Thr Arg Asp Arg Ala Phe Val
 305 310 315

<210> 47
 <211> 963
 <212> DNA
 <213> Rhizopus oryzae

<220>
 <221> CDS
 <222> (1)..(963)
 <223> R. oryzae LDH protein

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att att gtt gat gtt aat cct gac atc gtt caa gct caa gtc ctt gac Ile Ile Val Asp Val Asn Pro Asp Ile Val Gln Ala Gln Val Leu Asp 35 40 45	144
ctt gca gat gct gcc agt ata agt cac acg ccc atc cga gca ggt agc Leu Ala Asp Ala Ala Ser Ile Ser His Thr Pro Ile Arg Ala Gly Ser 50 55 60	192
gca gag gag gca ggg cag gca gat att gtt gtc atc acg gcc ggt gcg Ala Glu Glu Ala Gly Gln Ala Asp Ile Val Val Ile Thr Ala Gly Ala 65 70 75 80	240
aaa caa agg gaa ggt gag cct cgg aca aag ctc att gaa cga aac ttc Lys Gln Arg Glu Gly Glu Pro Arg Thr Lys Leu Ile Glu Arg Asn Phe 85 90 95	288
aga gtg ttg caa agt atc att ggt ggc atg caa ccc att cga cca gac Arg Val Leu Gln Ser Ile Ile Gly Gly Met Gln Pro Ile Arg Pro Asp 100 105 110	336
gca gtc atc ttg gtg gta gca aat cca gtc gat atc ttg aca cac att Ala Val Ile Leu Val Val Ala Asn Pro Val Asp Ile Leu Thr His Ile 115 120 125	384
gca aag acc ctc tct gga ctg cct cca aac cag gtc att ggc tcc ggt Ala Lys Thr Leu Ser Gly Leu Pro Pro Asn Gln Val Ile Gly Ser Gly 130 135 140	432
acc tac ctt gac acg acc cgt ctt cgc gtc cat ctt ggc gat gtc ttt Thr Tyr Leu Asp Thr Thr Arg Leu Arg Val His Leu Gly Asp Val Phe 145 150 155 160	480
gat gtc aat cct caa tcg gtc cat gct ttt gtc ttg ggt gaa cat ggg Asp Val Asn Pro Gln Ser Val His Ala Phe Val Leu Gly Glu His Gly 165 170 175	528
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aaa gcg ata tca ggt aaa gcg atg gag atc att cgt ttg aaa gga gcc Lys Ala Ile Ser Gly Lys Ala Met Glu Ile Ile Arg Leu Lys Gly Ala 210 215 220	672
acg ttt tat gga att ggt gcc tgt gca gcg gat tta gtg cac act atc	720

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tat	gga	gcc	act	ttt	tct	atg	cct	gct	aaa	ctt	gga	tgg	aga	ggt	gtt	816
Tyr	Gly	Ala	Thr	Phe	Ser	Met	Pro	Ala	Lys	Leu	Gly	Trp	Arg	Gly	Val	
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gaa	cag	atc	tat	gaa	gta	cca	ctg	acg	gaa	gaa	gaa	gaa	gcg	ttg	ctt	864
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Lys	Gln	Arg	Glu	Gly	Glu	Pro	Arg	Thr	Lys	Leu	Ile	Glu	Arg	Asn	Phe	
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Thr Tyr Leu Asp Thr Thr Arg Leu Arg Val His Leu Gly Asp Val Phe
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Asp Val Asn Pro Gln Ser Val His Ala Phe Val Leu Gly Glu His Gly
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 195 200 205

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Glu	Glu	Leu	Gly	Ile	Ile	Asp	Ile	Ala	Lys	Glu	His	Val	Glu	Gly	Asp
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Ala	Ile	Asp	Leu	Ala	Asp	Ala	Thr	Pro	Trp	Thr	Ser	Pro	Lys	Asn	Ile	50	55	60	
Tyr	Ala	Ala	Asp	Tyr	Pro	Asp	Cys	Lys	Asp	Ala	Asp	Leu	Val	Val	Ile	65	70	75	80
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Met Pro Leu Ser Asp Lys Glu Gln Glu Leu Met Thr Ala Ser Ala Asp
290 295 300

Gln Leu Lys Lys Val Met Asp Lys Ala Phe Lys Glu Thr Gly Val Lys
305 310 315 320

Val Arg Gln

We claim:

1. A recombinant nucleic acid construct comprising a nucleic acid having a nucleotide sequence encoding a lactate dehydrogenase protein, wherein the lactate dehydrogenase protein is operatively linked to a promoter functional in
5 a yeast cell from a species of genera *Candida*.
2. The recombinant nucleic acid construct of claim 1, wherein the nucleotide sequence encodes a lactate dehydrogenase protein from *Bacillus megaterium*.
10
3. The recombinant nucleic acid construct of claim 1, wherein the nucleotide sequence encodes a lactate dehydrogenase protein from *Lactobacillus helveticus*.
- 15 4. The recombinant nucleic acid construct of claim 1, wherein the nucleotide sequence encodes a lactate dehydrogenase protein from *Rhizopus oryzae*.
5. The recombinant nucleic acid construct of claim 1, wherein the
20 promoter is from a *Candida* species that is *Candida sonorensis*, *Candida parapsilosis*, *Candida naeodendra*, *Candida methanosorbosa*, *Candida entomophila*, *Candida krusei*, *Candida blankii*, or *Candida diddensiae*.
6. The recombinant nucleic acid construct of claim 1, further
25 comprising a gene coding for resistance to a selective agent.
7. The recombinant nucleic acid construct of claim 6, wherein the gene coding for resistance to a selective agent is a bacterial neomycin resistance gene, kanamycin resistance gene, hygromycin resistance gene, or zeocin resistance gene.
30
8. The recombinant nucleic acid construct of claim 1, further comprising a gene that encodes a protein that processes carbon sources other than monosaccharide hexoses.

9 The recombinant nucleic acid construct of claim 8, wherein the gene encodes an alpha-galactosidase.

5

10. The recombinant nucleic acid construct of claim 9, wherein the gene is yeast *MEL5*.

11. A genetically modified cell from genera *Candida*, comprising at least one exogenous LDH gene.

12. A cell from genera *Candida*, transformed with the recombinant nucleic acid construct of any of claims 1-10, wherein the cell expresses the lactate dehydrogenase protein.

15

13. The cell of claim 11, wherein the cell further expresses reduced pyruvate decarboxylase (PDC) activity.

14. The cell of claim 13, wherein the reduced PDC activity results from deletion of at least one pyruvate decarboxylase gene.

15. The cell of claim 13, wherein the reduced PDC activity results from genetic disruption of at least one pyruvate decarboxylase gene.

16. The cell of claim 11, that is a *Candida sonorensis*, *Candida parapsilosis*, *Candida naeodendra*, *Candida methanosorbosa*, *Candida entomophila*, *Candida krusei*, *Candida blankii*, or *Candida diddensiae* cell.

17. The cell according to claim 12, wherein the promoter is a promoter from the *Candida* species of the cell.

18. The cell according to claim 12, wherein the promoter is a promoter from a species other than the *Candida* species of the cell.

19. A *Candida* cell comprising a deletion at a *pdcl* gene locus, a disruption at a *pdcl* gene locus and two or more copies of lactate dehydrogenase genes in the cellular genome at each of the *pdcl* and *pdcl* loci.

5

20 The cell of claim 19, wherein the two or more copies of lactate dehydrogenase genes are each operably linked to a promoter that is transcriptionally active in the *Candida* cell.

10 21. A *Candida* cell genetically modified to contain non-functional or deleted *pdcl* or *pdcl* gene, characterized by at least a 10-fold reduction of ethanol production when cultured in the presence of a defined glucose or rich glucose medium.

15 22. The cell of claim 21 further comprising a gene encoding for a lactate dehydrogenase.

23. The cell of claim 22 wherein the lactate dehydrogenase is operably linked to a *pdcl* or *pdcl* promoter.

20

24. The *Candida* cell of claim 11, wherein the cell has increased lactic acid dehydrogenase activity relative to the *Candida* cell that is untransformed.

25. A method for producing lactic acid comprising the steps of

25 a) culturing a cell of claim 11 under conditions that allow the cell to proliferate; and

b) fermenting the cell culture of (a) in a nutrient medium comprising a sugar, under conditions whereby the amount of the sugar converted by the cell to lactic acid is increased, relative to the amount of the sugar converted to lactic acid
30 by an untransformed *Candida* cell.

26. The method according to claim 25, wherein the lactic acid is L-lactic acid.

27. The method of claim 25, wherein the cell is a *Candida sonorensis*
5 cell.

28. The method of claim 27, wherein the cell comprises at least one
lactate dehydrogenase gene that is a *L. helveticus*, *B. megaterium*, or *R. oryzae*
lactate dehydrogenase gene, or combinations thereof.
10

29. The method of claim 25, wherein the cell is a *Candida*
methanosorbosa cell.

30. The method of claim 29, wherein the cell comprises at least one
15 lactate dehydrogenase gene that is a *L. helveticus*, *B. megaterium*, or *R. oryzae*
lactate dehydrogenase gene, or combinations thereof.

31. The method of claim 25, wherein the cells are cultivated in a
medium that is a buffered medium, wherein the medium is buffered to maintain a
20 pH in the nutrient medium from about pH 5 to about pH 9.

32. The method of claim 25, wherein the final pH of the culture medium
after lactic acid production is from about pH 2.6 to about pH 5.

25 33. The method of claim 25, wherein fermenting step is performed under
an atmosphere that contains no more than 2% oxygen.

34. The method of claim 25, wherein the fermenting step is performed
under anaerobic conditions.
30

35. The method of claim 25, wherein the sugar in the nutrient medium is
one or a plurality of hexoses, one or a plurality of pentoses, or combinations
thereof.

36. The method of claim 25, wherein the sugar in the nutrient medium is glucose, xylose, or L-arabinose, or combinations thereof.

5 37. The method of claim 36, wherein the sugar in the nutrient medium is glucose, and wherein the yield of lactic acid relative to the amount of glucose consumed by the cell is at least 60% by weight.

10 38. The method of claim 36, wherein the sugar in the nutrient medium is xylose, and wherein the yield of lactic acid relative to the amount of xylose consumed by the cell is at least 15% by weight.

15 39. The method of claim 36, wherein the sugar in the nutrient medium is L-arabinose, and wherein the yield of lactic acid relative to the amount of L-arabinose consumed by the cell is at least 20% by weight.

20 40. The method of claim 25, wherein the *Candida* cell is a *Candida diddensiae*, *Candida parapsilosis*, *Candida naeodendra*, *Candida krusei*, *Candida blankii*, *Candida methanosorbosa* or *Candida entomophila* cell.

25 41. A method of reducing pyruvate decarboxylase activity in a cell from genera *Candida* comprising transforming the cell with the a recombinant nucleic acid construct, wherein the nucleic acid construct comprises a selectable gene flanked by 5' and 3' flanking sequences from at least one pyruvate decarboxylase gene native to the genera *Candida*.

30 42. The method of claim 41, wherein the at least one pyruvate decarboxylase gene is selected from the group consisting of pyruvate decarboxylase 1 (*pdcl*), pyruvate decarboxylase 2 (*pdcl2*), or both *pdcl* and *pdcl2*.

 43. The method of claim 41, wherein the flanking sequences are a promoter and a terminator for at least one pyruvate decarboxylase gene native to the genera *Candida*.

44. A genetically modified *Candida* cell made by the method of any of claims 41, 42 or 43.

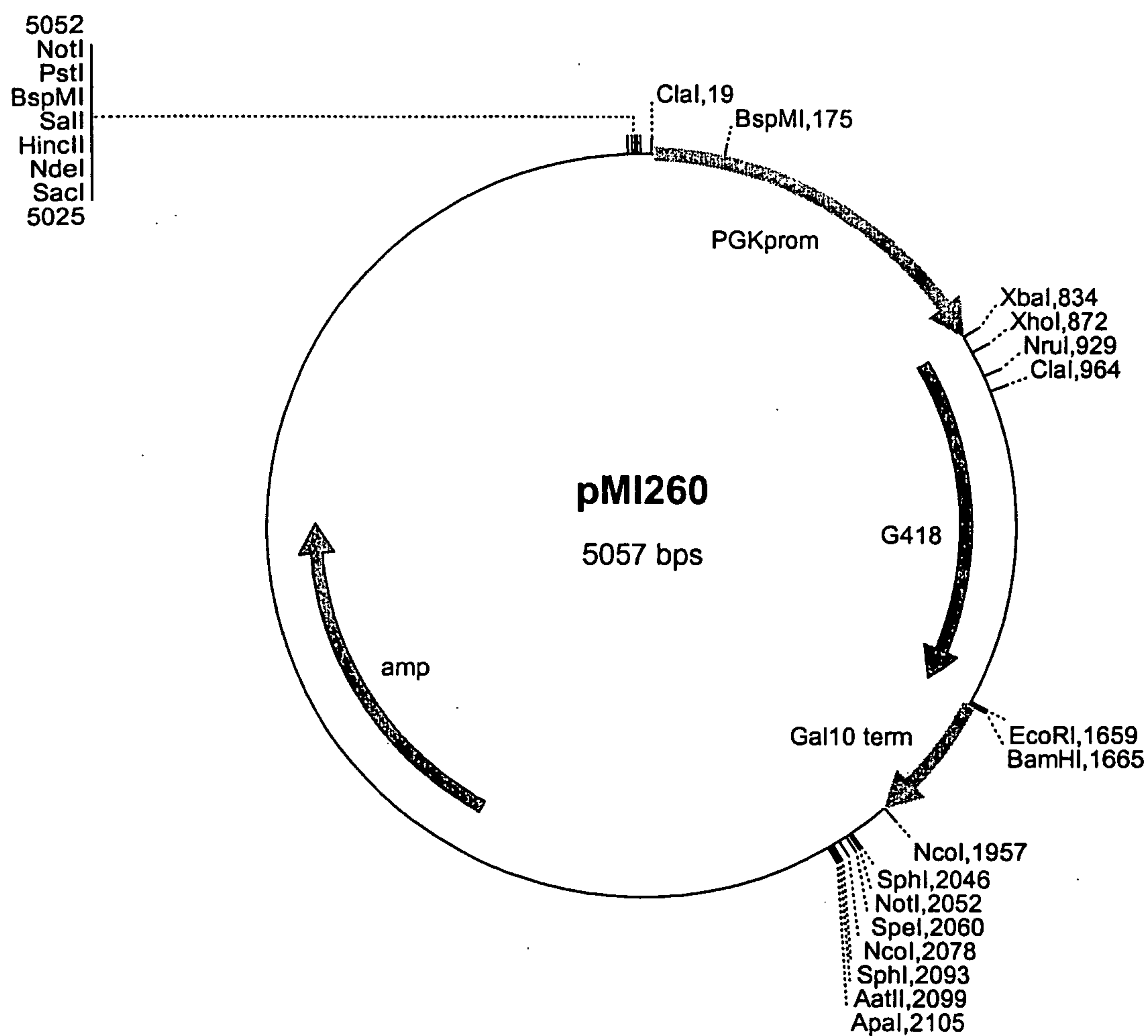
FIG. 1: G418 resistance vector pMI260.

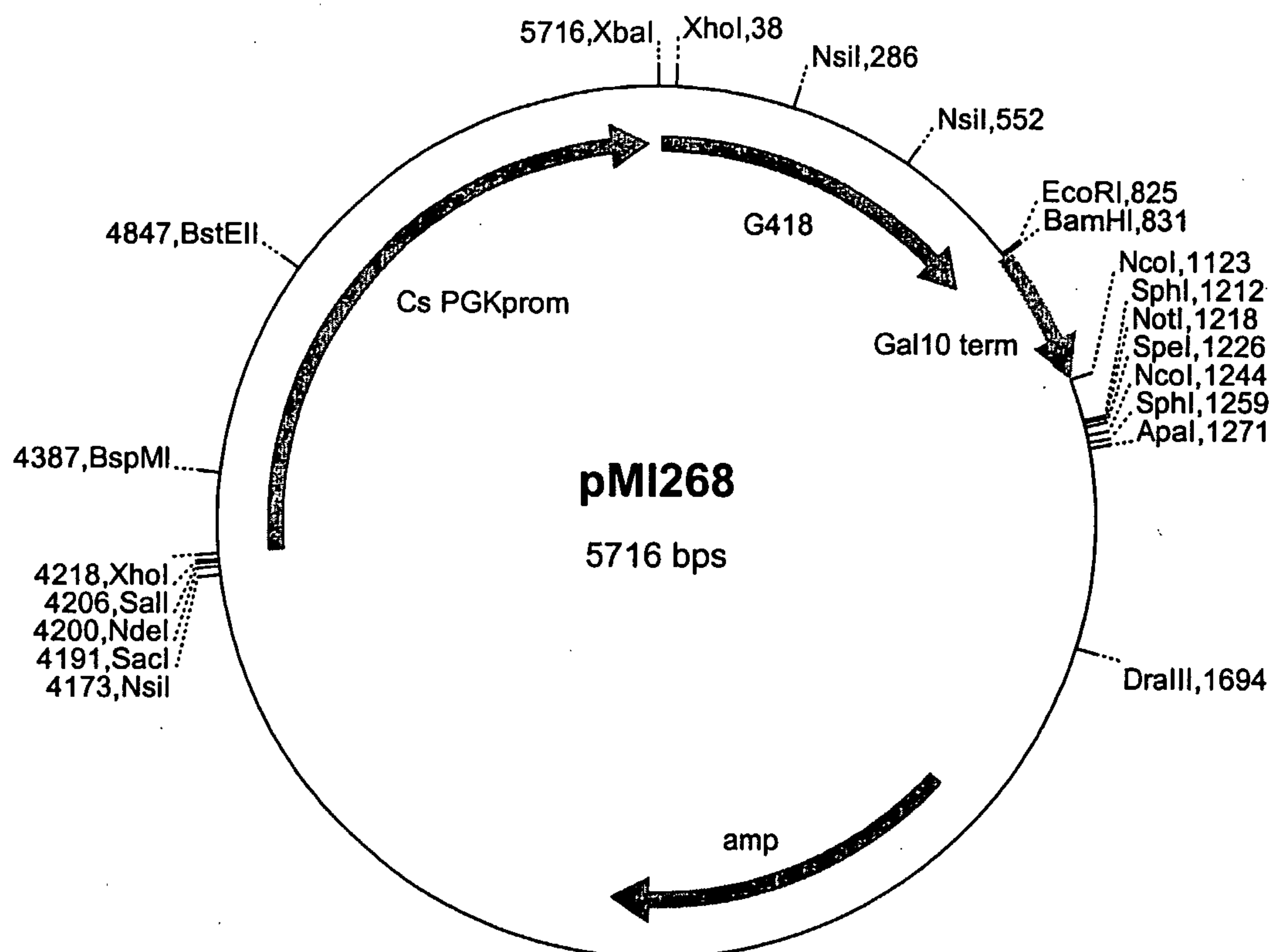
FIG. 2: G418 resistance vector pMI268.

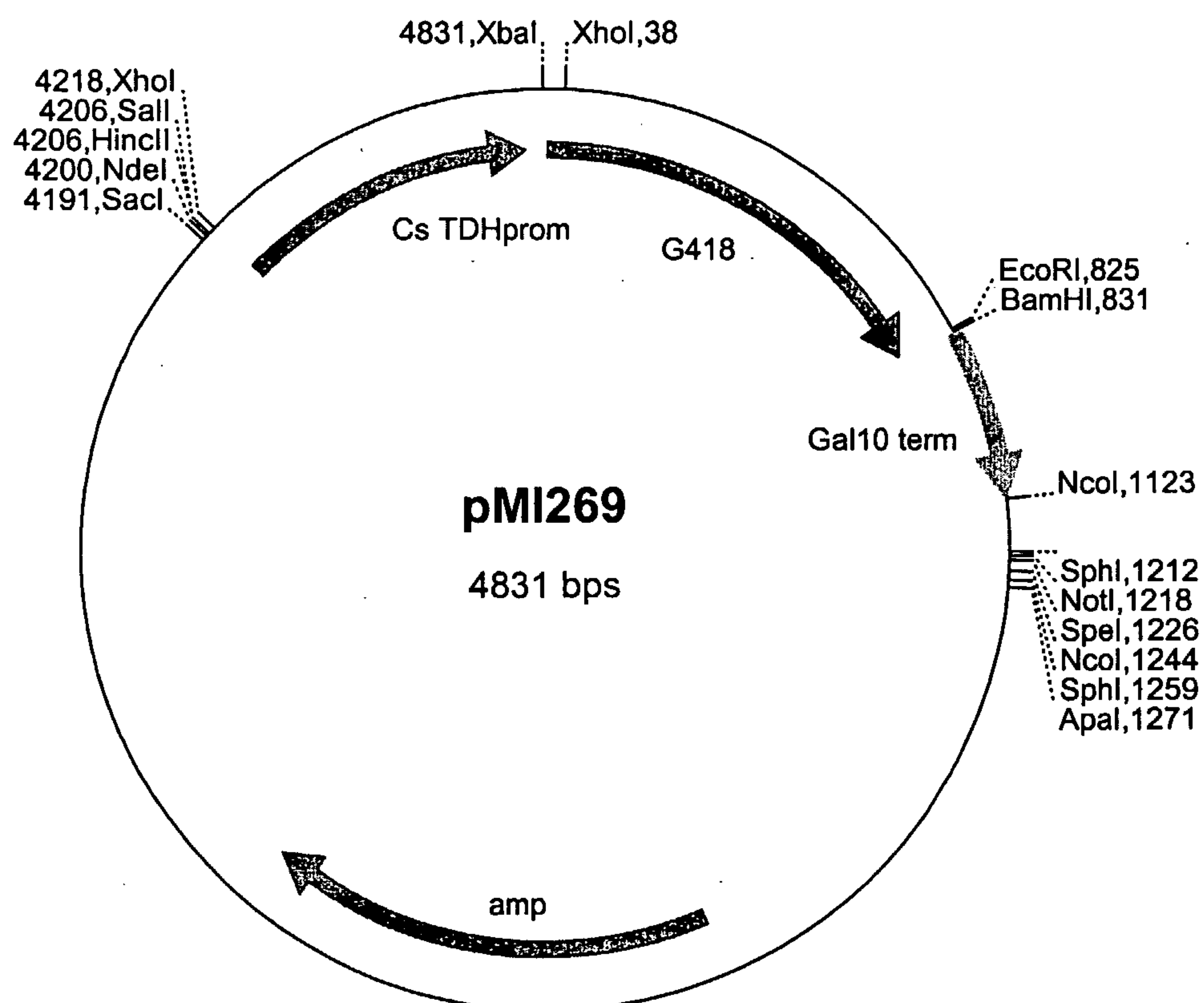
FIG. 3: G418 resistance vector pMI269.

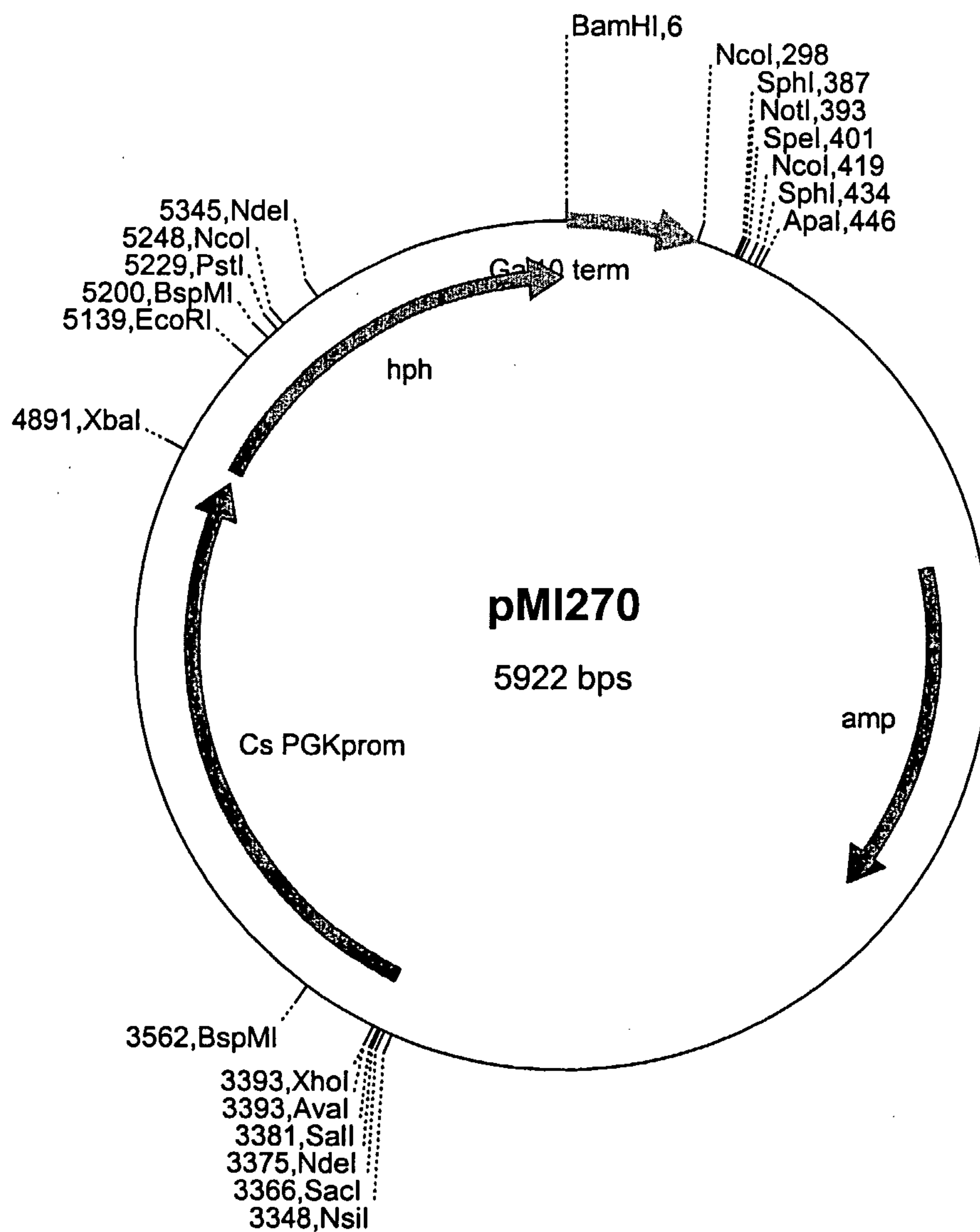
FIG. 4: Hygromycin resistance vector pMI270.

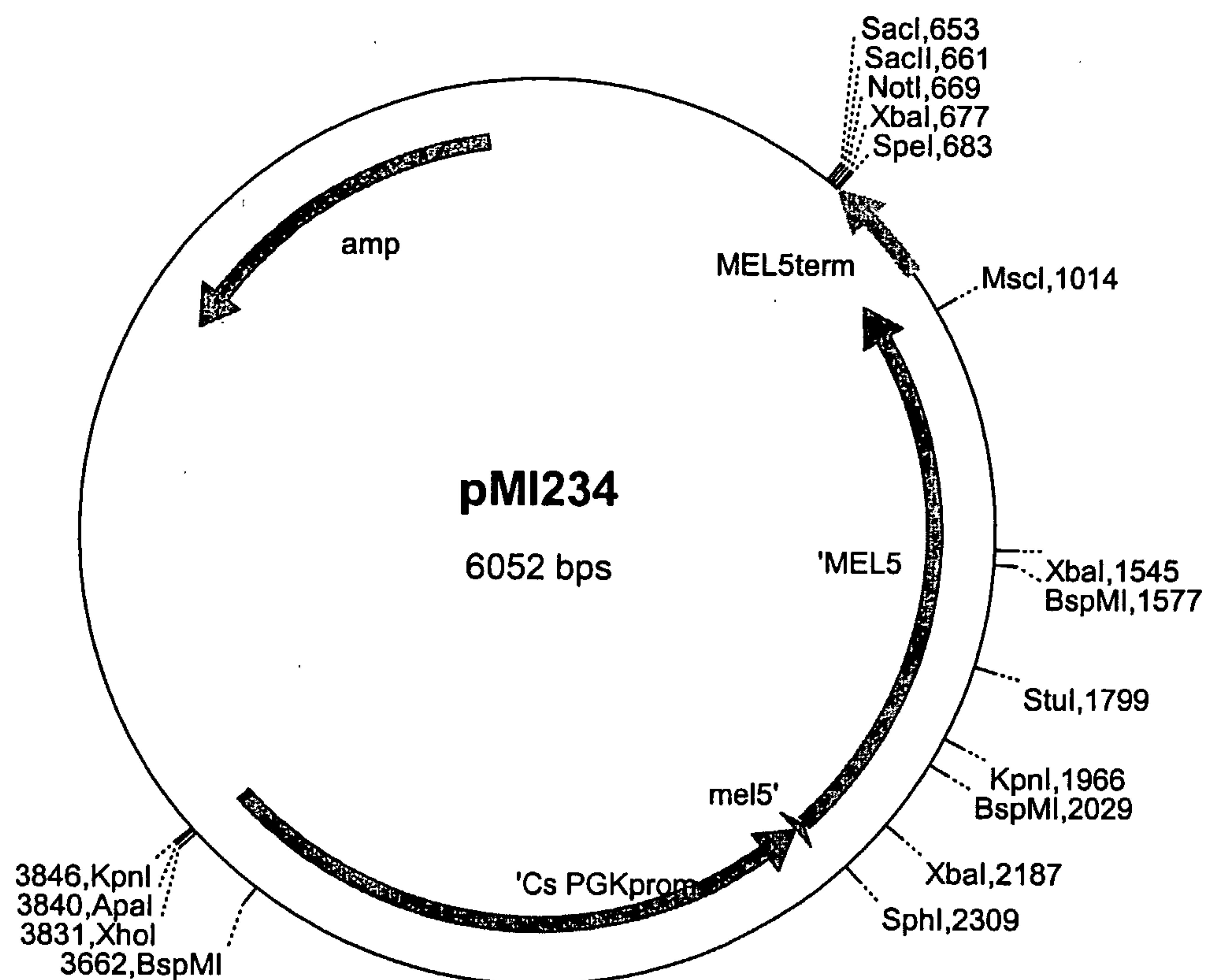
FIG. 5: Vector pMI234 for expression of *MEL5*.

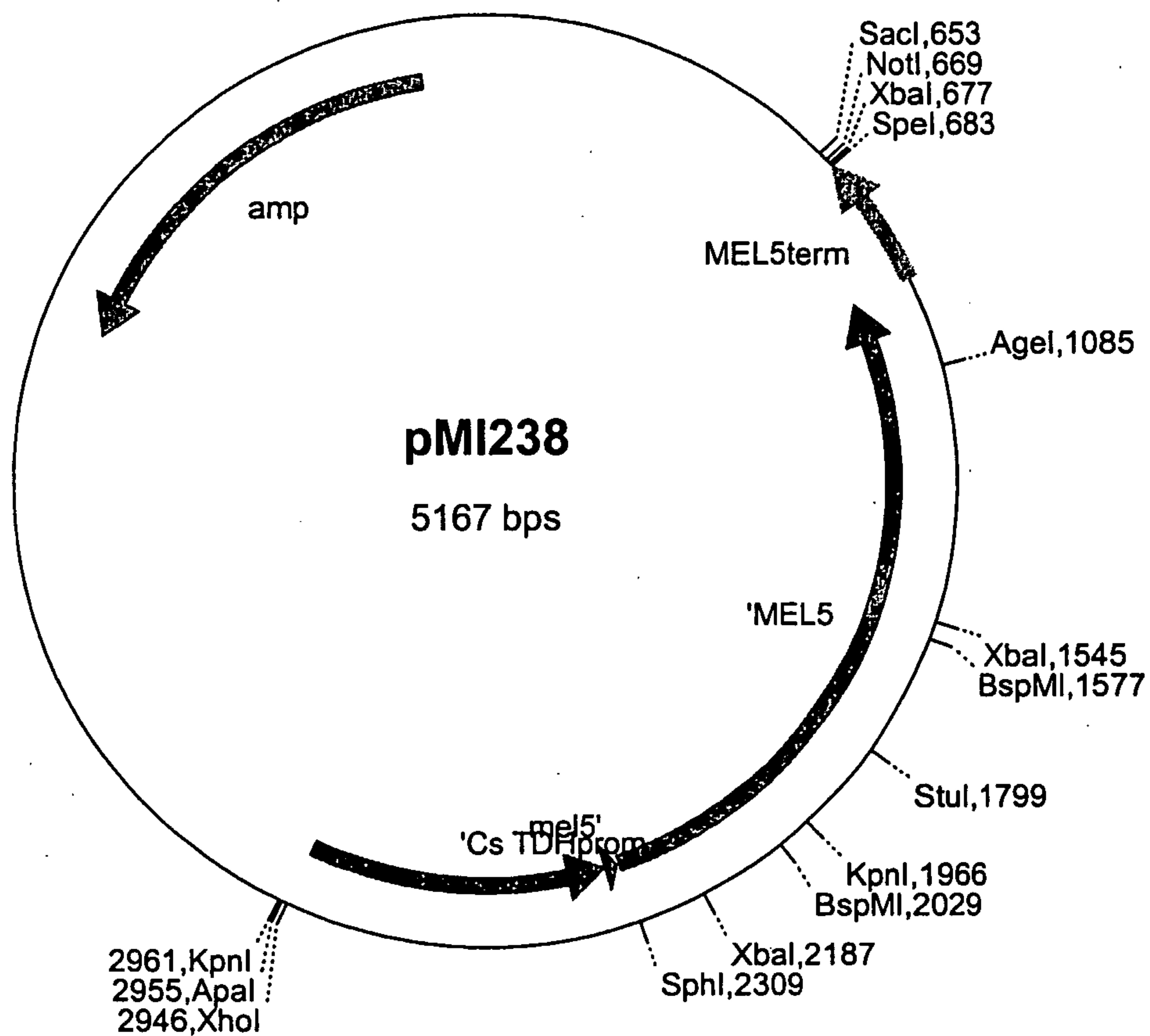
FIG. 6: Vector pMI238 for expression of *MEL5*.

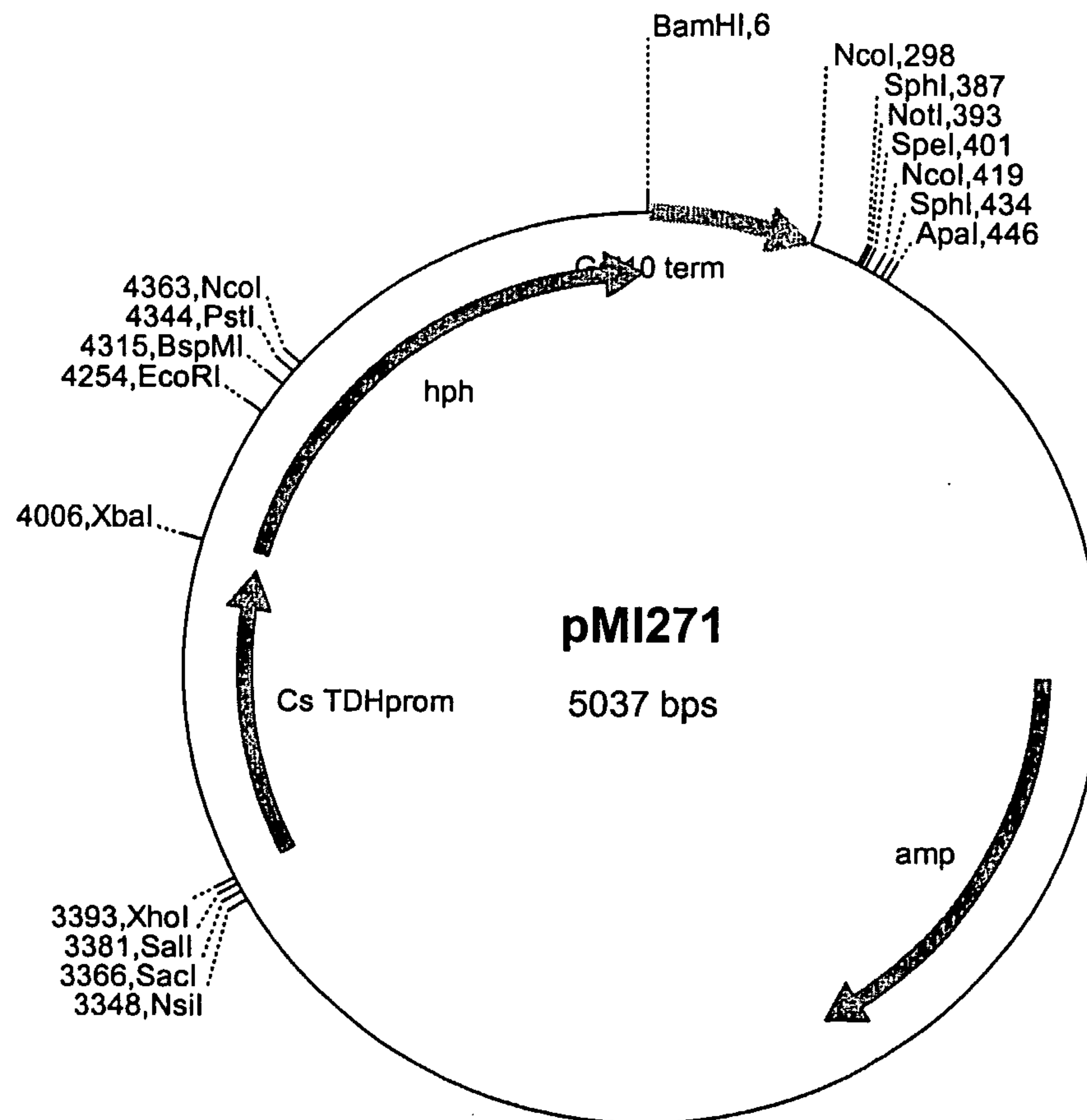
FIG. 7: Hygromycin resistance vector pMI271.

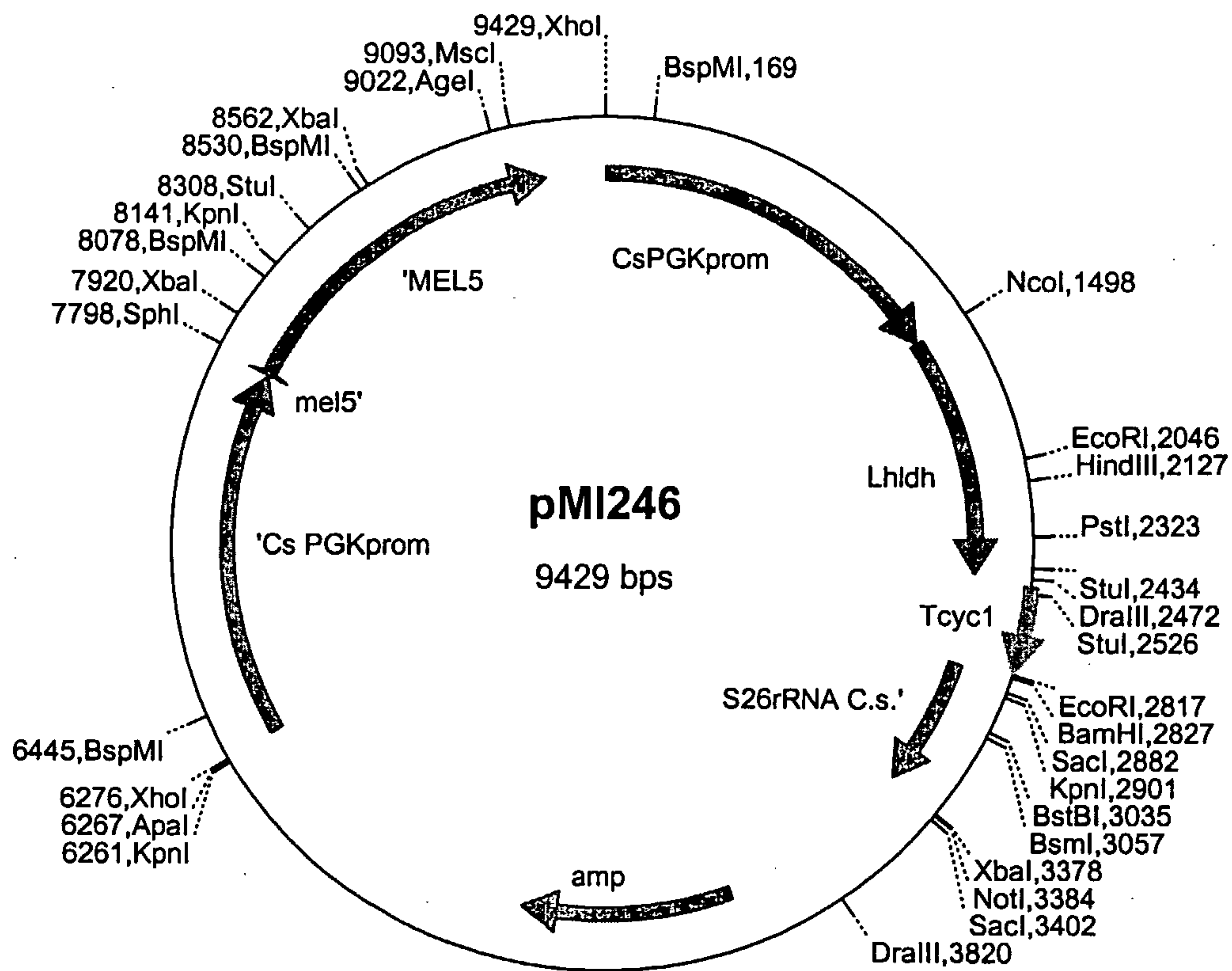
FIG. 8: Vector pMI246 for expression of *L. helveticus* LDH.

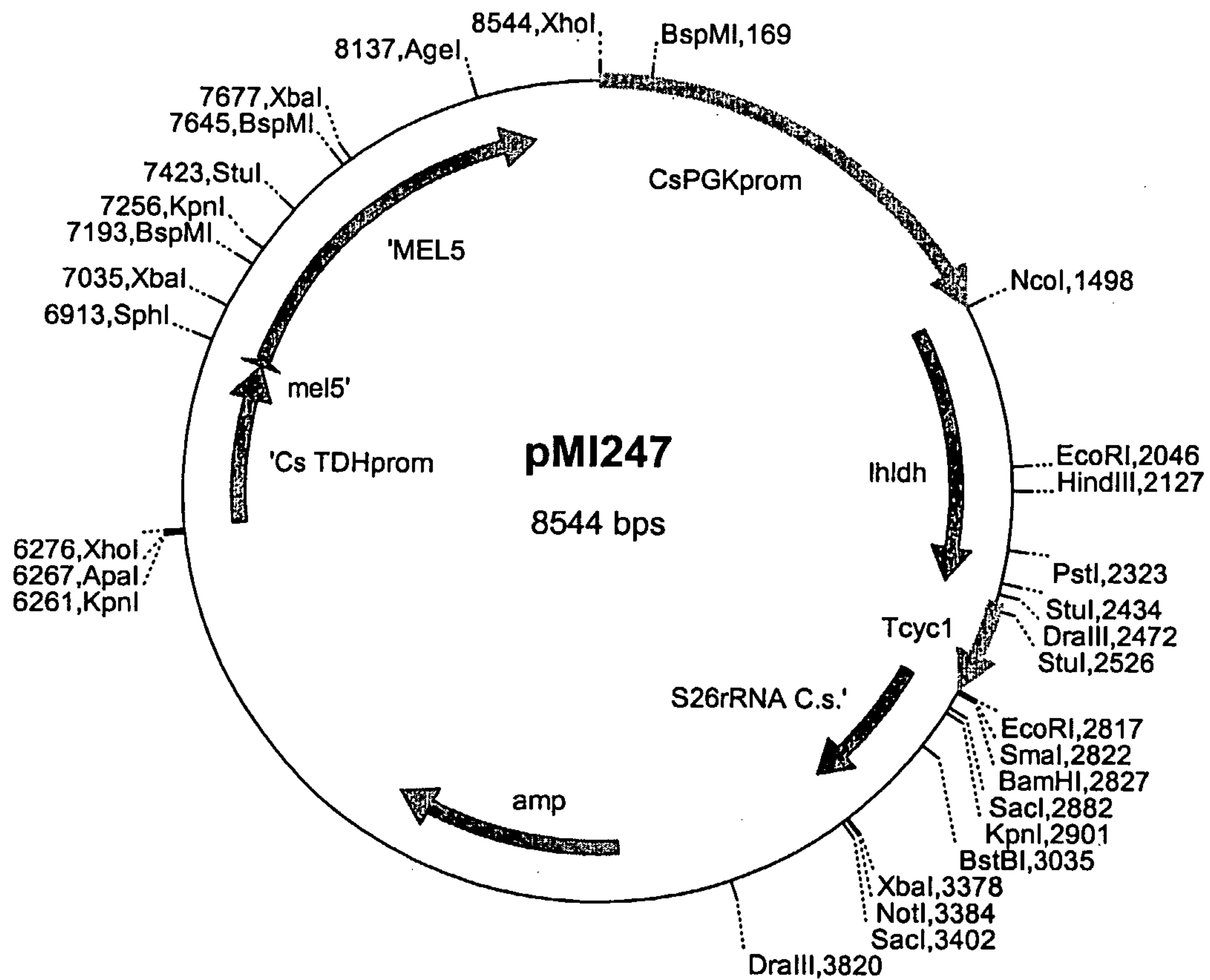
FIG. 9: Vector pMI247 for expression of *L. helveticus* LDH.

FIG. 10: Vector pMI257 for expression of *L. helveticus* LDH and for integration into the *PDC1* locus.

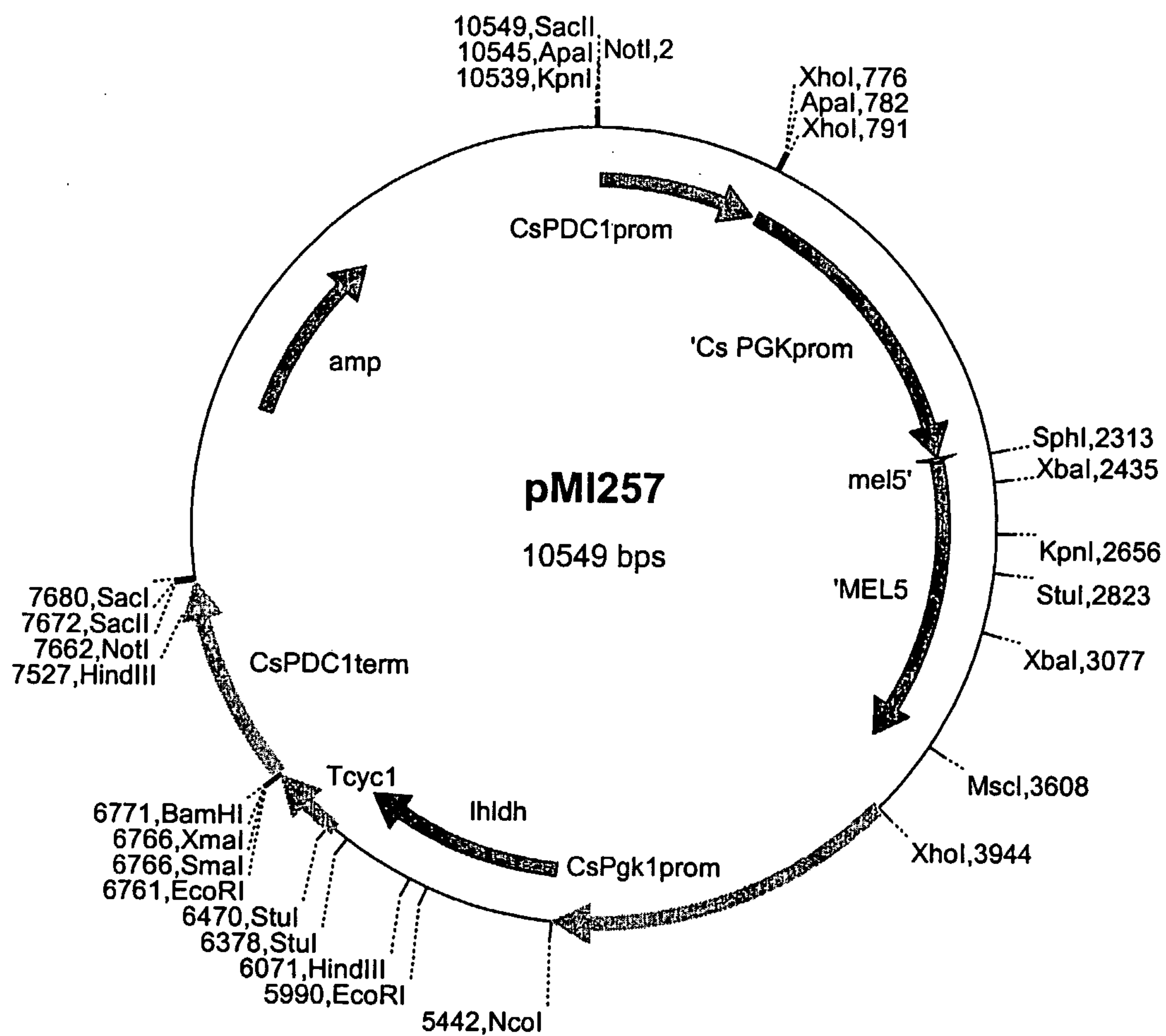


FIG. 11: Vector pMI265 for expression of *B. megaterium* LDH and for integration into the *PDC1* locus.

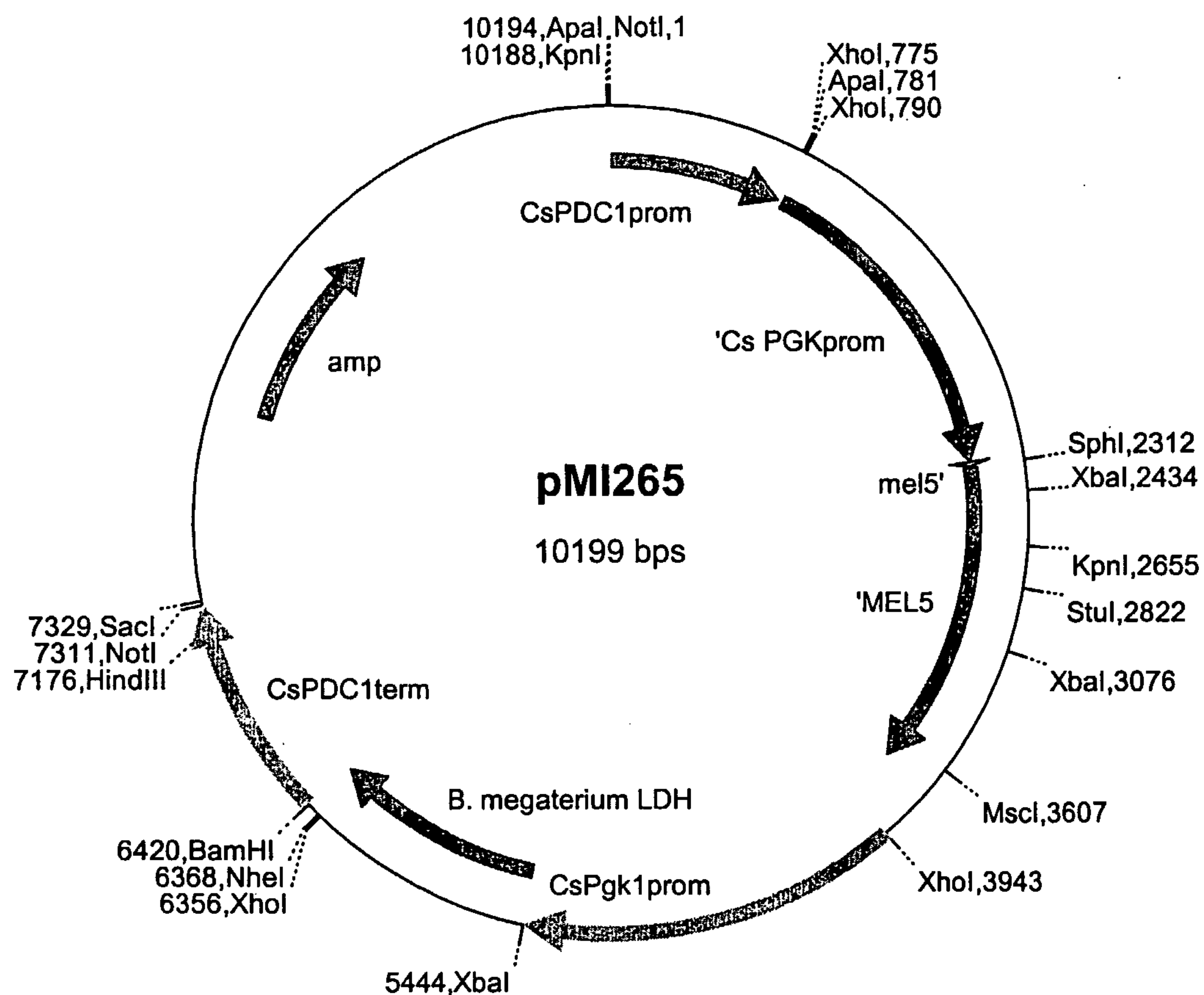


FIG. 12: Vector pMI266 for expression of *R. oryzae* LDH and for integration into the *PDC1* locus.

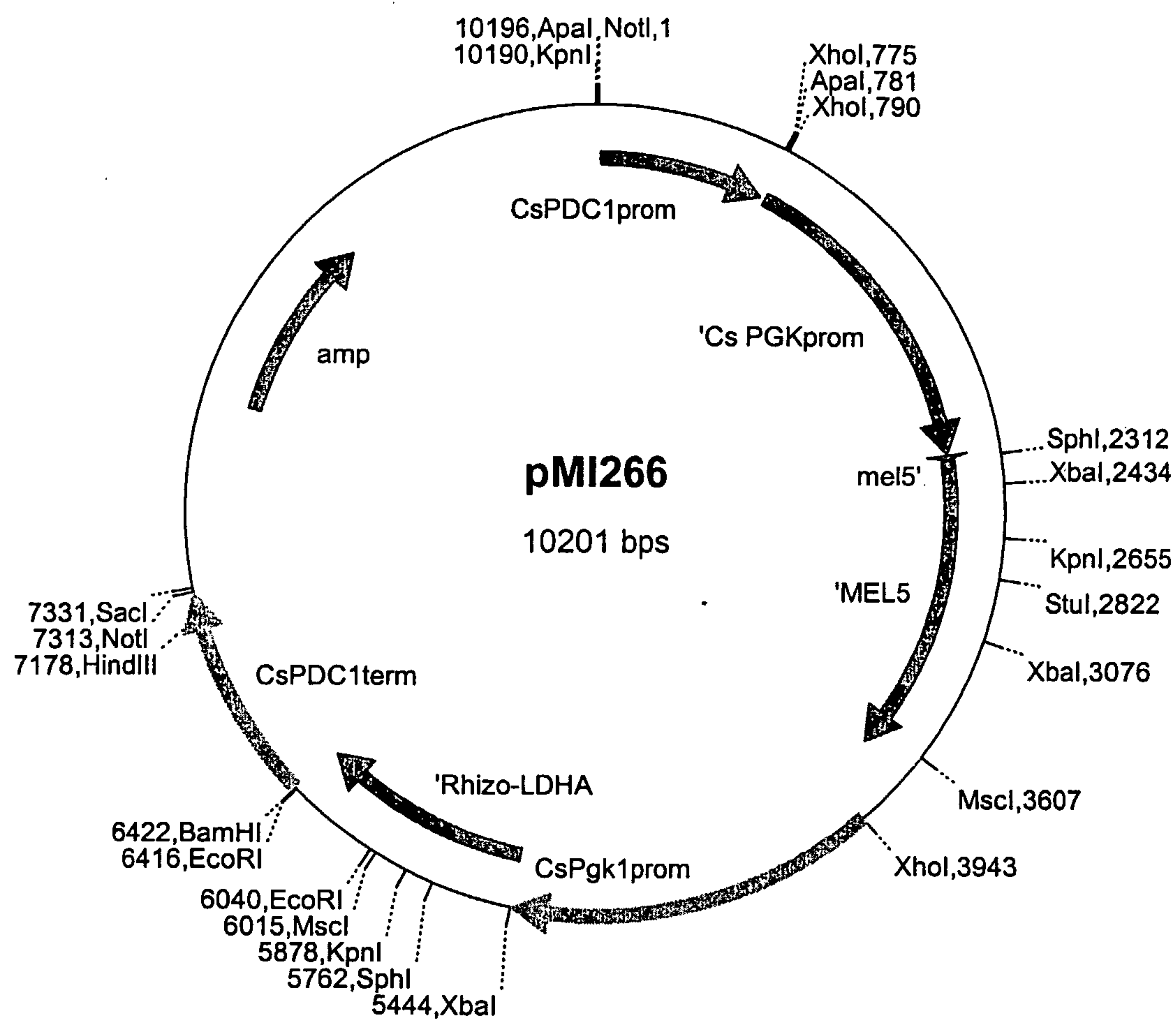


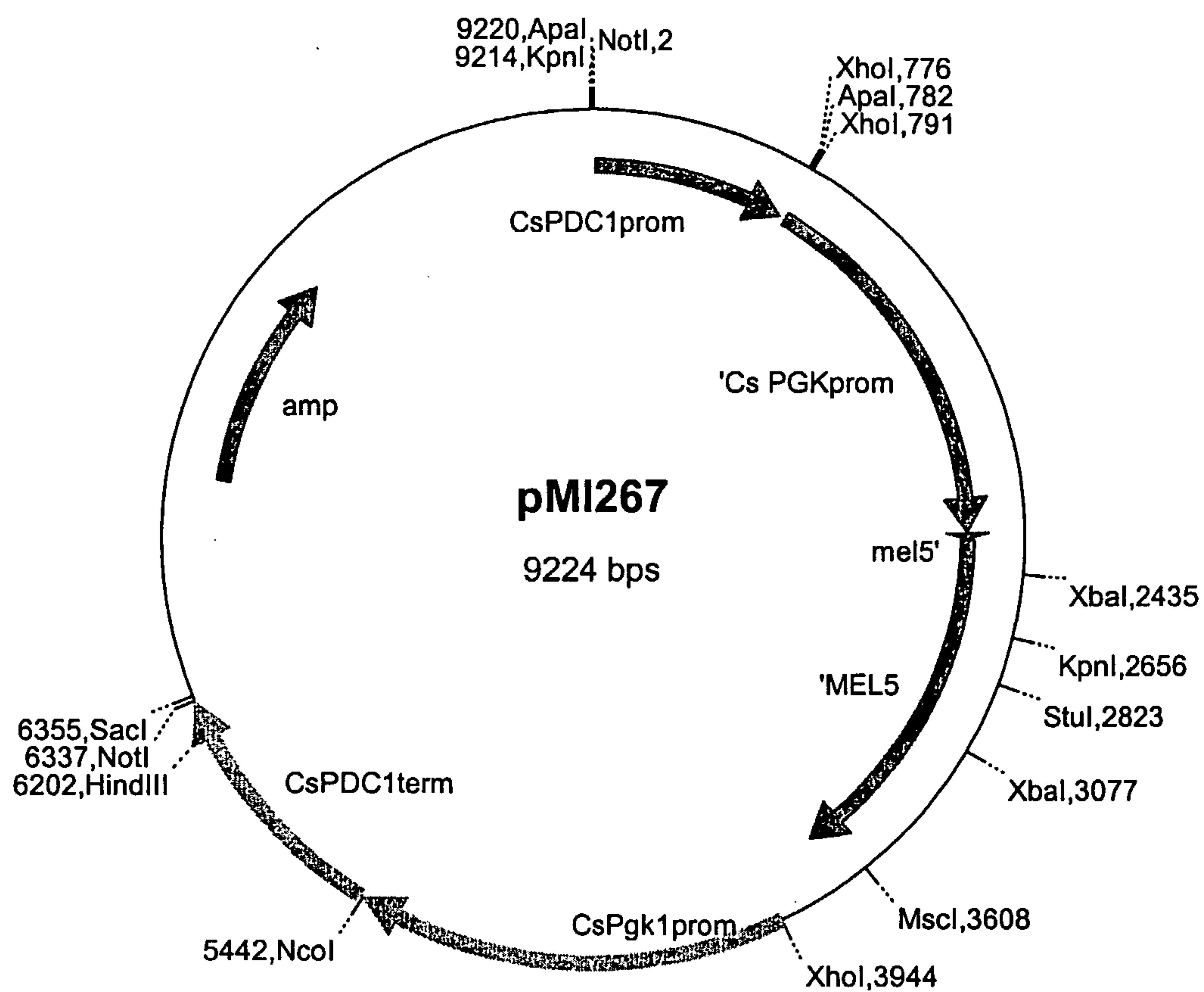
FIG. 13: Vector pMI267 for replacement of the *PDC1* locus.

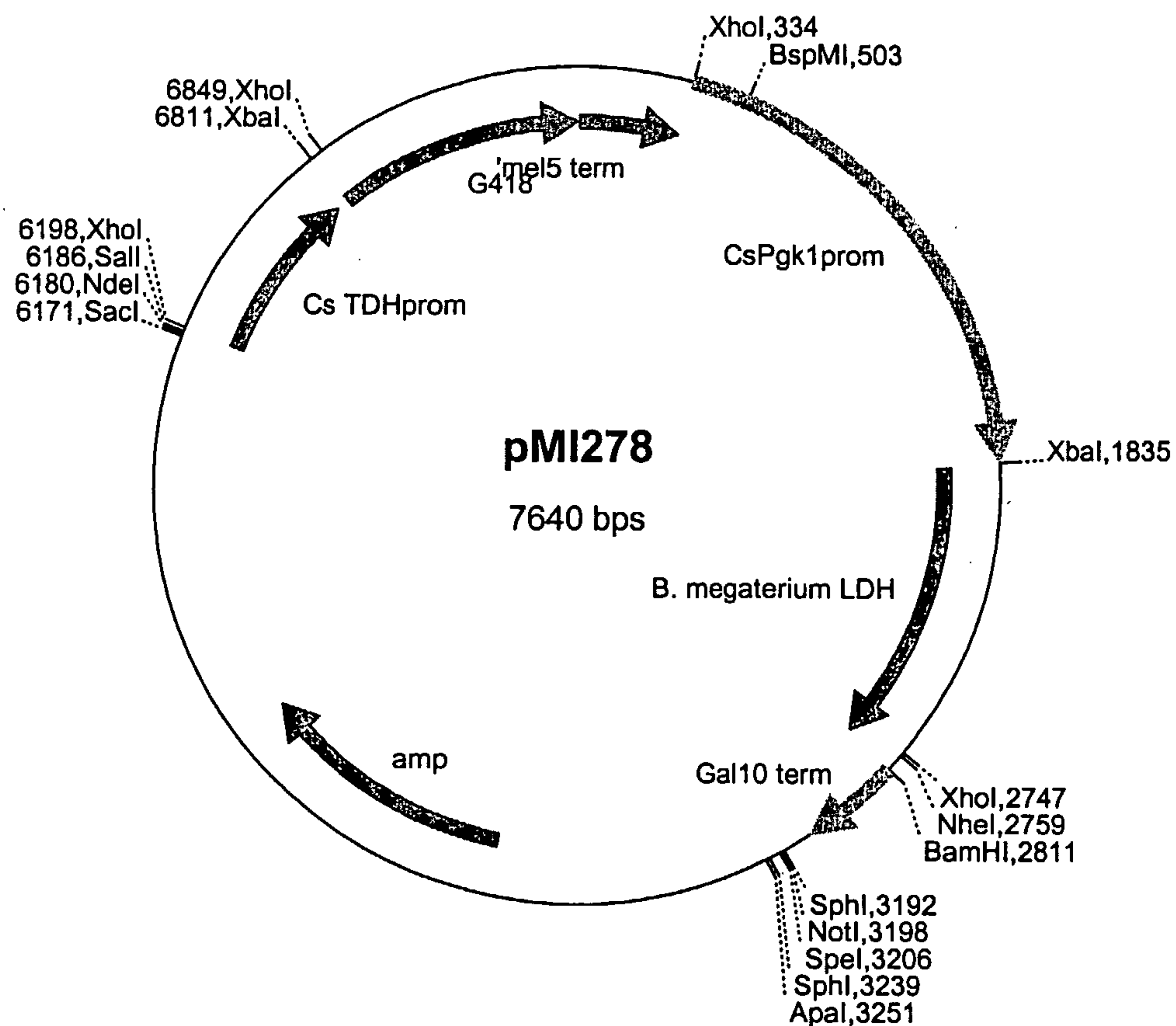
FIG. 14: Vector pMI278 for expression of *B. megaterium* LDH.

FIG. 15: Vector pMI286 for expression of *B. megaterium* LDH and for integration into the *PDC2* locus.

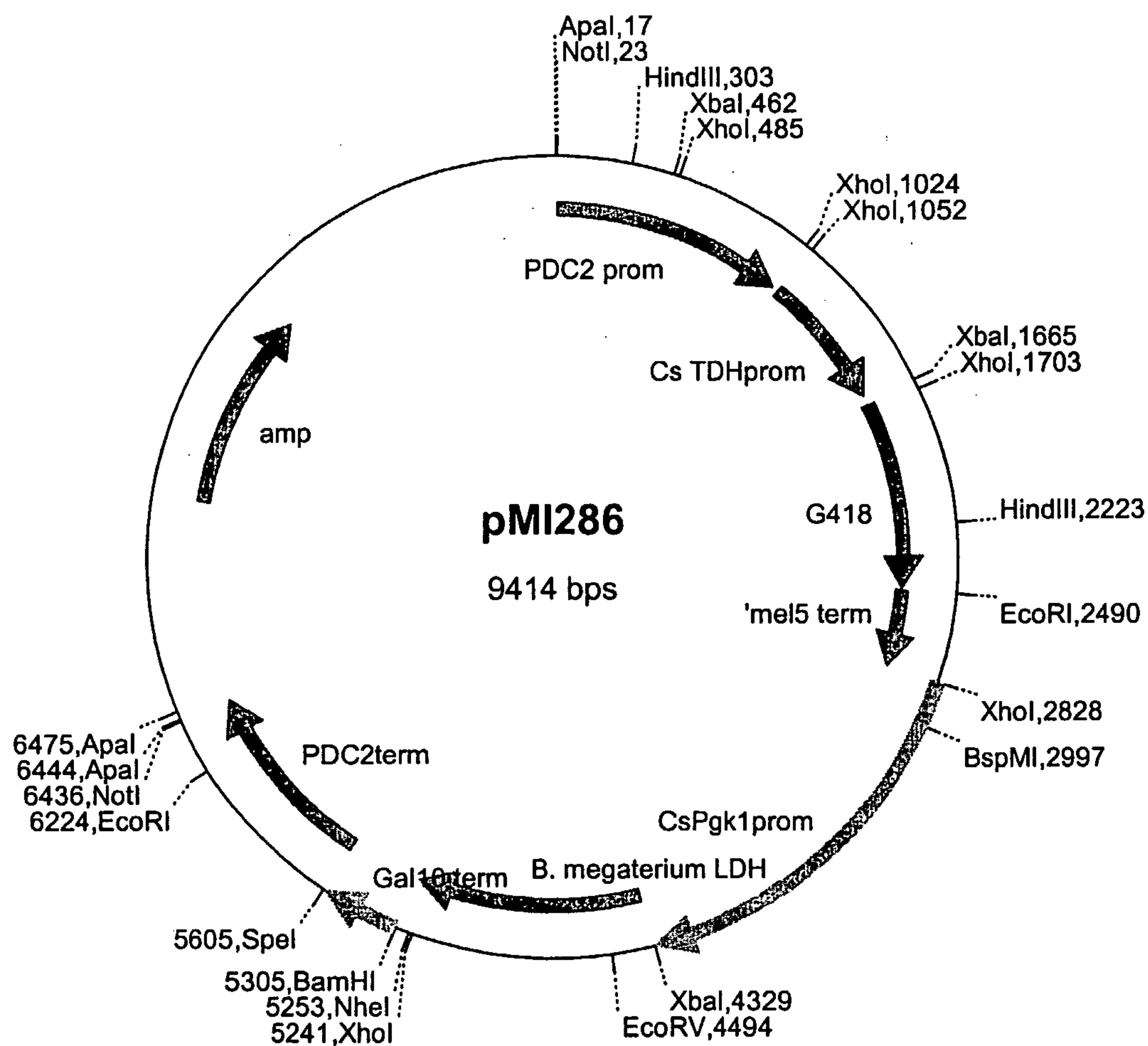


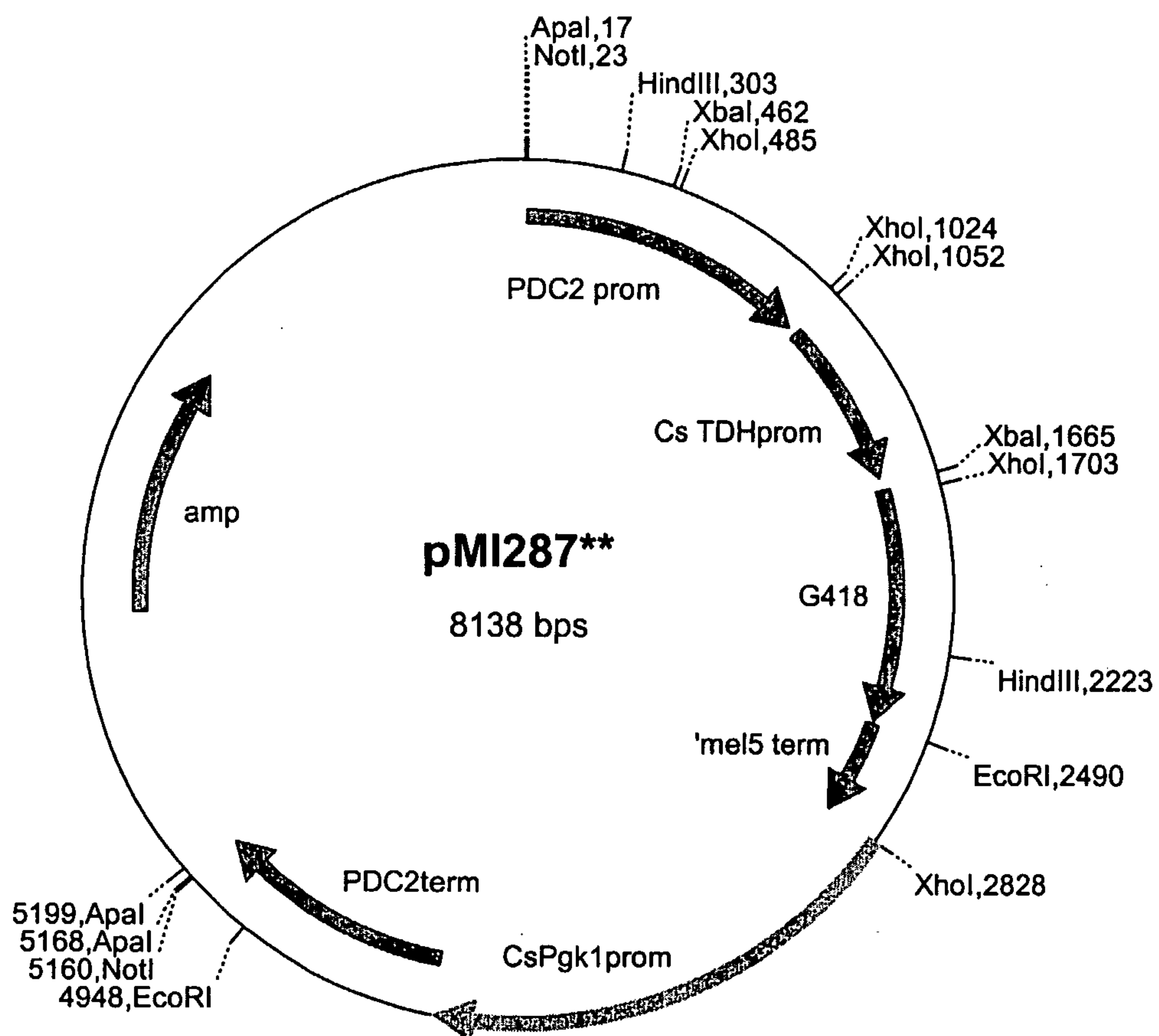
FIG. 16: Vector pMI287 for integration into the *PDC2* locus.

FIG. 17: Vector pMI288 for expression of *L. helveticus* LDH and for integration into the *PDC2* locus

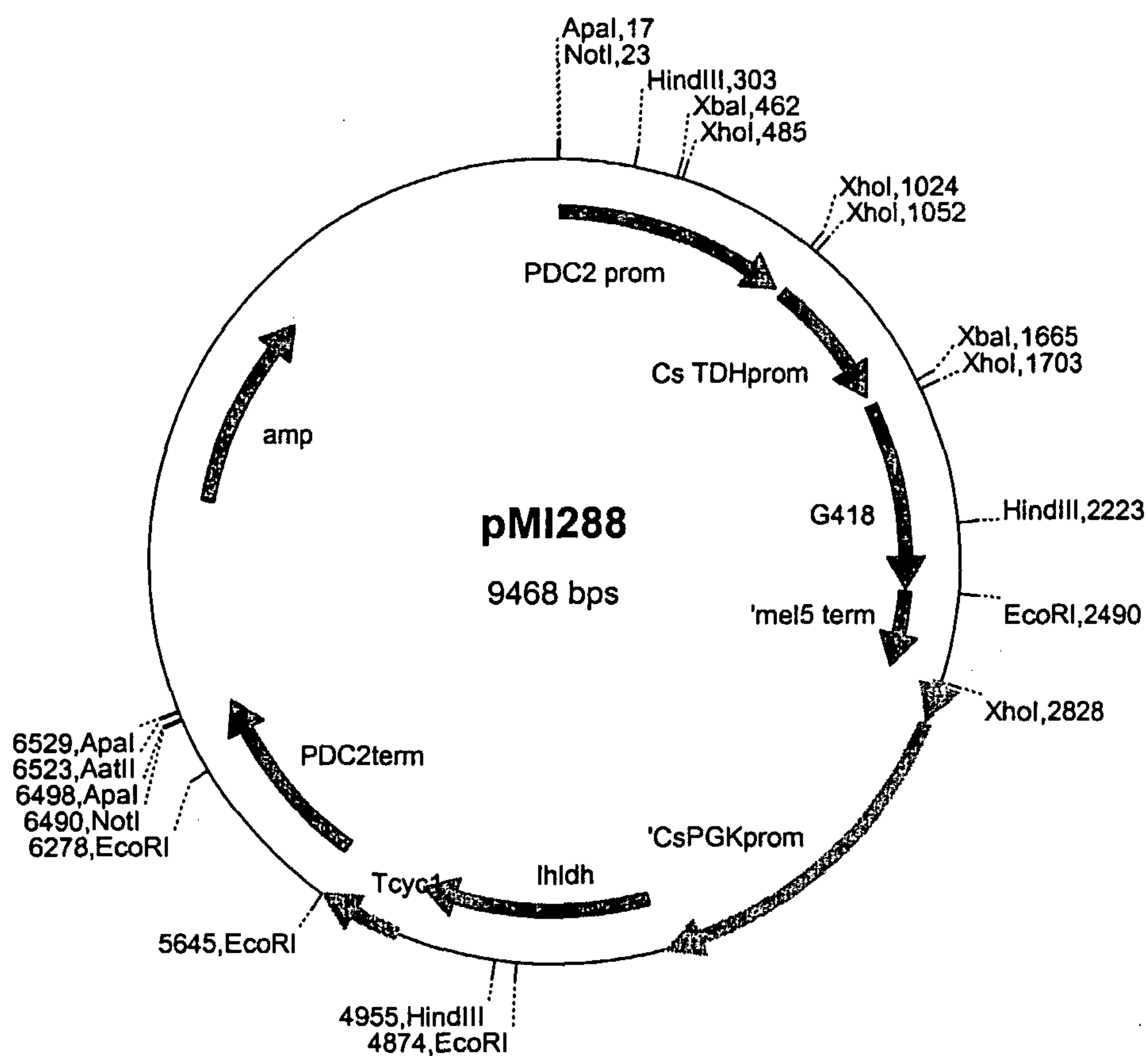


FIG. 18: Vector pMI256 for expression of *L. helveticus* LDH with *S. cerevisiae* MEL5 for integration into the *PDC1* locus

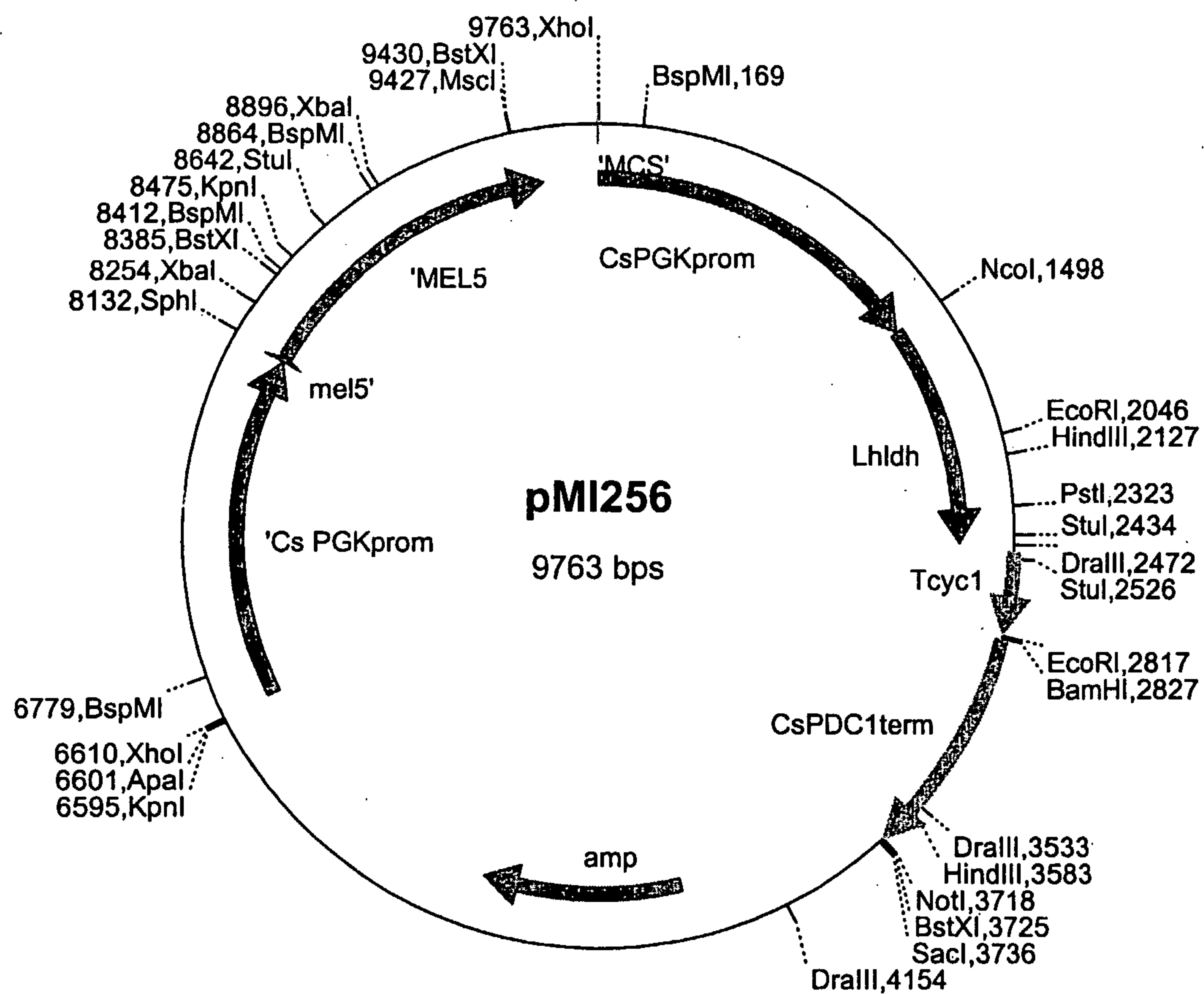


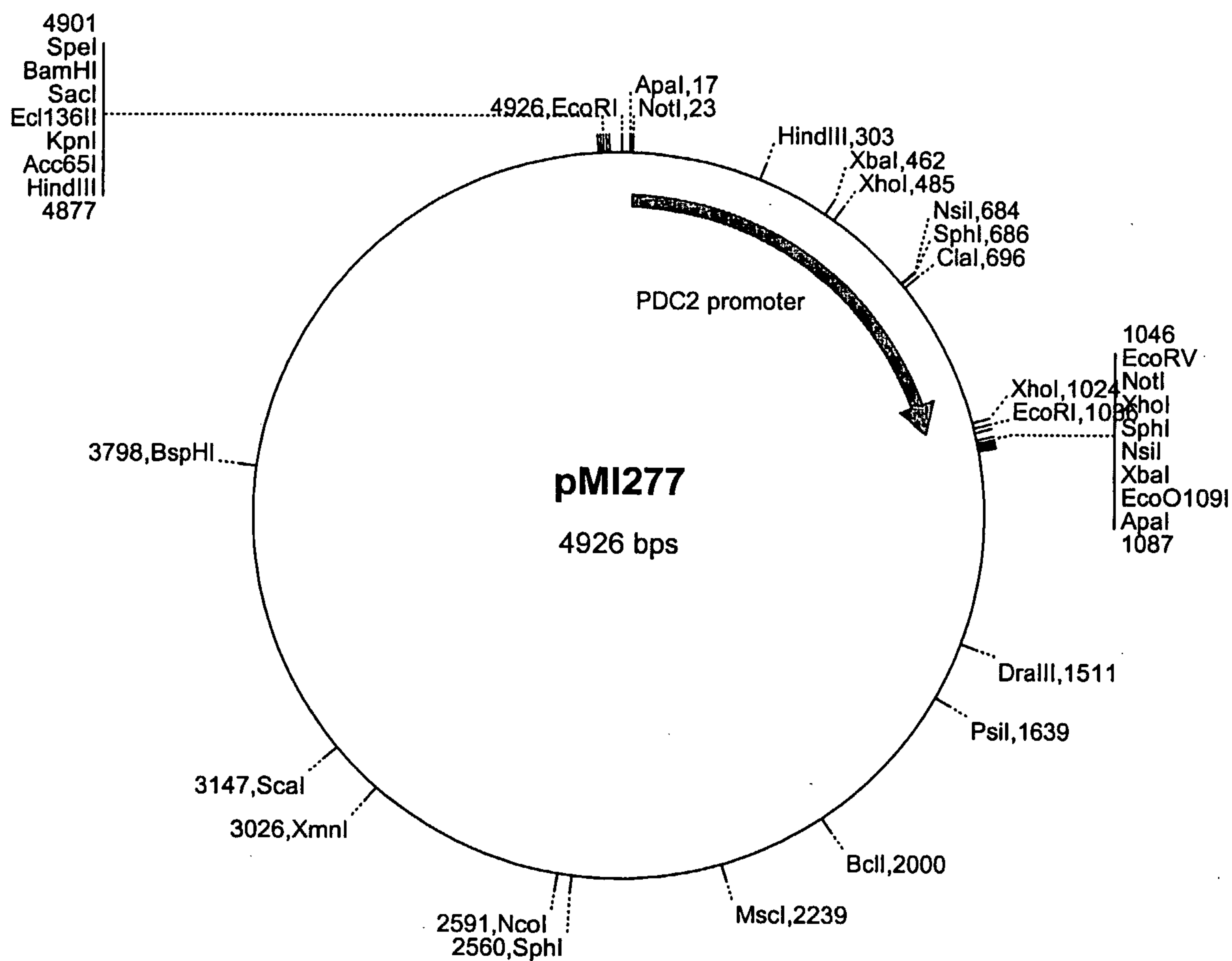
FIG. 19: Vector pMI277 containing the *PDC2* (*C. sonorensis*) promoter

FIG. 20: Vector pMI279 for expression of LDH (*B. megaterium*) containing the G418 resistance-coding gene for integration at the *PDC2* locus

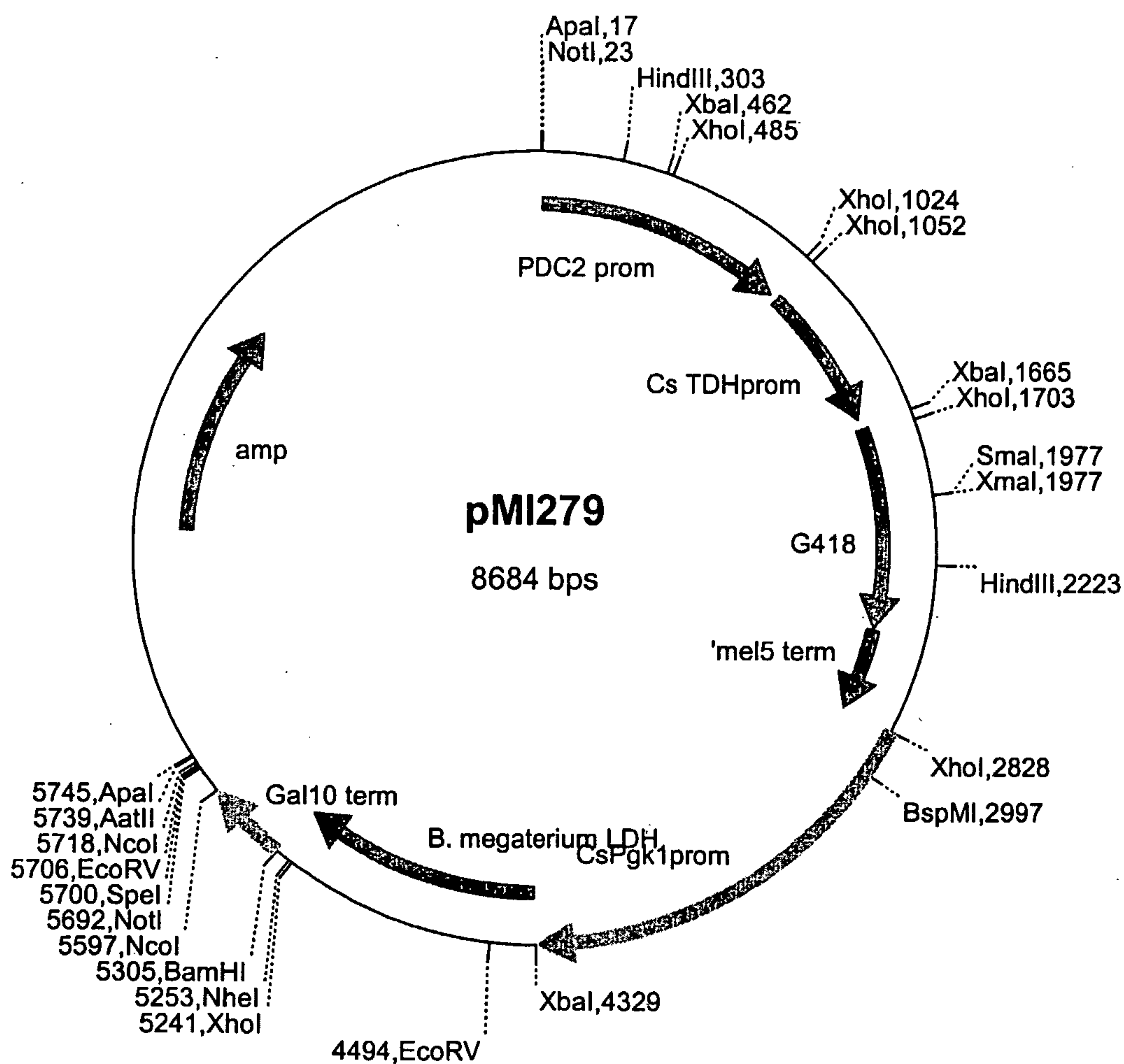


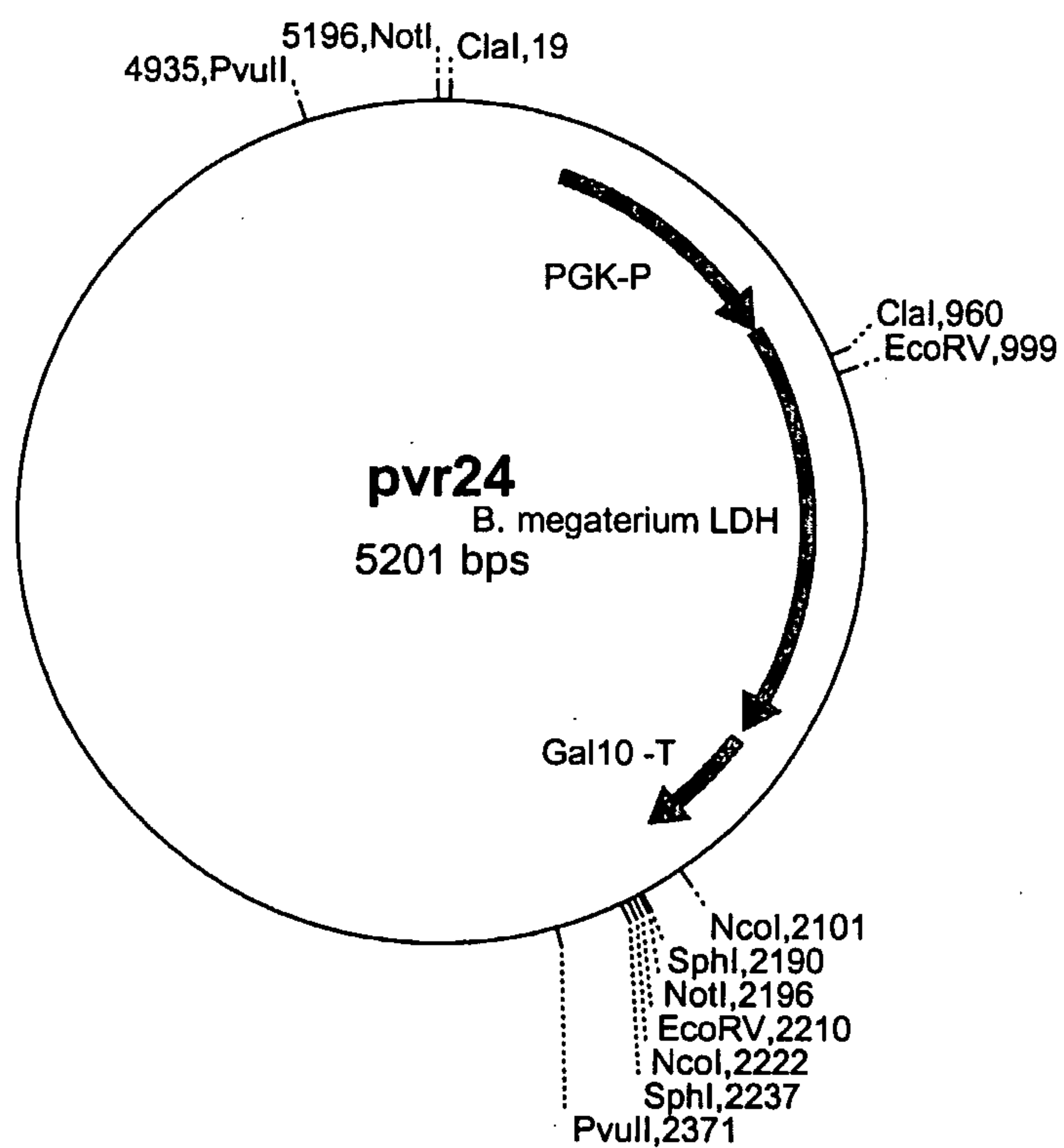
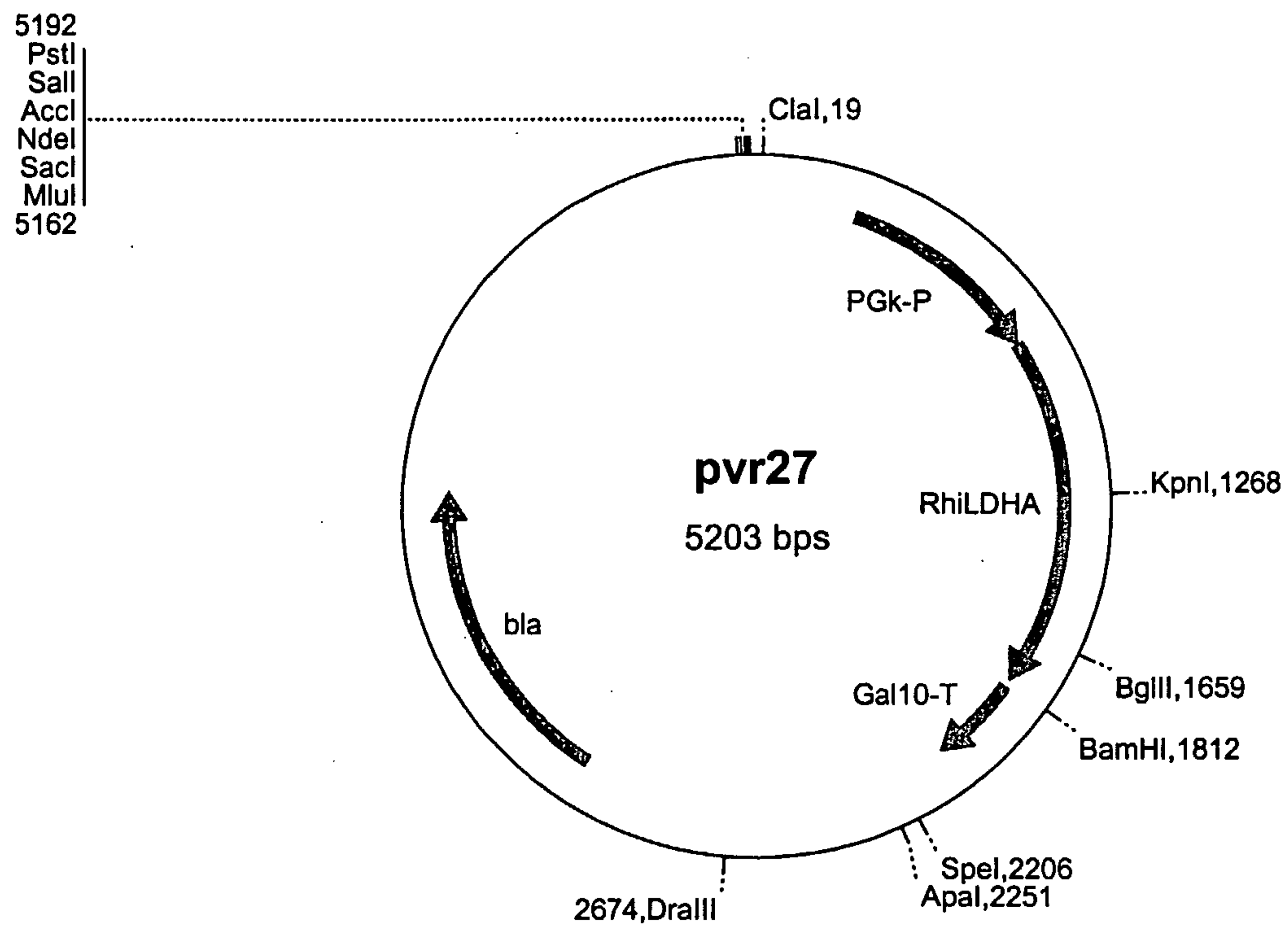
FIG. 21: Vector pVR24

FIG. 22: Vector pVR27

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SUBSTITUTE SHEET (RULE 26)



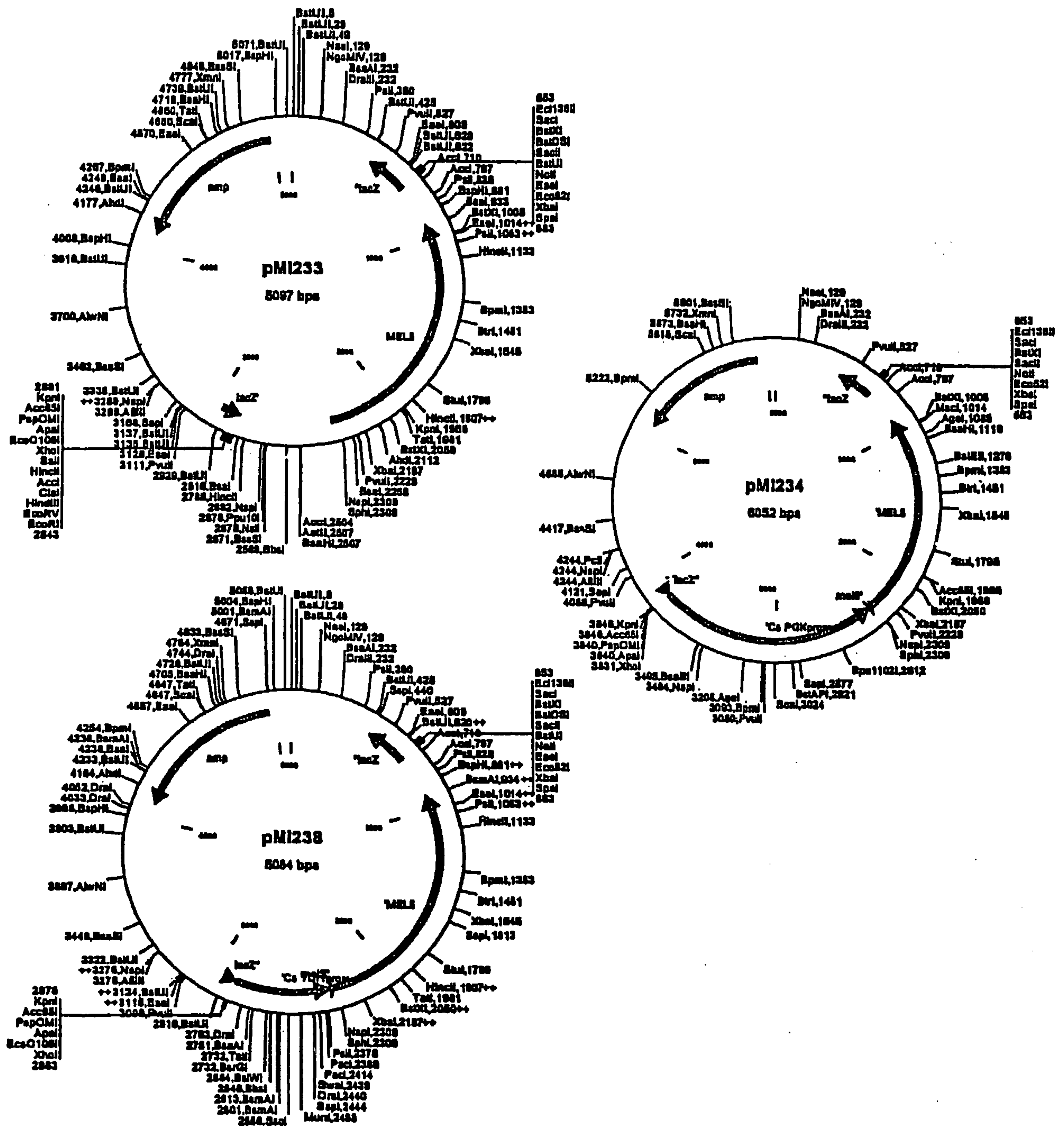


FIG. 23C