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(54) **Titre : MICROORGANISME MODIFIE PRESENTANT UN MEILLEUR COMPORTEMENT DE SEPARATION D'UNE BIOMASSE**
(54) **Title: MODIFIED MICROORGANISM WITH IMPROVED BIOMASS SEPARATION BEHAVIOUR**

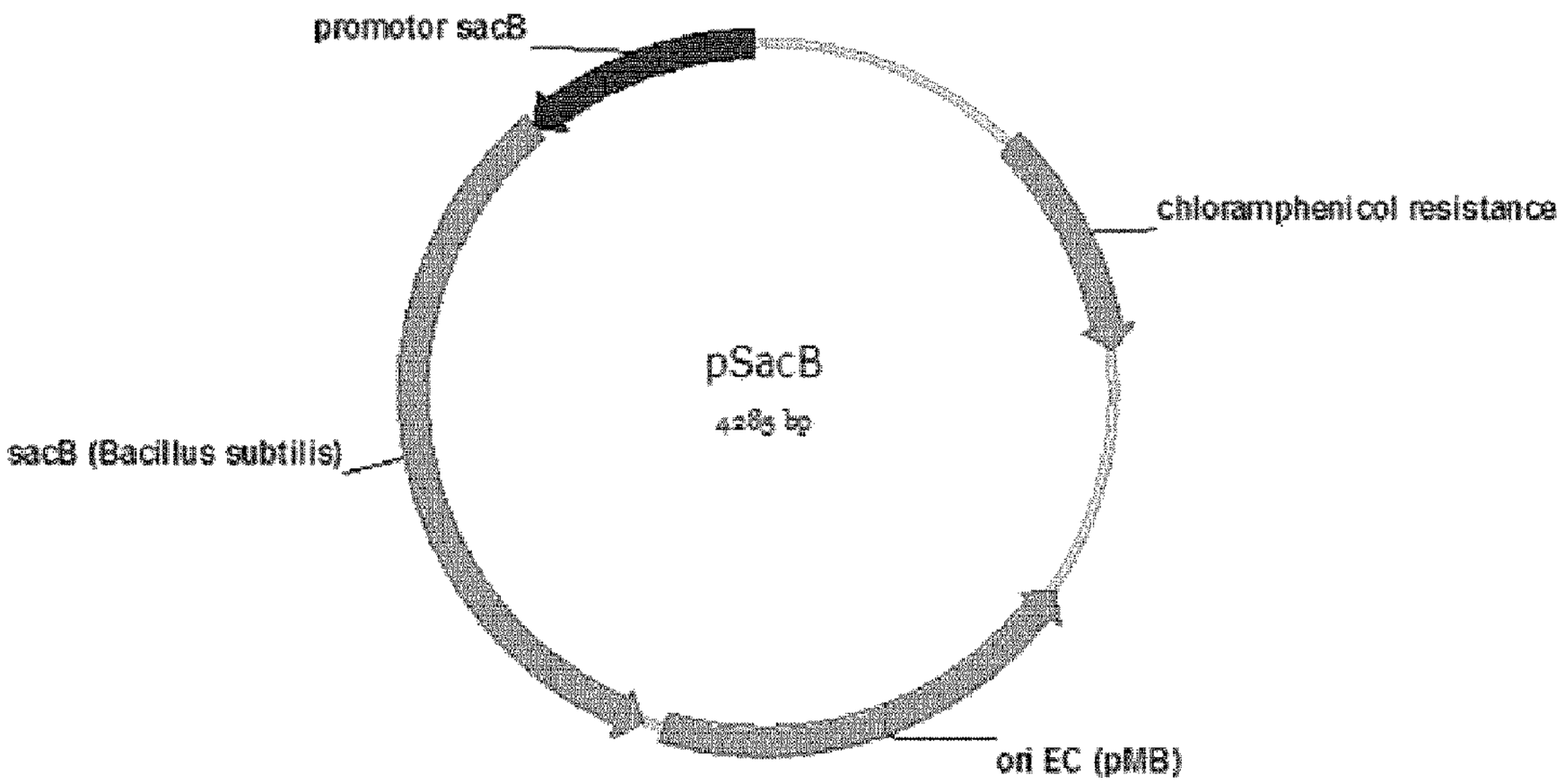

Fig. 1

(57) **Abrégé/Abstract:**
The present invention relates to a modified microorganism having, compared to its wild-type, a reduced activity of the enzyme that is encoded by the wcaJ-gene. The present invention also relates to a method for producing an organic compound and to the use of a modified microorganism.

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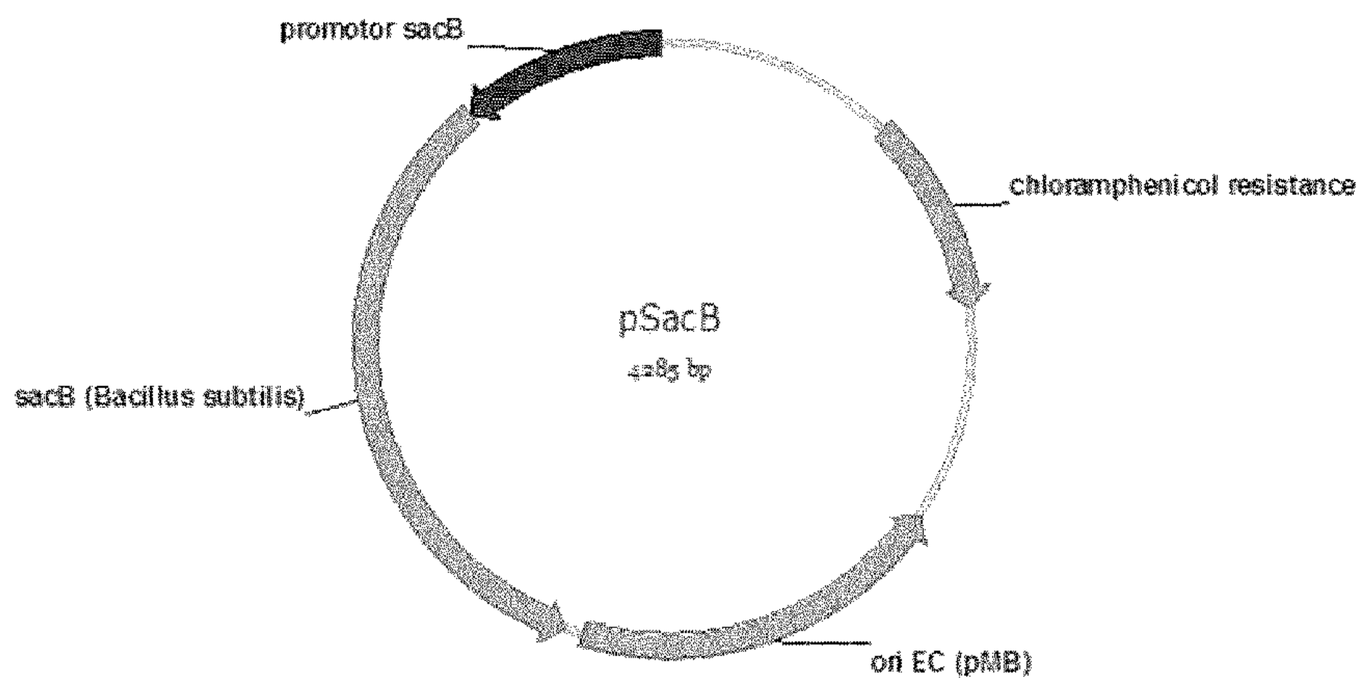

(57) **Abstract**: The present invention relates to a modified microorganism having, compared to its wild-type, a reduced activity of the enzyme that is encoded by the wcaJ-gene. The present invention also relates to a method for producing an organic compound and to the use of a modified microorganism.

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Modified microorganism with improved biomass separation behaviour

The present invention relates to a modified microorganism, to a method for producing an organic compound and to the use of a modified microorganism.

Organic compounds such as small dicarboxylic acids having 6 or fewer carbons are commercially significant chemicals with many uses. For example, the small diacids include 1,4-diacids, such as succinic acid, malic acid and tartaric acid, and the 5-carbon molecule itaconic acid. Other diacids include the two carbon oxalic acid, three carbon malonic acid, five carbon glutaric acid and the 6 carbon adipic acid and there are many derivatives of such diacids as well.

As a group the small diacids have some chemical similarity and their uses in polymer production can provide specialized properties to the resin. Such versatility enables them to fit into the downstream chemical infrastructure markets easily. For example, the 1,4-diacid molecules fulfill many of the uses of the large scale chemical maleic anhydride in that they are converted to a variety of industrial chemicals (tetrahydrofuran, butyrolactone, 1,4-butanediol, 2-pyrrolidone) and the succinate derivatives succindiamide, succinonitrile, diaminobutane and esters of succinate. Tartaric acid has a number of uses in the food, leather, metal and printing industries. Itaconic acid forms the starting material for production of 3-methylpyrrolidone, methyl-BDO, methyl-THF and others.

In particular, succinic acid or succinate – these terms are used interchangeably herein – has drawn considerable interest because it has been used as a precursor of many industrially important chemicals in the food, chemical and pharmaceutical industries. In fact, a report from the U.S. Department of Energy reports that succinic acid is one of 12 top chemical building blocks manufactured from biomass. Thus, the ability to make diacids in bacteria would be of significant commercial importance.

WO-A-2009/024294 discloses a succinic acid producing bacterial strain, being a member of the family of *Pasteurellaceae*, originally isolated from rumen, and capable of utilizing glycerol as a carbon source and variant and mutant strains derived there from retaining said capability, in particular, a bacterial strain designated DD1 as deposited with DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstr. 7B, D-38124 Braunschweig, Germany) having the deposit number DSM 18541 (ID 06-614) and having the ability to produce succinic acid. The DD1-strain belongs to the species *Basfia succiniciproducens* and the family of *Pasteurellaceae* as classified by Kuhnert et *al.*, 2010. Mutations of these strains, in which the *ldhA*-gene and/or the *pflD*- or the *pflA*-gene have been disrupted, are disclosed in WO-A-2010/092155, these mutant strains being characterized by a significantly increased production of succinic acid from carbon sources such as glycerol or mixtures of glycerol and carbohydrates such as maltose, under anaerobic conditions compared to the DD1-wild-type disclosed in WO-A-2009/024294.

However, when using bacterial strains such as those disclosed in WO-A-2009/024294 or WO-A-2010/092155 for the production or organic compounds such as succinic acid, the selectivity in which the carbon sources are converted into the desired organic compounds and also the yield of the desired organic compound is still improvable.

Furthermore, it has been observed that when using the bacterial strains of the prior art it is sometimes difficult to separate the biomass from the fermentation broth at the end of the fermentation process. Usually, the fermentative production of organic compounds such as succinic acid comprises the steps of cultivating the microorganisms under suitable culture conditions in a medium comprising at least one assimilable carbon source to allow the microorganism to produce the desired organic compound and the subsequent recovery of the organic compound from the fermentation broth, wherein in a first step of the recovery process the microorganisms (i. e. the biomass) are usually separated from the culture medium by, for example, sedimentation or centrifugation. When using the microorganisms of the prior art, the biomass can not be easily separated from the fermentation broth, which – as a part of the fermentation broth is somehow entrapped in the biomass – sometimes leads to a loss of a certain amount of the fermentation broth (and thus also a loss of a certain amount of the desired organic compound).

It was therefore an object of the present invention to overcome the disadvantages of the prior art.

In particular, it was an object of the present invention to provide microorganisms which can be used for the fermentative production of organic compounds such as succinic acid and which not only produce the desired organic products, such as succinic acid, from assimilable carbon sources such as glycerol, glucose, sucrose, xylose, lactose, fructose or maltose in large amounts, preferably with only low amounts of side products, but which can also easily be separated from the fermentation broth in the subsequent process of recovering the organic compound, with only a minor amount of the fermentation broth being entrapped in the biomass.

A contribution to achieving the abovementioned aims is provided by a modified microorganism having, compared to its wild-type, a reduced activity of the enzyme that is encoded by the *wcaJ*-gene. A contribution to achieving the abovementioned aims is in particular provided by a modified microorganism which the *wcaJ*-gene or parts thereof have been deleted or in which a regulatory element of the *wcaJ*-gene or at least a part thereof has been deleted or in which at least one mutation has been introduced into the *wcaJ*-gene.

Surprisingly, it has been discovered that a reduction of the activity of the enzyme that is encoded by the *wcaJ*-gene (this enzyme presumably being a glucose transferase), for example by a deletion of the *wcaJ*-gene or parts thereof, results in a microorganism that, after fermentative production of organic compounds such as succinic acid in an appropriate culture medium, can be separated – as the biomass – from the culture medium more easily compared to the corresponding microorganism in which the activity of this enzyme has not been decreased. When using the modified microorganism according to the present invention, less fermentation broth in

entrapped in the biomass that is separated in the purification process, which means that a higher amount of fermentation broth per gram of biomass used in the fermentation process (and thus a higher amount of the desired organic compound such as succinic acid) can be obtained from which the organic compound is isolated.

In context with the expression "*a modified microorganism having, compared to its wild-type, a reduced activity of the enzyme that is encoded by the x-gene*", wherein the *x*-gene is the *fruA*-gene and optionally, as described later, the *ldhA*-gene, the *pflA*-gene and/or the *pflD*-gene, the term "*wild-type*" refers to a microorganism in which the activity of the enzyme that is encoded by the *x*-gene has not been decreased, i. e. to a microorganism whose genome is present in a state as before the introduction of a genetic modification of the *x*-gene. Preferably, the expression "*wild-type*" refers to a microorganism (e.g., bacteria, yeast cell, fungal cell, etc.) whose genome, in particular whose x-gene, is present in a state as generated naturally as the result of evolution. The term is used both for the entire microorganism and for individual genes. As a consequence, the term "*wild-type*" preferably does not cover in particular those microorganisms, or those genes, whose gene sequences have at least in part been modified by man by means of recombinant methods. The term "*modified microorganism*" thus includes a microorganism which has been genetically altered, modified or engineered (e.g., genetically engineered) such that it exhibits an altered, modified or different genotype and/or phenotype (e. g., when the genetic modification affects coding nucleic acid sequences of the microorganism) as compared to the naturally-occurring wild-type microorganism from which it was derived. According to a particular preferred embodiment of the modified microorganism according to the present invention the modified microorganism is a recombinant microorganism, which means that the microorganism has been obtained using recombinant DNA. The expression "*recombinant DNA*" as used herein refers to DNA sequences that result from the use of laboratory methods (molecular cloning) to bring together genetic material from multiple sources, creating sequences that would not otherwise be found in biological organisms. An example of such a recombinant DNA is a plasmid into which a heterologous DNA-sequence has been inserted.

The wild-type from which the microorganisms according to the present invention are derived may yeasts, fungi or bacteria. Suitable bacteria, yeasts or fungi are in particular those bacteria, yeasts or fungi which have been deposited at the Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH (DSMZ), Brunswick, Germany, as bacterial, yeast or fungal strains. The expression "*a modified microorganism derived from a wild-type*", as used herein, refers to a microorganism that has been obtained from the wild-type by a controlled genetic modification (e.g. genetic engineering), by an uncontrolled (random) genetic modification (e.g. a treatment with a mutagenizing chemical agent, X-rays, UV light etc.) or by a combination of these methods. Further details of preparing the modified microorganism according to the present invention are given below.

Bacteria which are suitable according to the invention belong to the genera detailed under

http://www.dsmz.de/species/bacteria.htm,

yeasts which are suitable according to the invention belong to those genera which are detailed under

http://www.dsmz.de/species/yeasts.htm,

and fungi which are suitable according to the invention are those which are detailed under

http://www.dsmz.de/species/fungi.htm.

Preferably, the wild-type from which the modified microorganism according to the present invention has been derived is a bacterial cell. The term "*bacterial cell*" as used herein refers to a prokaryotic organism, i.e. a bacterium. Bacteria can be classified based on their biochemical and microbiological properties as well as their morphology. These classification criteria are well known in the art.

According to a preferred embodiment of the modified microorganism according to the present invention the wild-type from which the modified microorganism has been derived belongs to the family of *Enterobacteriaceae*, *Pasteurellaceae*, *Bacillaceae* or *Corynebacteriaceae*.

"*Enterobacteriaceae*" represent a large family of bacteria, including many of the more familiar bacteria, such as *Salmonella* and *Escherichia coli*. They belong to the Proteobacteria, and they are given their own order (Enterobacteriales). Members of the *Enterobacteriaceae* are rod-shaped. Like other Proteobacteria they have Gram-negative stains, and they are facultative anaerobes, fermenting sugars to produce lactic acid and various other end products such as succinic acid. Most also reduce nitrate to nitrite. Unlike most similar bacteria, *Enterobacteriaceae* generally lack cytochrome C oxidase. Most have many flagella used to move about, but a few genera are non-motile. They are non-spare forming, and mostly they are catalase-positive. Many members of this family are a normal part of the gut flora found in the intestines of humans and other animals, while others are found in water or soil, or are parasites on a variety of different animals and plants. *Escherichia coli*, better known as *E. coli*, is one of the most important model organisms, and its genetics and biochemistry have been closely studied. Most members of *Enterobacteriaceae* have peritrichous Type I fimbriae involved in the adhesion of the bacterial cells to their hosts. Examples for the *Enterobacteriaceae* are *E. coli*, *Proteus*, *Salmonella* and *Klebsiella*.

"*Pasteurellaceae*" comprise a large family of Gram-negative Proteobacteria with members ranging from bacteria such as *Haemophilus influenzae* to commensals of the animal and human mucosa. Most members live as commensals on mucosal surfaces of birds and mammals, especially in the upper respiratory tract. *Pasteurellaceae* are typically rod-shaped, and are a notable group of facultative anaerobes. They can be distinguished from the related *Enterobacteriaceae* by the presence of oxidase, and from most other similar bacteria by the absence of flagella. Bacteria in the family *Pasteurellaceae* have been classified into a number of genera based on metabolic properties and there sequences of the 16S RNA and 23S RNA. Many of the *Pas-*

teurellaceae contain pyruvate-formate-lyase genes and are capable of anaerobically fermenting carbon sources to organic acids.

"*Bacillaceae*" is a family of Gram-positive, heterotrophic, rod-shaped bacteria that may produce endospores. Motile members of this family are characterized by peritrichous flagellae. Some *Bacillaceae* are aerobic, while others are facultative or strict anaerobes. Most are non-pathogenic, but *Bacillus* species are known to cause disease in humans. This family also comprises the genus *Bacilli* which includes two orders, *Bacillales* and *Lactobacillales*. The bacillus species represents large cylindrical bacteria that can grow under aerobic conditions at 37°C. They are typically nonpathogenic. The genus *Bacillales* contains the species *Alicyclobacillaceae*, *Bacillaceae*, *Caryophanaceae*, *Listeriaceae*, *Paenibacillaceae*, *Planococcaceae*, *Sporolactobacillaceae*, *Staphylococcaceae*, *Thermoactinomycetaceae*, *Turicibacteraceae*. Many of the *Bacilli* contain pyruvate-formate-lyase genes and are capable of anaerobically fermenting carbon sources to organic acids.

"*Corynebacteriaceae*" is a large family of mostly Gram-positive and aerobic and nonmotile rod-shaped bacteria of the order *Eubacteriales*. This family also comprises the genus *Corynebacterium*, which is a genus of Gram-positive, rod-shaped bacteria. *Corynebacteria* are widely distributed in nature and are mostly innocuous. Some are useful in industrial settings such as *C. glutamicum*.

According to a particular preferred embodiment of the modified microorganism according to the present invention the wild-type from which the modified microorganism has been derived belongs to the family *Pasteurellaceae*. In this context it is furthermore preferred that the wild-type from which modified microorganism according to the present invention has been derived belongs to the genus *Basfia* and it is particularly preferred that the wild-type from which the modified microorganism has been derived belongs to the species *Basfia succiniciproducens*.

Most preferably, the wild-type from which the modified microorganism according to the present invention as been derived is *Basfia succiniciproducens*-strain DD1 deposited under the Budapest Treaty with DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, GmbH), Germany, having the deposit number DSM 18541. This strain has been originally isolated from the rumen of a cow of German origin. *Pasteurella* bacteria can be isolated from the gastrointestinal tract of animals and, preferably, mammals. The bacterial strain DD1, in particular, can be isolated from bovine rumen and is capable of utilizing glycerol (including crude glycerol) as a carbon source. Further strains of the genus *Basfia* that can be used for preparing the modified microorganism according to the present invention are the *Basfia*-strain that has been deposited under the deposit number DSM 22022 or the *Basfia*-strains that have been deposited with the Culture Collection of the University of Göteborg (CCUG), Sweden, having the deposit numbers CCUG 57335, CCUG 57762, CCUG 57763, CCUG 57764, CCUG 57765 or CCUG 57766. Said strains have been originally isolated from the rumen of cows of German or Swiss origin.

In this context it is particularly preferred that the wild-type from which the modified microorganism according to the present invention has been derived has a 16S rDNA of **SEQ ID NO: 1** or a sequence, which shows a sequence homology of at least 96 %, at least 97 %, at least 98 %, at least 99 % or at least 99.9% with **SEQ ID NO: 1**. It is also preferred that the wild-type from which the modified microorganism according to the present invention has been derived has a 23S rDNA of **SEQ ID NO: 2** or a sequence, which shows a sequence homology of at least 96 %, at least 97 %, at least 98 %, at least 99 % or at least 99.9 % with **SEQ ID NO: 2**.

The identity in percentage values referred to in connection with the various polypeptides or polynucleotides to be used for the modified microorganism according to the present invention is, preferably, calculated as identity of the residues over the complete length of the aligned sequences, such as, for example, the identity calculated (for rather similar sequences) with the aid of the program needle from the bioinformatics software package EMBOSS (Version 5.0.0, http://emboss.source-forge.net/what/) with the default parameters which are, i.e. gap open (penalty to open a gap): 10.0, gap extend (penalty to extend a gap): 0.5, and data file (scoring matrix file included in package): EDNAFUL.

It should be noted that the recombinant microorganisms according to the present invention can not only be derived from the above mentioned wild-type-microorganisms, especially from *Basfia succiniciproducens*-strain DD1, but also from variants of these strains. In this context the expression "*a variant of a strain*" comprises every strain having the same or essentially the same characteristics as the wild-type-strain. In this context it is particularly preferred that the 16 S rDNA of the variant has an identity of at least 90 %, preferably at least 95 %, more preferably at least 99 %, more preferably at least 99.5 %, more preferably at least 99.6 %, more preferably at least 99.7 %, more preferably at least 99.8% and most preferably at least 99.9 % with the wild-type from which the variant has been derived. It is also particularly preferred that the 23 S rDNA of the variant has an identity of at least 90 %, preferably at least 95 %, more preferably at least 99 %, more preferably at least 99.5 %, more preferably at least 99.6 %, more preferably at least 99.7 %, more preferably at least 99.8 % and most preferably at least 99.9 % with the wild-type from which the variant has been derived. A variant of a strain in the sense of this definition can, for example, be obtained by treating the wild-type-strain with a mutagenizing chemical agent, X-rays, or UV light.

The modified microorganism according to the present invention is characterized in that, compared to its wild-type, the activity of the enzyme that is encoded by the *wcaJ*-gene is reduced.

The reduction of the enzyme activity ($\Delta_{activity}$) is defined as follows:

$$\Delta_{activity} = 100\% - \left(\frac{\text{activity of the modified microorganism}}{\text{activity of the wildtype}} \times 100\% \right)$$

wherein, when determining $\Delta_{activity}$, the activity in the wild-type and the activity in the modified microorganism are determined under exactly the same conditions. Methods for the detection

and determination of the activity of the enzyme that is encoded by the *wcaJ*-gene can be found, for example, in Nothaft *et al.*: "*In vivo analysis of HPr reveals a fructose-specific phosphotransferase system that confers high-affinity uptake in Streptomyces coelicolor*", Journal of bacteriology, Vol. 185 (3), pages 929-937.

The reduced activity of the enzymes disclosed herein, in particular the reduced activity of the enzyme encoded by the *wcaJ*-gene, the lactate dehydrogenase and/or the pyruvate formate lyase, can be a reduction of the enzymatic activity by at least 50%, compared to the activity of said enzyme in the wild-type of the microorganism, or a reduction of the enzymatic activity by at least 90%, or more preferably a reduction of the enzymatic activity by at least 95%, or more preferably a reduction of the enzymatic activity by at least 98%, or even more preferably a reduction of the enzymatic activity by at least 99% or even more preferably a reduction of the enzymatic activity by at least 99.9%. The term "*reduced activity of the enzyme that is encoded by the wcaJ-gene*" or – as described below – "*a reduced lactate dehydrogenase activity*" or "*a reduced pyruvate formate lyase activity*", also encompasses a modified microorganism which has no detectable activity of these enzymes.

The term "*reduced activity of an enzyme*" includes, for example, the expression of the enzyme by said genetically manipulated (e.g., genetically engineered) microorganism at a lower level than that expressed by the wild-type of said microorganism. Genetic manipulations for reducing the expression of an enzyme can include, but are not limited to, deleting the gene or parts thereof encoding for the enzyme, altering or modifying regulatory sequences or sites associated with expression of the gene encoding the enzyme (e.g., by removing strong promoters or repressible promoters), modifying proteins (e.g., regulatory proteins, suppressors, enhancers, transcriptional activators and the like) involved in transcription of the gene encoding the enzyme and/or the translation of the gene product, or any other conventional means of decreasing expression of a particular gene routine in the art (including, but not limited to, the use of antisense nucleic acid molecules or iRNA or or other methods to knock-out or block expression of the target protein). Further on, one may introduce destabilizing elements into the mRNA or introduce genetic modifications leading to deterioration of ribosomal binding sites (RBS) of the RNA. It is also possible to change the codon usage of the gene in a way, that the translation efficiency and speed is decreased.

A reduced activity of an enzyme can also be obtained by introducing one or more gene mutations which lead to a reduced activity of the enzyme. Furthermore, a reduction of the activity of an enzyme may also include an inactivation (or the reduced expression) of activating enzymes which are necessary in order to activate the enzyme the activity of which is to be reduced. By the latter approach the enzyme the activity of which is to be reduced is preferably kept in an inactivated state

Microorganisms having a reduced activity of the enzyme encoded by the *wcaJ-gene* may occur naturally, i.e. due to spontaneous mutations. A microorganism can be modified to lack or to have significantly reduced activity of the enzyme that is encoded by the *wcaJ*-gene by various

techniques, such as chemical treatment or radiation. To this end, microorganisms will be treated by, e.g., a mutagenizing chemical agent, X-rays, or UV light. In a subsequent step, those microorganisms which have a reduced activity of the enzyme that is encoded by the *wcaJ*-gene will be selected. Modified microorganisms are also obtainable by homologous recombination techniques which aim to mutate, disrupt or excise the *wcaJ*-gene in the genome of the microorganism or to substitute the gene with a corresponding gene that encodes for an enzyme which, compared to the enzyme encoded by the wild-type-gene, has a reduced activity.

According to a preferred embodiment of the modified microorganism according to the present invention, a reduction of the activity of the enzyme encoded by the *wcaJ*-gene is achieved by a modification of the *wcaJ*-gene, wherein this gene modification is preferably realized by a deletion of the *wcaJ*-gene or at least a part thereof, a deletion of a regulatory element of the *wcaJ*-gene or at least a part thereof, such as a promotor sequence, or by an introduction of at least one mutation into the *wcaJ*-gene. In context with the introduction of at least one mutation into the *wcaJ*-gene it is particularly preferred that the at least one mutation leads to the expression of a truncated enzyme encoded by the *wcaJ*-gene. It is furthermore preferred that in the truncated enzyme at least 100 amino acids, preferably at least 125 amino acids, more preferred at least 150 amino acids and most preferred at least 160 amino acids of the wild-type enzyme encoded by the *wcaJ*-gene are deleted from the C-terminal end. Such a truncated enzyme encoded the *wcaJ*-gene can, for example, be obtained by inserting or deleting nucleotides at appropriate positions within the *wcaJ*-gene gene which leads to a frame shift mutation, wherein by means of this frame shift mutation a stop codon introduced. For example, insertion of a nucleotide in the codon that encodes of lysine between thymine at position 81 and adenine at position 82 leads to a frame shift mutation by means of which a stop codon is introduced as shown in **SEQ ID NO: 16**. Such mutations of the *wcaJ*-gene can be introduced, for example, by site-directed or random mutagenesis, followed by an introduction of the modified gene into the genome of the microorganism by recombination. Variants of the *wcaJ* -gene can be are generated by mutating the *wcaJ*-gene sequence **SEQ ID NO: 3** by means of PCR. The "*Quickchange Site-directed Mutagenesis Kit*" (Stratagene) can be used to carry out a directed mutagenesis. A random mutagenesis over the entire coding sequence, or else only part thereof, of **SEQ ID NO: 3** can be performed with the aid of the "*GeneMorph II Random Mutagenesis Kit*" (Stratagene).

In the following, a suitable technique for recombination, in particular for introducing a mutation or for deleting sequences, is described.

This technique is also sometimes referred to as the "Campbell recombination" herein (Leenhouts *et al.*, *Appl Env Microbiol.* (1989), Vol. 55, pages 394-400). "Campbell in", as used herein, refers to a transformant of an original host cell in which an entire circular double stranded DNA molecule (for example a plasmid) has integrated into a chromosome by a single homologous recombination event (a cross in event), and that effectively results in the insertion of a linearized version of said circular DNA molecule into a first DNA sequence of the chromosome that is homologous to a first DNA sequence of the said circular DNA molecule. "Campbelled in" refers to the linearized DNA sequence that has been integrated into the chromosome of a "Campbell in"

transformant. A "Campbell in" contains a duplication of the first homologous DNA sequence, each copy of which includes and surrounds a copy of the homologous recombination crossover point.

"Campbell out", as used herein, refers to a cell descending from a "Campbell in" transformant, in which a second homologous recombination event (a cross out event) has occurred between a second DNA sequence that is contained on the linearized inserted DNA of the "Campbelled in" DNA, and a second DNA sequence of chromosomal origin, which is homologous to the second DNA sequence of said linearized insert, the second recombination event resulting in the deletion (jettisoning) of a portion of the integrated DNA sequence, but, importantly, also resulting in a portion (this can be as little as a single base) of the integrated Campbelled in DNA remaining in the chromosome, such that compared to the original host cell, the "Campbell out" cell contains one or more intentional changes in the chromosome (for example, a single base substitution, multiple base substitutions, insertion of a heterologous gene or DNA sequence, insertion of an additional copy or copies of a homologous gene or a modified homologous gene, or insertion of a DNA sequence comprising more than one of these aforementioned examples listed above). A "Campbell out" cell is, preferably, obtained by a counter-selection against a gene that is contained in a portion (the portion that is desired to be jettisoned) of the "Campbelled in" DNA sequence, for example the *Bacillus subtilis sacB*-gene, which is lethal when expressed in a cell that is grown in the presence of about 5% to 10% sucrose. Either with or without a counter-selection, a desired "Campbell out" cell can be obtained or identified by screening for the desired cell, using any screenable phenotype, such as, but not limited to, colony morphology, colony color, presence or absence of antibiotic resistance, presence or absence of a given DNA sequence by polymerase chain reaction, presence or absence of an auxotrophy, presence or absence of an enzyme, colony nucleic acid hybridization, antibody screening, etc. The term "Campbell in" and "Campbell out" can also be used as verbs in various tenses to refer to the method or process described above.

It is understood that the homologous recombination events that leads to a "Campbell in" or "Campbell out" can occur over a range of DNA bases within the homologous DNA sequence, and since the homologous sequences will be identical to each other for at least part of this range, it is not usually possible to specify exactly where the crossover event occurred. In other words, it is not possible to specify precisely which sequence was originally from the inserted DNA, and which was originally from the chromosomal DNA. Moreover, the first homologous DNA sequence and the second homologous DNA sequence are usually separated by a region of partial non-homology, and it is this region of non-homology that remains deposited in a chromosome of the "Campbell out" cell.

Preferably, first and second homologous DNA sequence are at least about 200 base pairs in length, and can be up to several thousand base pairs in length. However, the procedure can be made to work with shorter or longer sequences. For example, a length for the first and second homologous sequences can range from about 500 to 2000 bases, and the obtaining of a "Campbell out" from a "Campbell in" is facilitated by arranging the first and second homologous

sequences to be approximately the same length, preferably with a difference of less than 200 base pairs and most preferably with the shorter of the two being at least 70% of the length of the longer in base pairs.

The *wcaJ*-gene the activity of which is reduced in the modified microorganism according to the present invention preferably comprises a nucleic acid selected from the group consisting of:

a) nucleic acids having the nucleotide sequence of **SEQ ID NO: 3**;

b) nucleic acids encoding the amino acid sequence of **SEQ ID NO: 4**;

c) nucleic acids which are at least 70%, at least 80 %, at least 85 %, at least 90 %, at least 95 %, at least 96 %, at least 97 %, at least 98 %, at least 99 %, at least 99.5 %, at least 99.6 %, at least 99.7 %, at least 99.8 % or at least 99.9 %, most preferably 100 % identical to the nucleic acid of a) or b), the identity being the identity over the total length of the nucleic acids of a) or b);

d) nucleic acid encoding an amino acid sequence which is at least 70%, at least 80 %, at least 85 %, at least 90 %, at least 95 %, at least 96 %, at least 97 %, at least 98 %, at least 99 %, at least 99.5 %, at least 99.6 %, at least 99.7 %, at least 99.8 % or at least 99.9 %, most preferably 100 % identical to the amino acid sequence encoded by the nucleic acid of a) or b), the identity being the identity over the total length of amino acid sequence encoded by the nucleic acids of a) or b)

e) nucleic acids capable of hybridizing under stringent conditions with a complementary sequence of any of the nucleic acids according to a) or b); and

f) nucleic acids encoding the same protein as any of the nucleic acids of a) or b), but differing from the nucleic acids of a) or b) above due to the degeneracy of the genetic code.

The term "*hybridization*" as used herein includes "*any process by which a strand of nucleic acid molecule joins with a complementary strand through base pairing*" (J. Coombs (1994) Dictionary of Biotechnology, Stockton Press, New York). Hybridization and the strength of hybridization (i.e., the strength of the association between the nucleic acid molecules) is impacted by such factors as the degree of complementarity between the nucleic acid molecules, stringency of the conditions involved, the Tm of the formed hybrid, and the G:C ratio within the nucleic acid molecules.

As used herein, the term "*Tm*" is used in reference to the "*melting temperature*". The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. The equation for calculating the Tm of nucleic acid molecules is well known in the art. As indicated by standard references, a simple estimate of the Tm value may be calculated by the equation: Tm = 81.5 + 0.41(% G+C), when a nucleic acid

molecule is in aqueous solution at 1 M NaCl (see e.g., Anderson and Young, Quantitative Filter Hybridization, in Nucleic Acid Hybridization (1985)). Other references include more sophisticated computations, which take structural as well as sequence characteristics into account for the calculation of Tm. Stringent conditions, are known to those skilled in the art and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6.

In particular, the term "*stringency conditions*" refers to conditions, wherein 100 contiguous nucleotides or more, 150 contiguous nucleotides or more, 200 contiguous nucleotides or more or 250 contiguous nucleotides or more which are a fragment or identical to the complementary nucleic acid molecule (DNA, RNA, ssDNA or ssRNA) hybridizes under conditions equivalent to hybridization in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO4, 1 mM EDTA at 50°C with washing in 2 × SSC, 0.1% SDS at 50°C or 65°C, preferably at 65°C, with a specific nucleic acid molecule (DNA; RNA, ssDNA or ss RNA). Preferably, the hybridizing conditions are equivalent to hybridization in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO4, 1 mM EDTA at 50°C with washing in 1 × SSC, 0.1% SDS at 50°C or 65°C, preferably 65°C, more preferably the hybridizing conditions are equivalent to hybridization in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO4, 1 mM EDTA at 50°C with washing in 0.1 × SSC, 0.1% SDS at 50°C or 65°C, preferably 65°C. Preferably, the complementary nucleotides hybridize with a fragment or the whole *wcaJ* nucleic acids. Alternatively, preferred hybridization conditions encompass hybridization at 65°C in 1 × SSC or at 42°C in 1 × SSC and 50% formamide, followed by washing at 65°C in 0.3 × SSC or hybridization at 50°C in 4 × SSC or at 40°C in 6 × SSC and 50% formamide, followed by washing at 50°C in 2 × SSC. Further preferred hybridization conditions are 0.1 % SDS, 0.1 SSD and 65°C.

The *wcaJ*-gene or parts of which that may be deleted by the above mentioned "Campbell recombination" or in which at least one mutation is introduced by the above mentioned "Campbell recombination" preferably comprises a nucleic acid as defined above.

Nucleic acid having the nucleotide sequence of **SEQ ID NO: 3** corresponds to the *wcaJ*-gene of *Basfia succiniciproducens*-strain DD1.

According to a preferred embodiment of the modified microorganism according to the present invention, this microorganism is not only characterized by a reduced activity of the enzyme encoded by the *wcaJ*-gene, but also, compared to the wild-type, by

i) a reduced pyruvate formate lyase activity,

ii) a reduced lactate dehydrogenase activity, or

iii) a reduced pyruvate formate lyase activity and a reduced lactate dehydrogenase activity.

Modified microorganisms being deficient in lactate dehydrogenase and/or being deficient in pyruvate formate lyase activity are disclosed in WO-A-2010/092155, US 2010/0159543 and WO-

A-2005/052135, the disclosure of which with respect to the different approaches of reducing the activity of lactate dehydrogenase and/or pyruvate formate lyase in a microorganism, preferably in a bacterial cell of the genus *Pasteurella*, particular preferred in *Basfia succiniciproducens* strain DD1, is incorporated herein by reference. Methods for determining the pyruvate formate lyase activity are, for example, disclosed by Asanuma N. and Hino T. in "*Effects of pH and Energy Supply on Activity and Amount of Pyruvate-Formate-Lyase in Streptococcus bovis*", Appl. Environ. Microbiol. (2000), Vol. 66, pages 3773-3777 and methods for determining the lactate dehydrogenase activity are, for example, disclosed by Bergmeyer, H.U., Bergmeyer J. and Grassl, M. (1983-1986) in "*Methods of Enzymatic Analysis*", 3rd Edition, Volume III, pages 126-133, Verlag Chemie, Weinheim.

In this context it is preferred that the reduction of the activity of lactate dehydrogenase is achieved by an inactivation of the *ldhA*-gene (which encodes the lactate dehydrogenase; LdhA; EC 1.1.1.27 or EC 1.1.1.28) and the reduction of the pyruvate formate lyase is achieved by an inactivation of the *pflA*-gene (which encodes for an activator of pyruvate formate lyase; PflA; EC 1.97.1.4) or the *pflD*-gene (which encodes the pyruvate formate lyase; PflD; EC 2.3.1.54), wherein the inactivation of these genes (i. e. *ldhA*, *pflA* and *pflD*) is preferably achieved by a deletion of theses genes or parts thereof, by a deletion of a regulatory element of these genes or at least a part thereof of by an introduction of at least one mutation into these genes, particular preferred by means of the "Campbell recombination" as described above.
The *ldhA*-gene the activity of which is reduced in the modified microorganism according to the present invention preferably comprises a nucleic acid selected from the group consisting of:

α1) nucleic acids having the nucleotide sequence of **SEQ ID NO: 10**;

α2) nucleic acids encoding the amino acid sequence of **SEQ ID NO: 11**;

α3) nucleic acids which are at least 70 %, at least 80 %, at least 85 %, at least 90 %, at least 95 %, at least 96 %, at least 97 %, at least 98 %, at least 99 %, at least 99.5 %, at least 99.6 %, at least 99.7 %, at least 99.8 % or at least 99.9 %, most preferably 100 % identical to the nucleic acid of α1) or α2), the identity being the identity over the total length of the nucleic acids of α1) or α2);

α4) nucleic acids encoding an amino acid sequence which is at least 70 %, at least 80 %, at least 85 %, at least 90 %, at least 95 %, at least 96 %, at least 97 %, at least 98 %, at least 99 %, at least 99.5 %, at least 99.6 %, at least 99.7 %, at least 99.8 % or at least 99.9 %, most preferably 100 % identical to the amino acid sequence encoded by the nucleic acid of α1) or α2), the identity being the identity over the total length of amino acid sequence encoded by the nucleic acids of α1) or α2);

α5) nucleic acids capable of hybridizing under stringent conditions with a complementary sequence of any of the nucleic acids according to α1) or α2); and

α6) nucleic acids encoding the same protein as any of the nucleic acids of α1) or α2), but differing from the nucleic acids of α1) or α2) above due to the degeneracy of the genetic code.

The *pflA*-gene the activity of which is reduced in the modified microorganism according to the present invention preferably comprises a nucleic acid selected from the group consisting of:

β1) nucleic acids having the nucleotide sequence of **SEQ ID NO: 12**;

β2) nucleic acids encoding the amino acid sequence of **SEQ ID NO: 13**;

β3) nucleic acids which are at least 70 %, at least 80 %, at least 85 %, at least 90 %, at least 95 %, at least 96 %, at least 97 %, at least 98 %, at least 99 %, at least 99.5 %, at least 99.6 %, at least 99.7 %, at least 99.8 % or at least 99.9 %, most preferably 100 % identical to the nucleic acid of β1) or β2), the identity being the identity over the total length of the nucleic acids of β1) or β2);

β4) nucleic acids encoding an amino acid sequence which is at least 70 %, at least 80 %, at least 85 %, at least 90 %, at least 95 %, at least 96 %, at least 97 %, at least 98 %, at least 99 %, at least 99.5 %, at least 99.6 %, at least 99.7 %, at least 99.8 % or at least 99.9 %, most preferably 100 % identical to the amino acid sequence encoded by the nucleic acid of β1) or β2), the identity being the identity over the total length of amino acid sequence encoded by the nucleic acids of β1) or β2);

β5) nucleic acids capable of hybridizing under stringent conditions with a complementary sequence of any of the nucleic acids according to β1) or β2); and

β6) nucleic acids encoding the same protein as any of the nucleic acids of β1) or β2), but differing from the nucleic acids of β1) or β2) above due to the degeneracy of the genetic code.

The *pflD*-gene the activity of which is reduced in the modified microorganism according to the present invention preferably comprises a nucleic acid selected from the group consisting of:

γ1) nucleic acids having the nucleotide sequence of **SEQ ID NO: 14**;

γ2) nucleic acids encoding the amino acid sequence of **SEQ ID NO: 15**;

γ3) nucleic acids which are at least 70 %, at least 80 %, at least 85 %, at least 90 %, at least 95 %, at least 96 %, at least 97 %, at least 98 %, at least 99 %, at least 99.5 %, at least 99.6 %, at least 99.7 %, at least 99.8 % or at least 99.9 %, most preferably 100 % identical to the nucleic acid of γ1) or γ2), the identity being the identity over the total length of the nucleic acids of γ1) or γ2);

γ4) nucleic acids encoding an amino acid sequence which is at least 70 %, at least 80 %, at least 85 %, at least 90 %, at least 95 %, at least 96 %, at least 97 %, at least 98 %, at least 99 %, at least 99.5 %, at least 99.6 %, at least 99.7 %, at least 99.8 % or at least 99.9 %, most preferably 100 % identical to the amino acid sequence encoded by the nucleic acid of γ1) or γ2), the identity being the identity over the total length of amino acid sequence encoded by the nucleic acids of γ1) or γ2);

γ5) nucleic acids capable of hybridizing under stringent conditions with a complementary sequence of any of the nucleic acids according to γ1) or γ2); and

γ6) nucleic acids encoding the same protein as any of the nucleic acids of γ1) or γ2), but differing from the nucleic acids of γ1) or γ2) above due to the degeneracy of the genetic code.

In this context it is preferred that the modified microorganism according to the present invention further comprises:

A) a deletion of the *ldhA*-gene or at least a part thereof, a deletion of a regulatory element of the *ldhA*-gene or at least a part thereof or an introduction of at least one mutation into the *ldhA*-gene;

B) a deletion of the *pflD*-gene or at least a part thereof, a deletion of a regulatory element of the *pflD*-gene or at least a part thereof or an introduction of at least one mutation into the *pflD*-gene;

C) a deletion of the *pflA*-gene or at least a part thereof, a deletion of a regulatory element of the *pflA*-gene or at least a part thereof or an introduction of at least one mutation into the *pflA*-gene;

D) a deletion of the *ldhA*-gene or at least a part thereof, a deletion of a regulatory element of the *ldhA*-gene or at least a part thereof or an introduction of at least one mutation into the *ldhA*-gene

and

a deletion of the *pflD*-gene or at least a part thereof, a deletion of a regulatory element of the *pflD*-gene or at least a part thereof or an introduction of at least one mutation into the *pflD*-gene;

or

E) a deletion of the *ldhA*-gene or at least a part thereof, a deletion of a regulatory element of the *ldhA*-gene or at least a part thereof or an introduction of at least one mutation into the *ldhA*-gene

and

a deletion of the *pflA*-gene or at least a part thereof, a deletion of a regulatory element of the *pflA*-gene or at least a part thereof or an introduction of at least one mutation into the *pflA*-gene.

Particular preferred embodiments of the modified microorganisms according to the present invention are:

- modified bacterial cells of the family *Pasteurellaceae*, in particular preferred of the genus *Basfia* and even more preferred of the species *Basfia succiniciproducens*, in which the *wcaJ*-gene or at least a part thereof has been deleted or wherein at least one mutation has been introduced in the *wcaJ*-gene, wherein the introduction of the at least one mutation preferably leads expression of an enzyme in which at least 100 amino acids, preferably at least 125 amino acids, more preferred at least 150 amino acids and most preferred at least 160 amino acids of the wild-type enzyme encoded by the *wcaJ*-gene are deleted from the C-terminal end;

- modified bacterial cells of the family *Pasteurellaceae*, in particular preferred of the genus *Basfia* and even more preferred of the species *Basfia succiniciproducens*, in which the *wcaJ*-gene or at least a part thereof has been deleted or wherein at least one mutation has been introduced in the *wcaJ*-gene, wherein the introduction of the at least one mutation preferably leads expression of an enzyme in which at least 100 amino acids, preferably at least 125 amino acids, more preferred at least 150 amino acids and most preferred at least 160 amino acids of the wild-type enzyme encoded by the *wcaJ*-gene are deleted from the C-terminal end, and in which, compared to the wild-type, the activity of the lactate dehydrogenase is reduced, preferably by a modification of the *ldhA*-gene, in particular by a modification of the *ldhA*-gene having the nucleic acid sequence according to **SEQ ID NO: 10** and encoding for LdhA having the amino acid sequence according to **SEQ ID NO: 11**;

- modified bacterial cells of the family *Pasteurellaceae*, in particular preferred of the genus *Basfia* and even more preferred of the species *Basfia succiniciproducens*, in which the *wcaJ*-gene or at least a part thereof has been deleted or wherein at least one mutation has been introduced in the *wcaJ*-gene, wherein the introduction of the at least one mutation preferably leads expression of an enzyme in which at least 100 amino acids, preferably at least 125 amino acids, more preferred at least 150 amino acids and most preferred at least 160 amino acids of the wild-type enzyme encoded by the *wcaJ*-gene are deleted from the C-terminal end, and in which, compared to the wild-type, the activity of the py-

ruvate formate lyase is reduced, preferably by a modification of the *pflA*-gene or the *pflD*-gene, in particular by a modification of the *pflA*-gene having the nucleic acid sequence according to **SEQ ID NO: 12** and encoding for PflA having the amino acid sequence according to **SEQ ID NO: 13** or by a modification of the *pflD*-gene having the nucleic acid sequence according to **SEQ ID NO: 14** and encoding for PflD having the amino acid sequence according to **SEQ ID NO: 15**;

- modified bacterial cells of the family *Pasteurellaceae*, in particular preferred of the genus *Basfia* and even more preferred of the species *Basfia succiniciproducens*, in which the *wcaJ*-gene or at least a part thereof has been deleted or wherein at least one mutation has been introduced in the *wcaJ*-gene, wherein the introduction of the at least one mutation preferably leads expression of an enzyme in which at least 100 amino acids, preferably at least 125 amino acids, more preferred at least 150 amino acids and most preferred at least 160 amino acids of the wild-type enzyme encoded by the *wcaJ*-gene are deleted from the C-terminal end, and in which, compared to the wild-type, the activity of the lactate dehydrogenase and the pyruvate formate lyase is reduced, preferably by a modification of the *ldhA*-gene and the *pflA*-gene, preferably by a modification of the *ldhA*-gene and the *pflA*-gene, in particular by a modification of the *ldhA*-gene having the nucleic acid sequence according to **SEQ ID NO: 10** and encoding for LdhA having the amino acid sequence according to **SEQ ID NO: 11** or by a modification of the *pflA*-gene having the nucleic acid sequence according to **SEQ ID NO: 12** and encoding for PflA having the amino acid sequence according to **SEQ ID NO: 13**, or a modification of the *ldhA*-gene and the *pflD*-gene, in particular by a modification of the *ldhA*-gene having the nucleic acid sequence according to **SEQ ID NO: 10** and encoding for LdhA having the amino acid sequence according to **SEQ ID NO: 11** or by a modification of the *pflD*-gene having the nucleic acid sequence according to **SEQ ID NO: 14** and encoding for PflD having the amino acid sequence according to **SEQ ID NO: 15**.

A contribution to solving the problems mentioned at the outset is furthermore provided by a method of producing an organic compound comprising:

I) cultivating the modified microorganism according to the present invention in a culture medium comprising at least one assimilable carbon source to allow the modified microorganism to produce the organic compound, thereby obtaining a fermentation broth comprising the organic compound;

II) recovering the organic compound from the fermentation broth obtained in process step I).

In process step I) the modified microorganism according to the present invention is cultured in a culture medium comprising at least one assimilable carbon source to allow the modified microorganism to produce the organic compound, thereby obtaining a fermentation broth comprising the organic compound. Preferred organic compounds that can be produced by the process according to the present invention comprise carboxylic acids such as formic acid, lactic acid, pro-

pionic acid, 2-hydroxypropionic acid, 3-hydroxypropionic acid, 3-hydroxybutyric acid, acrylic acid, pyruvic acid or salts of these carboxylic acids, dicarboxylic acids such as malonic acid, succinic acid, malic acid, tartaric acid, glutaric acid, itaconic acid, adipic acid or salts thereof, tricarboxylic acids such as citric acid or salts thereof, alcohols such as methanol or ethanol, amino acids such as L-asparagine, L-aspartic acid, L-arginine, L-isoleucine, L-glycine, L-glutamine, L-glutamic acid, L-cysteine, L-serine, L-tyrosine, L-tryptophan, L-threonine, L-valine, L-histidine, L-proline, L-methionine, L-lysine, L-leucine, etc..

According to a preferred embodiment of the process according to the present invention the organic compound is succinic acid. The term "*succinic acid*", as used in the context of the present invention, has to be understood in its broadest sense and also encompasses salts thereof (i. e. succinate), as for example alkali metal salts, like Na^+ and K^+-salts, or earth alkali salts, like Mg^{2+} and Ca^{2+}-salts, or ammonium salts or anhydrides of succinic acid.

The modified microorganism according to the present invention is, preferably, incubated in the culture medium at a temperature in the range of about 10 to 60°C or 20 to 50°C or 30 to 45°C at a pH of 5.0 to 9.0 or 5.5 to 8.0 or 6.0 to 7.0.

Preferably, the organic compound, especially succinic acid, is produced under anaerobic conditions. Anaerobic conditions may be established by means of conventional techniques, as for example by degassing the constituents of the reaction medium and maintaining anaerobic conditions by introducing carbon dioxide or nitrogen or mixtures thereof and optionally hydrogen at a flow rate of, for example, 0.1 to 1 or 0.2 to 0.5 vvm. Aerobic conditions may be established by means of conventional techniques, as for example by introducing air or oxygen at a flow rate of, for example, 0.1 to 1 or 0.2 to 0.5 vvm. If appropriate, a slight over pressure of 0.1 to 1.5 bar may be applied in the process.

The assimilable carbon source is preferably selected from sucrose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, D-fructose, D-glucose, D-xylose, L-arabinose, D-galactose, D-mannose, glycerol and mixtures thereof or compositions containing at least one of said compounds, or is selected from decomposition products of starch, cellulose, hemicellulose and/or lignocellulose. Preferably, the assimilable carbon source comprises D-glucose, maltose, sucrose, glycerol or a mixture of at least two of these compounds, wherein mixtures of glycerol and D-glucose, glycerol and sucrose, glycerol and D-xylose, glycerol and maltose and D-glucose and fructose are particularly preferred.

The initial concentration of the assimilable carbon source is, preferably adjusted to a value in a range of 5 to 100 g/l, preferably 5 to 75 g/l and more preferably 5 to 50 g/l and may be maintained in said range during cultivation. The pH of the reaction medium may be controlled by addition of suitable bases as for example, gaseous ammonia, NH_4HCO_3, $(NH_4)_2CO_3$, NaOH, Na_2CO_3, $NaHCO_3$, KOH, K_2CO_3, $KHCO_3$, $Mg(OH)_2$, $MgCO_3$, $Mg(HCO_3)_2$, $Ca(OH)_2$, $CaCO_3$, $Ca(HCO_3)_2$, CaO, $CH_6N_2O_2$, C_2H_7N and/or mixtures thereof. These alkaline neutralization agents are especially required if the organic compounds that are formed in the course of the

fermentation process are carboxylic acids or dicarboxylic acids. In the case of succinic acid as the organic compound, $Mg(OH)_2$ and $MgCO_3$ area particular preferred bases.

The fermentation step I) according to the present invention can, for example, be performed in stirred fermenters, bubble columns and loop reactors. A comprehensive overview of the possible method types including stirrer types and geometric designs can be found in Chmiel: "*Bioprozesstechnik: Einführung in die Bioverfahrenstechnik*", Volume 1. In the process according to the present invention, typical variants available are the following variants known to those skilled in the art or explained, for example, in Chmiel, Hammes and Bailey: "*Biochemical Engineering*", such as batch, fed-batch, repeated fed-batch or else continuous fermentation with and without recycling of the biomass. Depending on the production strain, sparging with air, oxygen, carbon dioxide, hydrogen, nitrogen or appropriate gas mixtures may be effected in order to achieve good yield (YP/S).

Particularly preferred conditions for producing the organic acid, especially succinic acid, in process step I) are:

Assimilable carbon source:	glycerol, sucrose, D-glucose, maltose, glycerol + D-glucose, glycerol + sucrose, glycerol + maltose, glycerol + D-xylose, D-glucose + fructose
Temperature:	30 to 45°C
pH:	5.5 to 7.0
Supplied gas:	CO_2

It is furthermore preferred in process step I) that the assimilable carbon source is converted to the organic compound, preferably to succinic acid, with a carbon yield YP/S of at least 0.5 g/g up to about 1.28 g/g; as for example a carbon yield YP/S of at least 0,6 g/g, of at least 0.7 g/g, of at least 0.75 g/g, of at least 0.8 g/g, of at least 0.85 g/g, of at least 0.9 g/g, of at least 0.95 g/g, of at least 1.0 g/g, of at least 1.05 g/g, of at least 1.1 g/g, of at least 1.15 g/g, of at least 1.20 g/g, of at least 1.22 g/g, or of at least 1.24 g/g (organic compound/carbon, preferably succinic acid/carbon).

It is furthermore preferred in process step I) that the assimilable carbon source is converted to the organic compound, preferably to succinic acid, with a specific productivity yield of at least 0.6 g g $DCW^{-1}h^{-1}$ organic compound, preferably succinic acid, or of at least of at least 0.65 g g $DCW^{-1}h^{-1}$, of at least 0.7 g g $DCW^{-1}h^{-1}$, of at least 0.75 g g $DCW^{-1}h^{-1}$ or of at least 0.77 g g $DCW^{-1}h^{-1}$ organic compound, preferably succinic acid.

It is furthermore preferred in process step I) that the assimilable carbon source is converted to the organic compound, preferably to succinic acid, with a space time yield for the organic compound, preferably for succinic acid, of at least 2.2 g/(L×h) or of at least 2.5 g/(L×h) , at least 2.75 g/(L×h), at least 3 g/(L×h), at least 3.25 g/(L×h), at least 3.5 g/(L×h), at least 3.7 g/(L×h), at least 4.0 g/(L×h) at least 4.5 g/(L×h) or at least 5.0 g/(L×h) of the organic compound, preferably

succinic acid. According to another preferred embodiment of the process according to the present invention in process step I) the modified microorganism is converting at least 20 g/L, more preferably at least 25 g/l and even more preferably at least 30 g/l of the assimilable carbon source, preferably an assimilable carbon source selected from sucrose, maltose, D-fructose, D-glucose, D-xylose, L-arabinose, D-galactose, D-mannose, and/or glycerol, to at least 20 g/l, more preferably to at least 25 g/l and even more preferably at least 30 g/l of the organic compound, preferably succinic acid.

The different yield parameters as described herein ("*carbon yield*" or "*YP/S*"; "*specific productivity yield*"; or "*space-time-yield (STY)*") are well known in the art and are determined as described for example by Song and Lee, 2006. "*Carbon yield*" and "*YP/S*" (each expressed in mass of organic compound produced/mass of assimilable carbon source consumed) are herein used as synonyms. The specific productivity yield describes the amount of a product, like succinic acid, that is produced per h and L fermentation broth per g of dry biomass. The amount of dry cell weight stated as "*DCW*" describes the quantity of biologically active microorganism in a biochemical reaction. The value is given as g product per g DCW per h (i.e. g g $DCW^{-1}h^{-1}$). The space-time-yield (STY) is defined as the ratio of the total amount of organic compound formed in the fermentation process to the volume of the culture, regarded over the entire time of cultivation. The space-time yield is also known as the "*volumetric productivity*".

In process step II) the organic compound, preferably succinic acid, is recovered from the fermentation broth obtained in process step I).

Usually, the recovery process comprises the step of separating the recombinant microorganims from the fermentation broth as the so called "biomass". Processes for removing the biomass are known to those skilled in the art, and comprise filtration, sedimentation, flotation or combinations thereof. Consequently, the biomass can be removed, for example, with centrifuges, separators, decanters, filters or in a flotation apparatus. For maximum recovery of the product of value, washing of the biomass is often advisable, for example in the form of a diafiltration. The selection of the method is dependent upon the biomass content in the fermentation broth and the properties of the biomass, and also the interaction of the biomass with the organic compound (e. the product of value). In one embodiment, the fermentation broth can be sterilized or pasteurized. In a further embodiment, the fermentation broth is concentrated. Depending on the requirement, this concentration can be done batch wise or continuously. The pressure and temperature range should be selected such that firstly no product damage occurs, and secondly minimal use of apparatus and energy is necessary. The skillful selection of pressure and temperature levels for a multistage evaporation in particular enables saving of energy.

The recovery process may further comprise additional purification steps in which the organic compound, preferably succinic acid, is further purified. If, however, the organic compound is converted into a secondary organic product by chemical reactions as described below, a further purification of the organic compound is, depending on the kind of reaction and the reaction conditions, not necessarily required. For the purification of the organic compound obtained in pro-

cess step II), preferably for the purification of succinic acid, methods known to the person skilled in the art can be used, as for example crystallization, filtration, electrodialysis and chromatography. In the case of succinic acid as the organic compound, for example, succinic acid may be isolated by precipitating it as a calcium succinate product by using calcium hydroxide, -oxide, -carbonate or hydrogen carbonate for neutralization and filtration of the precipitate. The succinic acid is recovered from the precipitated calcium succinate by acidification with sulfuric acid followed by filtration to remove the calcium sulfate (gypsum) which precipitates. The resulting solution may be further purified by means of ion exchange chromatography in order to remove undesired residual ions. Alternatively, if magnesium hydroxide, magnesium carbonate or mixtures thereof have been used to neutralize the fermentation broth, the fermentation broth obtained in process step I) may be acidified to transform the magnesium succinate contained in the medium into the acid form (i. e. succinic acid), which subsequently can be crystallized by cooling down the acidified medium. Examples of further suitable purification processes are disclosed in EP-A-1 005 562, WO-A-2008/010373, WO-A-2011/082378, WO-A-2011/043443, WO-A-2005/030973, WO-A-2011/123268 and WO-A-2011/064151 and EP-A-2 360 137.

According to a preferred embodiment of the process according to the present invention the process further comprises the process step:

III) conversion of the organic compound contained in the fermentation broth obtained in process step I) or conversion of the recovered organic compound obtained in process step II) into a secondary organic product being different from the organic compound by at least one chemical reaction.

In case of succinic acid as the organic compound preferred secondary organic products are selected from the group consisting of succinic acid esters and polymers thereof, tetrahydrofuran (THF), 1,4-butanediol (BDO), gamma-butyrolactone (GBL) and pyrrolidones.

According to a preferred embodiment for the production of THF, BDO and/or GBL this process comprises:

b1) either the direct catalytic hydrogenation of the succinic acid obtained in process steps I) or II) to THF and/or BDO and/or GBL or

b2) the chemical esterification of succinic acid and/or succinic acid salts obtained in process steps I) or II) into its corresponding di-lower alkyl ester and subsequent catalytic hydrogenation of said ester to THF and/or BDO and/or GBL.

According to a preferred embodiment for the production of pyrrolidones this process comprises:

b) the chemical conversion of succinic acid ammonium salts obtained in process steps I) or II) to pyrrolidones in a manner known per se.

For details of preparing these compounds reference is made to US-A-2010/0159543 and WO-A-2010/092155.

A contribution to solving the problems mentioned at the outset is furthermore provided by the use of the modified microorganism according to the present invention for the fermentative production of organic compounds. Preferred organic compounds are those compounds that have already been mentioned in connection with the process according to the present invention, succinic acid being the most preferred organic compound. Furthermore, preferred conditions for the fermentative production of organic compounds, preferably of succinic acid, are those conditions that have already been described in connection with process step I) of the process according to the present invention.

The invention is now explained in more detail with the aid of figures and non-limiting examples.

Figure 1 shows a schematic map of plasmid pSacB (**SEQ ID NO: 5**).

Figure 2 shows a schematic map of plasmid pSacB Δ*ldhA* (**SEQ ID NO: 6**).

Figure 3 shows a schematic map of plasmid pSacB Δ*pflA* (**SEQ ID NO: 7**).

Figure 4 shows a schematic map of plasmid pSacB Δ*wcaJ* (**SEQ ID NO: 8**).

Figure 5 shows a schematic map of plasmid pSacB Δ*pflD* (**SEQ ID NO: 9**).

Figure 6 shows a pellet of cells as obtained by sedimentation of different culture media.

Examples

Example 1: General method for the transformation of *Basfia succiniciproducens*

Strain
Wild-type DD1 (deposit DSM18541)
DD1 Δ*wcaJ*
DD1 Δ*ldhA*
DD1 Δ*ldhA* Δ*pflD*
DD1 Δ*ldhA* Δ*pflD* Δ*wcaJ*
DD1 Δ*ldhA* Δ*pflA*
DD1 Δ*ldhA* Δ*pflA* Δ*wcaJ*

Table 1: Nomenclature of the DD1-wild-type and mutants referred to in the examples

Basfia succiniciproducens DD1 (wild-type) was transformed with DNA by electroporation using the following protocol:

For preparing a pre-culture DD1 was inoculated from frozen stock into 40 ml BHI (brain heart infusion; Becton, Dickinson and Company) in 100 ml shake flask. Incubation was performed over night at 37°C; 200 rpm. For preparing the main-culture 100 ml BHI were placed in a 250 ml shake flask and inoculated to a final OD (600 nm) of 0.2 with the pre-culture. Incubation was performed at 37°C, 200 rpm. The cells were harvested at an OD of approximately 0.5, 0.6 and 0.7, pellet was washed once with 10% cold glycerol at 4°C and re-suspended in 2 ml 10% glycerol (4°C).

100 µl of competent cells were the mixed with 2-8 µg Plasmid-DNA and kept on ice for 2 min in an electroporation cuvette with a width of 0.2 cm. Electroporation under the following conditions: 400 Ω; 25 µF; 2.5 kV (Gene Pulser, Bio-Rad). 1 ml of chilled BHI was added immediately after electroporation and incubation was performed for approximately 2 h at 37°C.

Cells were plated on BHI with 5 mg/L chloramphenicol and incubated for 2-5 d at 37°C until the colonies of the transformants were visible. Clones were isolated and restreaked onto BHI with 5 mg/l chloramphenicol until purity of clones was obtained.

Example 2: Generation of deletion constructs

Mutation/deletion plasmids were constructed based on the vector pSacB (**SEQ ID NO: 5**). Figure 1 shows a schematic map of plasmid pSacB. 5'- and 3'- flanking regions (approx. 1500 bp each) of the chromosomal fragment, which should be deleted were amplified by PCR from chromosomal DNA of *Basfia succiniciproducens* and introduced into said vector using standard techniques. Normally, at least 80 % of the ORF were targeted for a deletion. In such a way, the deletion plasmids for the lactate dehydrogenase *ldh*A, pSacB_delta_*ldhA* (**SEQ ID NO: 6**), the pyruvate formate lyase activating enzyme *pflA*, pSacB_delta_*pflA* (**SEQ ID No: 7**), the putative fructose-specific transporter *wcaJ*, pSacB_delta_*wcaJ* (**SEQ ID No: 8**) and pyruvate formate lyase *pflD,* pSacB_delta_ *pflD* (**SEQ ID No: 9**) were constructed. Figures 2, 3, 4 and 5 show schematic maps of plasmid pSacB_delta_*ldhA*, pSacB_delta_*pflA*, pSacB_delta_*wcaJ* and pSacB_delta_*pflD*, respectively.

In the plasmid sequence of pSacB (**SEQ ID NO: 5**) the *sacB*-gene is contained from bases 2380-3801. The *sacB*-promotor is contained from bases 3802-4264. The chloramphenicol gene is contained from base 526-984. The origin of replication for *E. coli* (ori EC) is contained from base 1477-2337 (see fig. 1).

In the plasmid sequence of pSacB_delta_*ldhA* (**SEQ ID NO: 6**) the 5' flanking region of the *ldhA* gene, which is homologous to the genome of *Basfia succiniciproducens*, is contained from bases 1519-2850, while the 3' flanking region of the *ldhA*-gene, which is homologous to the genome of *Basfia succiniciproducens*, is contained from bases 62-1518. The *sacB*-gene is contained from bases 5169-6590. The *sacB*-promoter is contained from bases 6591-7053. The chloramphenicol gene is contained from base 3315-3773. The origin of replication for *E. coli* (ori EC) is contained from base 4266-5126 (see fig. 2).

In the plasmid sequence of pSacB_delta_*pflA* (**SEQ ID NO: 7**) the 5' flanking region of the *pflA*-gene, which is homologous to the genome of *Basfia succiniciproducens*, is contained from bases 1506-3005, while the 3' flanking region of the *pflA*-gene, which is homologous to the genome of *Basfia succiniciproducens*, is contained from bases 6-1505. The *sacB*-gene is contained from bases 5278-6699. The *sacB*-promoter is contained from bases 6700-7162. The chloramphenicol gene is contained from base 3424-3882. The origin of replication for *E. coli* (ori EC) is contained from base 4375-5235 (see fig. 3).

In the plasmid sequence of pSacB_delta_*wcaJ* (**SEQ ID NO: 8**) the 5' flanking region of the *wcaJ* gene, which is homologous to the genome of *Basfia succiniciproducens*, is contained from bases 1506-3122, while the 3' flanking region of the *wcaJ*-gene, which is homologous to the genome of *Basfia succiniciproducens*, is contained from bases 6-1505. The *sacB*-gene is contained from bases 5395-6816. The *sacB*-promoter is contained from bases 6817-7279. The chloramphenicol gene is contained from base 3541-3999. The origin of replication for *E. coli* (ori EC) is contained from base 4492-5352 (see fig. 4).

In the plasmid sequence of pSacB_delta_*pflD* (**SEQ ID NO: 9**) the 5' flanking region of the *pflD*-gene, which is homologous to the genome of *Basfia succiniciproducens*, is contained from bases 1533-2955, while the 3' flanking region of the *pflD*-gene, which is homologous to the genome of *Basfia succiniciproducens*, is contained from bases 62-1532. The *sacB*-gene is contained from bases 5256-6677. The *sacB*-promoter is contained from bases 6678-7140. The chloramphenicol gene is contained from base 3402-3860. The origin of replication for *E. coli* (ori EC) is contained from base 4353-5213 (see fig. 5).

Example 3: Generation of improved succinate producing strains

a) *Basfia succiniciproducens* DD1 was transformed as described above with the pSacB_delta_*ldhA* and "Campbelled in" to yield a "Campbell in" strain. Transformation and integration into the genome of *Basfia succiniciproducens* was confirmed by PCR yielding bands for the integrational event of the plasmid into the genome of *Basfia succiniciproducens*.

The "Campbell in" strain was then "Campbelled out" using agar plates containing sucrose as a counter selection medium, selecting for the loss (of function) of the *sacB* gene. Therefore, the "Campbell in" strains were incubated in 25-35 ml of non selective medium (BHI containing no antibiotic) at 37°C, 220 rpm over night. The overnight culture was then streaked onto freshly prepared BHI containing sucrose plates (10%, no antibiotics) and incubated overnight at 37°C ("first sucrose transfer"). Single colony obtained from first transfer were again streaked onto freshly prepared BHI containing sucrose plates (10%) and incubated overnight at 37°C ("second sucrose transfer"). This procedure was repeated until a minimal completion of five transfers ("third, forth, fifth sucrose transfer") in sucrose. The term "first to fifth sucrose transfer" refers to the transfer of a strain after chromosomal integration of a vector containing a *sacB* -evan-sucrase gene onto sucrose and growth

medium containing agar plates for the purpose of selecting for strains with the loss of the sacB gene and the surrounding plasmid sequences. Single colony from the fifth transfer plates were inoculated onto 25-35 ml of non selective medium (BHI containing no antibiotic) and incubated at 37°C, 220 rpm over night. The overnight culture was serially diluted and plated onto BHI plates to obtain isolated single colonies.

The "Campbelled out" strains containing the mutation/deletion of the *ldhA*-gene were confirmed by chloramphenicol sensitivity. The mutation/deletion mutants among these strains were identified and confirmed by PCR analysis. This led to the *ldhA*-deletion mutant *Basfia succiniciproducens* DD1 Δ*ldhA*.

b) *Basfia succiniciproducens* DD1 Δ*ldhA* was transformed with pSacB_delta_*pflD* as described above and "Campbelled in" to yield a "Campbell in" strain. Transformation and integration was confirmed by PCR. The "Campbell in" strain was then "Campbelled out" as described previously. The deletion mutants among these strains were identified and confirmed by PCR analysis. This led to the *ldhA pflD*-double deletion mutant *Basfia succiniciproducens* DD1 Δ*ldhA* Δ*pflD*.

c) *Basfia succiniciproducens* DD1 Δ*ldhA* Δ*pflD* was transformed with pSacB_delta_*wcaJ* as described above and "Campbelled in" to yield a "Campbell in" strain. Transformation and integration was confirmed by PCR. The "Campbell in" strain was then "Campbelled out" as described previously. The deletion mutants among these strains were identified and confirmed by PCR analysis. This led to the *ldhA pflD wcaJ*-triple deletion mutant *Basfia succiniciproducens* DD1 Δ*ldhA* Δ*pflD* Δ*wcaJ*.

d) *Basfia succiniciproducens* DD1 Δ*ldhA* was transformed with pSacB_delta_*pflA* as described above and "Campbelled in" to yield a "Campbell in" strain. Transformation and integration was confirmed by PCR. The "Campbell in" strain was then "Campbelled out" as described previously. The deletion mutants among these strains were identified and confirmed by PCR analysis. This led to the *ldhA pflA*-double deletion mutant *Basfia succiniciproducens* DD1 Δ*ldhA* Δ*pflA*.

e) *Basfia succiniciproducens* DD1 Δ*ldhA* Δ*pflA* was transformed with pSacB_delta_*wcaJ* as described above and "Campbelled in" to yield a "Campbell in" strain. Transformation and integration was confirmed by PCR. The "Campbell in" strain was then "Campbelled out" as described previously. The deletion mutants among these strains were identified and confirmed by PCR analysis. This led to the *ldhA pflA wcaJ*-triple deletion mutant *Basfia succiniciproducens* DD1 Δ*ldhA* Δ*pflA* Δ*wcaJ*.

f) *Basfia succiniciproducens* DD1 was transformed with pSacB_delta_*wcaJ* as described above and "Campbelled in" to yield a "Campbell in" strain. Transformation and integration was confirmed by PCR. The "Campbell in" strain was then "Campbelled out" as described previously. The deletion mutants among these strains were identified and confirmed by

PCR analysis. This led to the *wcaJ*-deletion mutant *Basfia succiniciproducens* DD1 *ΔwcaJ*.

Example 4: Cultivation of various DD1-strains on glucose and sucrose

The productivity of the DD1 was compared with the productivity of the mutant strain DD1 Δ*wcaJ* in the presence of glucose or sucrose as a carbon source.

The productivity of the DD1 Δ*ldhA* Δ*pflD* was compared with the productivity of the mutant strain DD1 Δ*ldhA* Δ*pflD* Δ*wcaJ* in the presence of glucose or sucrose as a carbon source.

The productivity of the DD1 Δ*ldhA* Δ*pflA* was compared with the productivity of the mutant strain DD1 Δ*ldhA* Δ*pflA* Δ*wcaJ* in the presence of glucose or sucrose as a carbon source.

Productivity was analyzed utilizing media and incubation conditions described below.

1. Medium preparation

The composition and preparation of the cultivation medium is as described in the following table 2, 3, 4 and 5.

Trace element solution	
Compound	Final concentration
citric acid	3.5 g/L
$ZnSO_4 \times 7\ H_2O$	1851 mg/L
$CaSO_4 \times 2\ H_2O$	10 mg/L
$FeSO_4 \times 7\ H_2O$	2040 mg/L
$CaCl_2 \times 2\ H_2O$	12460 mg/L
$MnCl_2 \times 4\ H_2O$	1200 mg/L
$Na_2MoO_4 \times 2\ H_2O$	38 mg/L
$CuCl_2 \times 2\ H_2O$	188 mg/L
$NiCl_2 \times 6\ H_2O$	32 mg/L
$CoCl_2 \times 6\ H_2O$	101 mg/L

Table 2: Composition of trace element solution.

Vitamin solution	
Compound	Final concentration
Thiamin HCl (B1)	500 mg/L
Nicotinic acid (B3)	500 mg/L
Riboflavin (B2)	20 mg/L
Biotin (B7)	5 mg/L
Pantothenic acid (B5)	100 mg/L
Pyridoxine (B6)	500 mg/L
Cyanocobalamin (B12)	5 mg/L
Lipoic acid	5 mg/L

Table 3: Composition of vitamin solution.

Compound	Volume/ Mass	Stock concentration	Final concentration
Medium 1			
$MgCO_3$	2.5 g	100%	50.00 g/L
Water	28 mL	-	-
Medium 2			
Succinic acid	2.5 mL	50 g/L	2.50 g/L
Glucose	4.00 mL	650 g/L	52.00 g/L
$(NH_4)_2SO_4$	0.25 mL	500 g/L	2.50 g/L
$(NH_4)_2HPO_4$	0.5 mL	200 g/L	2.00 g/L
K_2CO_3	0.50 mL	200 g/L	2.00 g/L
KH_2PO_4	0.50 mL	100 g/L	1.00 g/L
Na_2CO_3	0.50 mL	200 g/L	2.00 g/L
vitamin solution	0.50 mL	25 g/L	0.25 g/L
trace element solution	0.50 mL	21 g/L	0.21 g/L

Table 4: Composition of LSM medium for cultivation on glucose.

Compound	Volume/ Mass	Stock concentration	Final concentration
Medium 1			
$MgCO_3$	2.5 g	100%	50.00 g/L
Water	28 mL	-	-
Medium 2			
Succinic acid	2.5 mL	50 g/L	2.50 g/L
Sucrose	3.85 mL	650 g/L	50.00 g/L
$(NH_4)_2SO_4$	0.25 mL	500 g/L	2.50 g/L
$(NH_4)_2HPO_4$	0.5 mL	200 g/L	2.00 g/L
K_2CO_3	0.50 mL	200 g/L	2.00 g/L
KH_2PO_4	0.50 mL	100 g/L	1.00 g/L
Na_2CO_3	0.50 mL	200 g/L	2.00 g/L
vitamin solution	0.50 mL	25 g/L	0.25 g/L
trace element solution	0.50 mL	21 g/L	0.21 g/L

Table 5: Composition of LSM medium for cultivation on sucrose.

2. Cultivations and analytics

For growing the main culture bacteria from a freshly grown BHI-agar plate was used to inoculate to OD600 = 0.75 a 100 ml-serum bottle with gas tight butyl rubber stopper containing 50 ml of the liquid medium described in table 2 and 3 with a CO_2-atmosphere. The bottles were incubated at 37°C and 160 rpm (shaking diameter: 2.5 cm). Consumption of the C-sources and production of carboxylic acids was quantified via HPLC (HPLC methods are described in table 10and 11) after 24h or 48h. Cell growth was measured by measuring the absorbance at 600 nm (OD600) using a spectrophotometer (Ultrospec3000, Amersham Biosciences, Uppsala Sweden).

3. Results

The results of the cultivation experiments with different DD1-strains are shown in table 6, 7 and 8. As can be seen from these results, a reduction of the activity of the enzyme that is encoded by the Δ*wcaJ*-cell leads to increased production of succinic acid.

Moreover, samples obtained by cultivation of the DD1 Δ*ldhA* Δ*pflD*/DD1 Δ*ldhA* Δ*pflA*-strain and the DD1Δ*ldhA* Δ*pflD* Δ*wcaJ*/DD1ΔldhA ΔpflA Δ*wcaJ*-strain after 24h or 48 h of incubation have been transferred into a 15 ml tube to measure supernatant volumes obtained after centrifugation. As shown in table 9, a reduction of the activity of the enzyme that is encoded by the Δ*wcaJ*-cell leads to modified microorganisms that show a significantly improved sedimentation behavior (a denser cell pellet is obtained after centrifugation) and that can thus easier be removed from the culture medium in the subsequent purification process.

	DD1	DD1Δ*wcaJ*	DD1	DD1Δ*wcaJ*
substrate	glucose	glucose	sucrose	sucrose
tc [h][a]	24	24	48	48
$\Delta C_{substrate}$ [g/l][b]	48.4	48.4	48.6	48.1
ΔC_{SA}[g/l][c] (succinic acid)	26.1	28.2	22.0	24.3
ΔC_{LA} [g/L][c, h] (lactic acid)	5.1	3.3	11.8	10.0
ΔC_{FA}[g/l][c,h] (formic acid)	4.8	4.2	4.1	4.2
ΔC_{AA}[g/l][c,h] (acetic acid)	7.2	7.2	6.2	6.9
ΔC_{PA}[g/l][c,h] (pyruvic acid)	0.0	0.0	0.0	0.0
ΔC_{P}[g/l][c,h] (propionic acid)	0.0	0.0	0.0	0.0
ΔC_{E}[g/l][c] (ethanol)	0.3	0.2	0.3	0.2
SA Yield (SA/S) [g/g][g]	0.54	0.58	0.45	0.50

Table 6: Cultivation of the DD1 and the DD1 Δ*wcaJ*-strain on glucose and sucrose.

a cultivation time

b consumption of substrate (glucose or sucrose)

c formation of succinic acid, lactic acid, formic acid, acetic acid, pyruvic acid, propionic acid and ethanol

g SA yield (ration of SA per consumed substrate)

h Detection limits for acetic acid, lactic acid, malic acid, and formic acid were found to be lower than 0.01g/l in the given HPLC method

	DD1 Δ*ldhA*Δ*pflD*	DD1 Δ*ldhA* Δ*pflD*Δ*wcaJ*	DD1 Δ*ldhA*Δ*pflD*	DD1 Δ*ldhA* Δ*pflD*Δ*wcaJ*
substrate	glucose	glucose	sucrose	sucrose
tc [h][a]	24	24	48	48
$\Delta C_{substrate}$ [g/l][b]	49.95	49.95	50.40	50.40
ΔC_{SA}[g/l][c] (succinic acid)	31.45	32.80	28.82	36.94
ΔC_{LA} [g/L][c, h] (lactic acid)	0.22	0.16	0.35	0.22
ΔC_{FA}[g/l][c,h] (formic acid)	0.00	0.00	0.00	0.00
ΔC_{AA}[g/l][c,h] (acetic acid)	2.89	4.48	1.39	5.45
ΔC_{PA}[g/l][c,h] (pyruvic acid)	1.87	0.94	2.70	0.23
ΔC_{P}[g/l][c,h] (propionic acid)	0.00	0.00	0.00	0.00
ΔC_{E}[g/l][c] (ethanol)	0.00	0.00	0.00	0.00
SA Yield (SA/S) [g/g][g]	0.63	0.66	0.57	0.73

Table 7: Cultivation of the DD1 Δ*ldhA* Δ*pflD*-strain and the DD1 Δ*ldhA* Δ*pflD* Δ*wcaJ*-strain on glucose and sucrose.

[a] cultivation time
[b] consumption of substrate (glucose or sucrose)
[c] formation of succinic acid, lactic acid, formic acid, acetic acid, pyruvic acid, propionic acid and ethanol
[g] SA yield (ration of SA per consumed substrate)
[h] Detection limits for acetic acid, lactic acid, malic acid, and formic acid were found to be lower than 0.01g/l in the given HPLC method

	DD1 Δ*ldhA*Δ*pflA*	DD1 Δ*ldhA* Δ*pflA*Δ*wcaJ*	DD1 Δ*ldhA*Δ*pflA*	DD1 Δ*ldhA* Δ*pflA*Δ*wcaJ*
substrate	glucose	glucose	sucrose	sucrose
tc [h][a]	24	24	48	48
$\Delta C_{substrate}$ [g/l][b]	49.95	49.95	50.40	50.40
ΔC_{SA}[g/l][c] (succinic acid)	32.37	31.77	29.36	31.36
ΔC_{LA} [g/L][c, h] (lactic acid)	0.23	0.28	0.37	0.37
ΔC_{FA}[g/l][c,h] (formic acid)	0.00	0.00	0.00	0.00
ΔC_{AA}[g/l][c,h] (acetic acid)	2.68	2.40	0.90	1.87
ΔC_{PA}[g/l][c,h] (pyruvic acid)	1.96	2.05	3.16	2.49
ΔC_{P}[g/l][c,h] (propionic acid)	0.00	0.00	0.00	0.00
ΔC_{E}[g/l][c] (ethanol)	0.00	0.00	0.00	0.00
SA Yield (SA/S) [g/g][g]	0.65	0.64	0.58	0.62

Table 8: Cultivation of the DD1 Δ*ldhA* Δ*pflA*-strain and the DD1 Δ*ldhA* Δ*pflA* Δ*wcaJ*-strain on glucose and sucrose

a cultivation time

b consumption of substrate (glucose or sucrose)

c formation of succinic acid, lactic acid, formic acid, acetic acid, pyruvic acid, propionic acid and ethanol

g SA yield (ration of SA per consumed substrate)

h Detection limits for acetic acid, lactic acid, malic acid, and formic acid were found to be lower than 0.01g/l in the given HPLC method

Strain	Substrate	Cultivation time	Supernatant volume
DD1 Δ*ldhA* Δ*pflD*	glucose	24 h	8,0 mL
DD1 Δ*ldhA* Δ*pflD* Δ*wcaJ*	glucose	24 h	8,8 mL
DD1 Δ*ldhA* Δ*pflA*	glucose	24 h	8,0 mL
DD1 Δ*ldhA* Δ*pflA* Δ*wcaJ*	glucose	24 h	8,4 mL
DD1 Δ*ldhA* Δ*pflD*	sucrose	48 h	8,3 mL
DD1 Δ*ldhA* Δ*pflD* Δ*wcaJ*	sucrose	48 h	9,0 mL
DD1 Δ*ldhA* Δ*pflA*	sucrose	48 h	8,3 mL
DD1 Δ*ldhA* Δ*pflA* Δ*wcaJ*	sucrose	48 h	8,6 mL

Table 9: Supernatant volumes obtained after centrifugation (4,600 rpm, 10 min) of 10 mL of bacterial cultures.

HPLC column	Aminex HPX-87 H, 300 × 7.8 mm (BioRad)			
Precolumn	Cation H			
Temperature	50 °C			
Eluent flow rate	0.50 ml/min			
Injection volume	5.0 µl			
Diode array detector	RI-Detector			
Runtime	28 min			
max. pressure	140 bar			
Eluent A	5 mM H_2SO_4			
Eluent B	5 mM H_2SO_4			
Gradient	Time [min]	A[%]	B[%]	Flow [ml/min]
	0.0	50	50	0.50
	28.0	50	50	0.50

Table 10: HPLC method (ZX-THF50) for analysis of glucose, succinic acid, formic acid, lactic acid, acetic acid, pyruvic acid and ethanol

HPLC column	Fast Carbohydrate, 100 × 7.8 mm (Biorad)			
Precolumn	Deashing Refill Cartridges (30 °C)			
Temperature	75 °C			
Eluent flow rate	1.00 ml/min			
Injection volume	1.0 µl			
Diode array detector	RI-Detector			
Runtime	8 min			
max. pressure	150 bar			
Eluent A	water			
Eluent B	water			
Gradient	Time [min]	A[%]	B[%]	Flow [ml/min]
	0.0	50	50	1.00
	8.0	50	50	1.00

Table 11: HPLC method (Fast-CH) for analysis of glucose and sucrose

SEQUENCES

SEQ ID NO: 1 (nucleotide sequence of 16 S rDNA of strain DD1)

tttgatcctggctcagattgaacgctggcggcaggcttaacacatgcaagtcgaacggtagcgggaggaaagcttgctttctttgccga
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SEQ ID NO: 2 (nucleotide sequence of 23 S rDNA of strain DD1)

agtaataacgaacgacacaggtataagaatacttgaggttgtatggttaagtgactaagcgtacaaggtggatgccttggcaatcaga
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SEQ ID NO: 3 (nucleotide sequence of *wcaJ*-gene from strain DD1)
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SEQ ID NO: 4 (amino acid sequence of the enzyme encoded by the above *wcaJ*-gene)
MIKRLFDIVVALIALILFSPLYLFVAYKVKQNLGSPVLFKQTRPGLHGKPFEMIKFRTMKDGADEN
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SEQ ID NO: 5 (complete nucleotide sequence of plasmid pSacB)
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SEQ ID NO: 6 (complete nucleotide sequence of plasmid pSacB_delta_*ldhA*)
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aatatctcatttcactaaataatagtgaacggcaggtatatgtgatgggttaaaaaggatcggcggccgctcgatttaaatc

SEQ ID NO: 9 (complete nucleotide sequence of plasmid pSacB_*pflD*)

tcgagaggcctgacgtcgggcccggtaccacgcgtcatatgactagttcggacctagggatgggatcgagctcttttccttgccgaca
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gtgaactcggatcccaccgcccatgccgaaattattgcgttgcgtaacgccgcgcagaaaatccaaaattaccgcctgctcaatacc
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SEQ ID NO: 10 (nucleotide sequence of *ldhA*-gene from strain DD1)
ttgacaaaatcagtatgtttaaataaggagctaactatgaaagttgccgtttacagtactaaaaattatgatcgcaaacatctggatttgg
cgaataaaaaatttaattttgagcttcatttctttgatttttacttgatgaacaaaccgcgaaaatggcggagggcgccgatgccgtctgta
ttttcgtcaatgatgatgcgagccgcccggtgttaacaaagttggcgcaaatcggagtgaaaattatcgctttacgttgtgccggttttaat
aatgtggatttggaggcggcaaaagagctgggattaaaagtcgtacgggtgcctgcgtattcgccggaagccgttgccgagcatgcg
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gtgcgaaaatagcgttgaaggctaa

SEQ ID NO: 11 (amino acid sequence of LdhA from strain DD1)
MTKSVCLNKELTMKVAVYSTKNYDRKHLDLANKKFNFELHFFDFLLDEQTAKMAEGADAVCIFV
NDDASRPVLTKLAQIGVKIIALRCAGFNNVDLEAAKELGLKVVRVPAYSPEAVAEHAIGLMLTLN
RRIHKAYQRTRDANFSLEGLVGFNMFGKTAGVIGTGKIGLAAIRILKGFGMDVLAFDPFKNPAAE
ALGAKYVGLDELYAKSHVITLHCPATADNYHLLNEAAFNKMRDGVMIINTSRGVLIDSRAAIEAL
KRQKIGALGMDVYENERDLFFEDKSNDVITDDVFRRLSSCHNVLFTGHQAFLTEEALNNIADVT
LSNIQAVSKNATCENSVEG

SEQ ID NO: 12 (nucleotide sequence of *pflA*-gene from strain DD1)
atgtcggttttaggacgaattcattcatttgaaacctgcgggacagttgacgggccgggaatccgctttatttttatttttacaaggctgcttaa
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acctatcgccattttatgaacgcctcgggcggcggagttaccgcttccggcggtgaagctattttacaggcggaatttgtacgggactgg

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gacacggatcttgtgttgcttgacctgaaagaaatgaatgaacgggttcacgaaagcctgattggcgtgccgaataaaagagtgctcg
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gtactcggcgataaatacgagcttgaagatgtaaaaccgccgacaaaagaattaatggagcatgttaaggggttgcttgcaggctac
gggcttaatgtgacatattag

SEQ ID NO: 13 (amino acid sequence of PflA from strain DD1)
MSVLGRIHSFETCGTVDGPGIRFILFLQGCLMRCKYCHNRDTWDLHGGKEISVEELMKEVVTY
RHFMNASGGGVTASGGEAILQAEFVRDWFRACHKEGINTCLDTNGFVRHHDHIIDELIDDTDLV
LLDLKEMNERVHESLIGVPNKRVLEFAKYLADRNQRTWIRHVVVPGYTDSDEDLHMLGNFIKD
MKNIEKVELLPYHRLGAHKWEVLGDKYELEDVKPPTKELMEHVKGLLAGYGLNVTY

SEQ ID NO: 14 (nucleotide sequence of *pflD*-gene from strain DD1)
atggctgaattaacagaagctcaaaaaaaagcatgggaaggattcgttcccggtgaatggcaaaacggcgtaaatttacgtgacttt
atccaaaaaaactatactccgtatgaaggtgacgaatcattcttagctgatgcgactcctgcaaccagcgagttgtggaacagcgtga
tggaaggcatcaaaatcgaaaacaaaactcacgcacctttagatttcgacgaacatactccgtcaactatcacttctcacaagcctgg
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cacttgaatgttaacgttcttaaccgtgaaatgttgttagacgcgatggaaaatccggaaaaaatacccgcaattaaccattcgtgtttcag
gttacgcggttcgtttcaactcattaactaaagagcaacaacaagacgtcatcactcgtacgtttacacaatcaatgtaa

SEQ ID NO: 15 (amino acid of PflD from strain DD1)
MAELTEAQKKAWEGFVPGEWQNGVNLRDFIQKNYTPYEGDESFLADATPATSELWNSVMEGI

KIENKTHAPLDFDEHTPSTITSHKPGYINKDLEKIVGLQTDAPLKRAIMPYGGIKMIKGSCEVYGR
KLDPQVEFIFTEYRKTHNQGVFDVYTPDILRCRKSGVLTGLPDAYGRGRIIGDYRRLAVYGIDYL
MKDKKAQFDSLQPRLEAGEDIQATIQLREEIAEQHRALGKIKEMAASYGYDISGPATNAQEAIQ
WTYFAYLAAVKSQNGAAMSFGRTSTFLDIYIERDLKRGLITEQQAQELMDHLVMKLRMVRFLRT
PEYDQLFSGDPMWATETIAGMGLDGRPLVTKNSFRVLHTLYTMGTSPEPNLTILWSEQLPEAF
KRFCAKVSIDTSSVQYENDDLMRPDFNNDDYAIACCVSPMVVGKQMQFFGARANLAKTMLYAI
NGGIDEKNGMQVGPKTAPITDEVLNFDTVIERMDSFMDWLATQYVTALNIIHFMHDKYAYEAAL
MAFHDRDVFRTMACGIAGLSVAADSLSAIKYAKVKPIRGDIKDKDGNVVASNVAIDFEIEGEYPQ
FGNNDPRVDDLAVDLVERFMKKVQKHKTYRNATPTQSILTITSNVVYGKKTGNTPDGRRAGAP
FGPGANPMHGRDQKGAVASLTSVAKLPFAYAKDGISYTFSIVPNALGKDDEAQKRNLAGLMDG
YFHHEATVEGGQHLNVNVLNREMLLDAMENPEKYPQLTIRVSGYAVRFNSLTKEQQQDVITRT
FTQSM

SEQ ID NO: 16 (nucleotide sequence of *wcaJ*-gene from strain DD1 with insertion of cytosine between nucleotides 81 and 82)

Atgataaaacgcctttcgatattgttgtcgcattgatagcattgattttgttttcgcccttatatttgtttgtggcttatcaaggtaaaacaaaatt
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cgcagatgaaaacggtaatattttgccggatgcggagcgcttaacacctttcggcaaaatgttgcgcgctaccagtctggacgagttgc
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PCT

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(This sheet is not part of and does not count as a sheet of the international application)

0-1	**Form PCT/RO/134 Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)**	
0-1-1	Prepared Using	PCT Online Filing Version 3.5.000.241e MT/FOP 20141031/0.20.5.20
0-2	**International Application No.**	
0-3	**Applicant's or agent's file reference**	B74822PC

1	**The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:**	
1-1	**page**	1
1-2	**line**	32-34
1-3	**Identification of deposit**	
1-3-1	Name of depositary institution	DSMZ Leibniz-Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ)
1-3-2	Address of depositary institution	Inhoffenstr. 7B, 38124 Braunschweig, Germany
1-3-3	Date of deposit	11 August 2006 (11.08.2006)
1-3-4	Accession Number	DSMZ 18541
1-4	**Additional Indications**	With respect to the designation of the EPO the applicant hereby declares under Rule 32(1) EPC that the biological material is to be made available only by the issue of a sample to an expert.
1-5	**Designated States for Which Indications are Made**	

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0-4	**This form was received with the international application:** (yes or no)	yes
0-4-1	Authorized officer	Kuiper-Cristina, Nathalie

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0-5-1	Authorized officer	

CLAIMS

1. A modified microorganism having, compared to its wild-type, a reduced activity of the enzyme that is encoded by the *wcaJ*-gene.

2. Modified microorganism according to claim 1, wherein the wild-type from which the modified microorganism has been derived belongs to the family of *Enterobacteriaceae*, *Pasteurellaceae*, *Bacillaceae* or *Corynebacteriaceae*.

3. Modified microorganism according to claim 3, wherein the wild-type from which the modified microorganism has been derived has a 16S rDNA of **SEQ ID NO: 1** or a sequence, which shows a sequence homology of at least 96 with **SEQ ID NO: 1**.

4. Modified microorganism according to claim 1 or 2, wherein the wild-type from which the modified microorganism has ben derived belongs to the genus *Basfia*.

5. Modified microorganism according to claim 3, wherein the wild-type from which the modified microorganism has been derived belongs to the species *Basfia succiniciproducens*.

6. Modified microorganism according to claim 4, wherein the wild-type from which the modified microorganism has been derived is *Basfia succiniciproducens* strain DD1 as deposited under DSM 18541 with the DSMZ, Germany.

7. Modified microorganism according to anyone of claims 1 to 6, wherein the *wcaJ*-gene comprises a nucleic acid selected from the group consisting of:
   a) nucleic acids having the nucleotide sequence of **SEQ ID NO: 3**;
   b) nucleic acids encoding the amino acid sequence of **SEQ ID NO: 4**;
   c) nucleic acids which are at least 70% identical to the nucleic acid of a) or b), the identity being the identity over the total length of the nucleic acids of a) or b);
   d) nucleic acids encoding an amino acid sequence which is at least 70% identical to the amino acid sequence encoded by the nucleic acid of a) or b), the identity being the identity over the total length of amino acid sequence encoded by the nucleic acids of a) or b);
   e) nucleic acids capable of hybridizing under stringent conditions with a complementary sequence of any of the nucleic acids according to a) or b); and
   f) nucleic acids encoding the same protein as any of the nucleic acids of a) or b), but differing from the nucleic acids of a) or b) above due to the degeneracy of the genetic code.

8. Modified microorganism according to anyone of claims 1 to 7, wherein the *wcaJ*-gene is modified

9. Modified microorganism according to claim 8, wherein the modification of the *wcaJ*-gene is achieved by a deletion of the *wcaJ*-gene or at least a part thereof, a deletion of a regu-

latory element of the *wcaJ*-gene or at least a part thereof or an introduction of at least one mutation into the *wcaJ*-gene.

10. Modified microorganism according to claim 9, wherein the at least one mutation in the *wcaJ*-gene leads to the expression of a truncated enzyme encoded by the *wcaJ*-gene.

11. Modified microorganism according to claim 10, wherein in the truncated enzyme at least 100 amino acids of the wild-type enzyme encoded by the *wcaJ*-gene are deleted from the C-terminal end.

12. Modified microorganism according to anyone of claims 1 to 11, wherein the microorganism further has, compared to its wild-type,
i) a reduced pyruvate formate lyase activity,
ii) a reduced lactate dehydrogenase activity, or
iii) a reduced pyruvate formate lyase activity and a reduced lactate dehydrogenase activity.

13. Modified microorganism according to claim 12, wherein the microorganism comprises:
A) a deletion of the *ldhA*-gene or at least a part thereof, a deletion of a regulatory element of the *ldhA*-gene or at least a part thereof or an introduction of at least one mutation into the *ldhA*-gene;
B) a deletion of the *pflD*-gene or at least a part thereof, a deletion of a regulatory element of the *pflD*-gene or at least a part thereof or an introduction of at least one mutation into the *pflD*-gene;
C) a deletion of the *pflA*-gene or at least a part thereof, a deletion of a regulatory element of the *pflA*-gene or at least a part thereof or an introduction of at least one mutation into the *pflA*-gene;
D) a deletion of the *ldhA*-gene or at least a part thereof, a deletion of a regulatory element of the *ldhA*-gene or at least a part thereof or an introduction of at least one mutation into the *ldhA*-gene
and
a deletion of the *pflD*-gene or at least a part thereof, a deletion of a regulatory element of the *pflD*-gene or at least a part thereof or an introduction of at least one mutation into the *pflD*-gene;
or
E) a deletion of the *ldhA*-gene or at least a part thereof, a deletion of a regulatory element of the *ldhA*-gene or at least a part thereof or an introduction of at least one mutation into the *ldhA*-gene
and
a deletion of the *pflA*-gene or at least a part thereof, a deletion of a regulatory element of the *pflA*-gene or at least a part thereof an introduction of at least one mutation into the *pflA*-gene.

14. A method of producing an organic compound comprising:
    I) cultivating the modified microorganism according to anyone of claims 1 to 13 in a culture medium comprising at least one assimilable carbon source to allow the modified microorganism to produce the organic compound, thereby obtaining a fermentation broth comprising the organic compound;
    II) recovering the organic compound from the fermentation broth obtained in process step I).

15. Method according to claim 14, wherein the organic compound is succinic acid.

16. Method according to claims 14 or 15, wherein the assimilable carbon source is selected from the group consisting of sucrose, maltose, D-glucose, glycerol, mixtures of glycerol and D-glucose, mixtures of glycerol and sucrose, mixtures of glycerol and D-xylose, mixtures of glycerol and mixtures of maltose and D-glucose and fructose.

17. Method according to anyone of claims 14 to 16, wherein the process further comprises the process step:
    III) conversion of the organic compound contained in the fermentation broth obtained in process step I) or conversion of the recovered organic compound obtained in process step II) into a secondary organic product being different from the organic compound by at least one chemical reaction.

18. Method according to claim 17, wherein the organic compound is succinic acid and wherein the secondary organic product is selected from the group consisting of succinic acid esters or polymers thereof, tetrahydrofuran (THF), 1,4-butanediol (BDO), gamma-butyrolactone (GBL) and pyrrolidones.

19. Use of a modified microorganism according to anyone of claims 1 to 13 for the fermentative production of an organic compound

20. Use according to claim 19, wherein the organic compound is succinic acid.

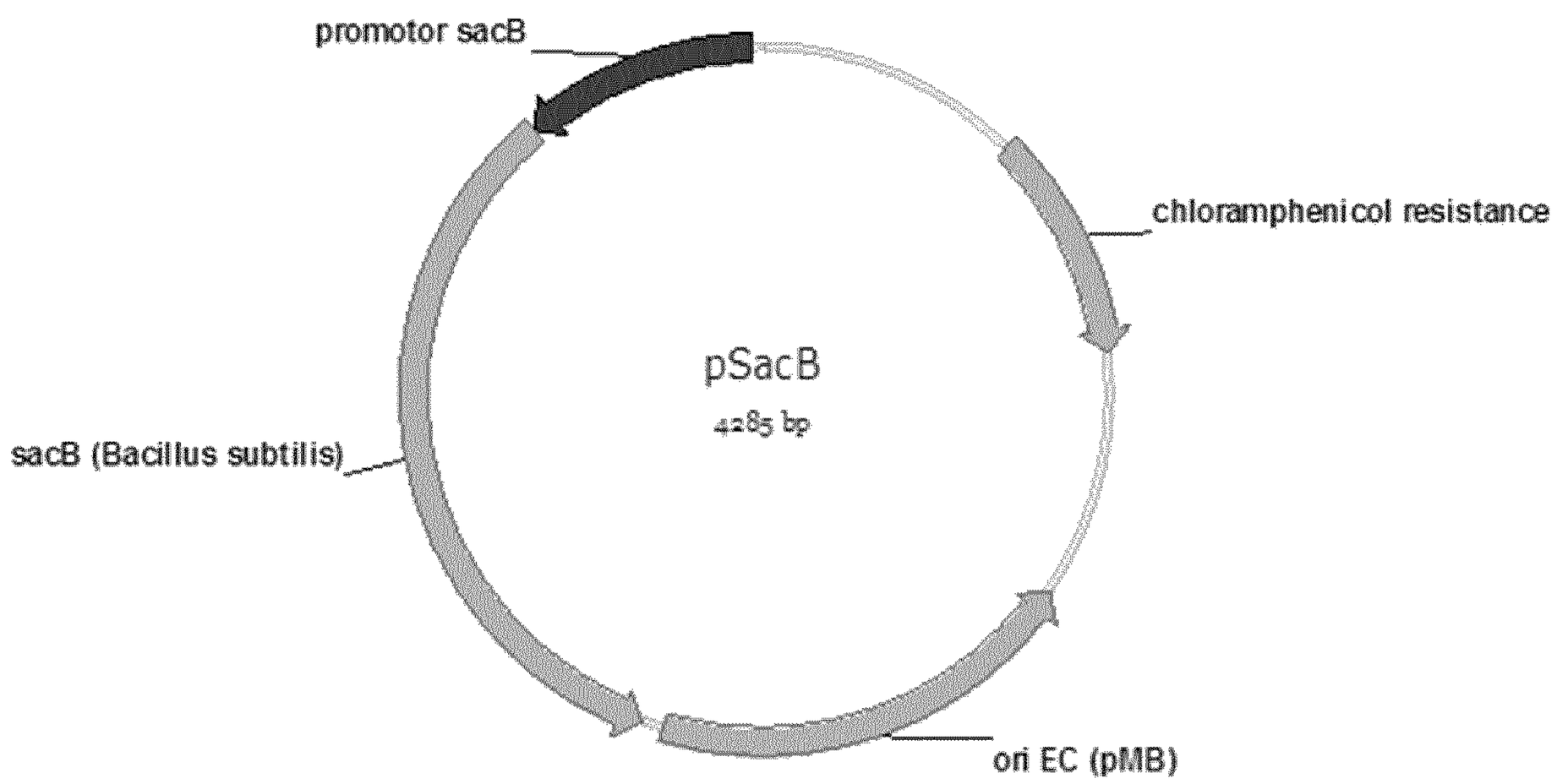
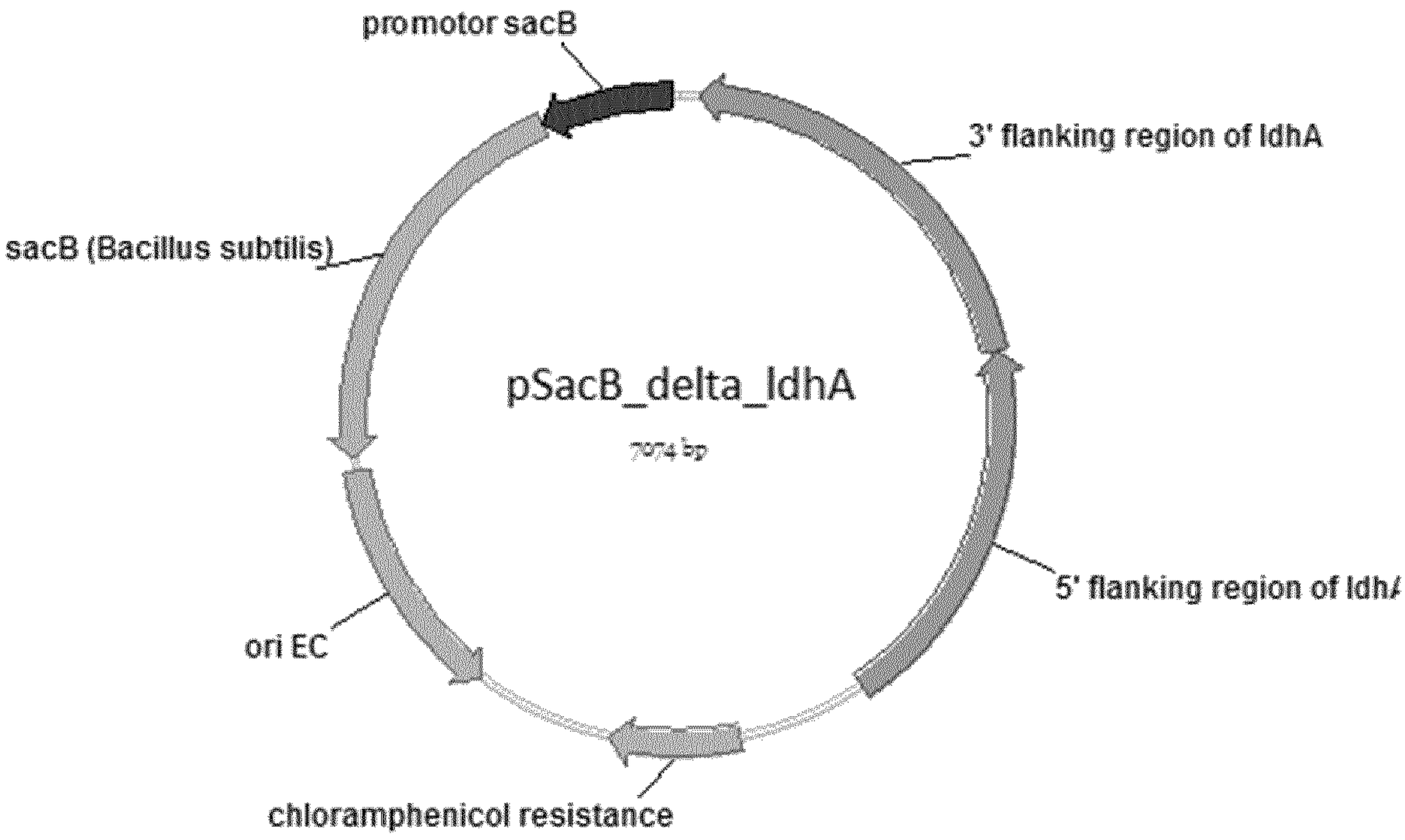

promotor sacB
chloramphenicol resistance
pSacB
4285 bp
sacB (Bacillus subtilis)
ori EC (pMB)

Fig. 1


promotor sacB
3' flanking region of ldhA
sacB (Bacillus subtilis)
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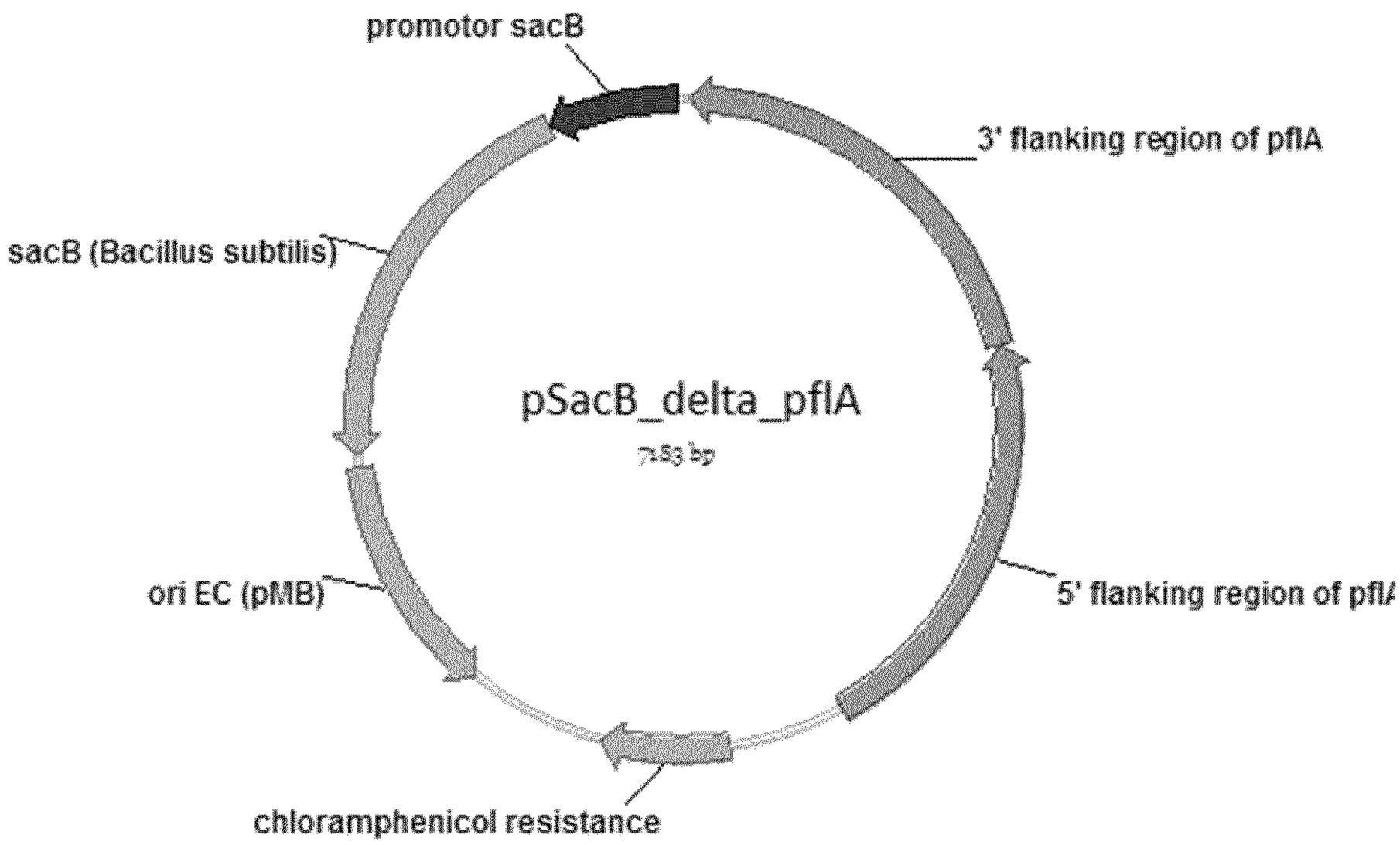
7074 bp
5' flanking region of ldhA
ori EC
chloramphenicol resistance

Fig. 2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3' flanking region of pflA
sacB (Bacillus subtilis)
pSacB_delta_pflA
7853 bp
ori EC (pMB)
5' flanking region of pflA
chloramphenicol resistance

Fig. 3

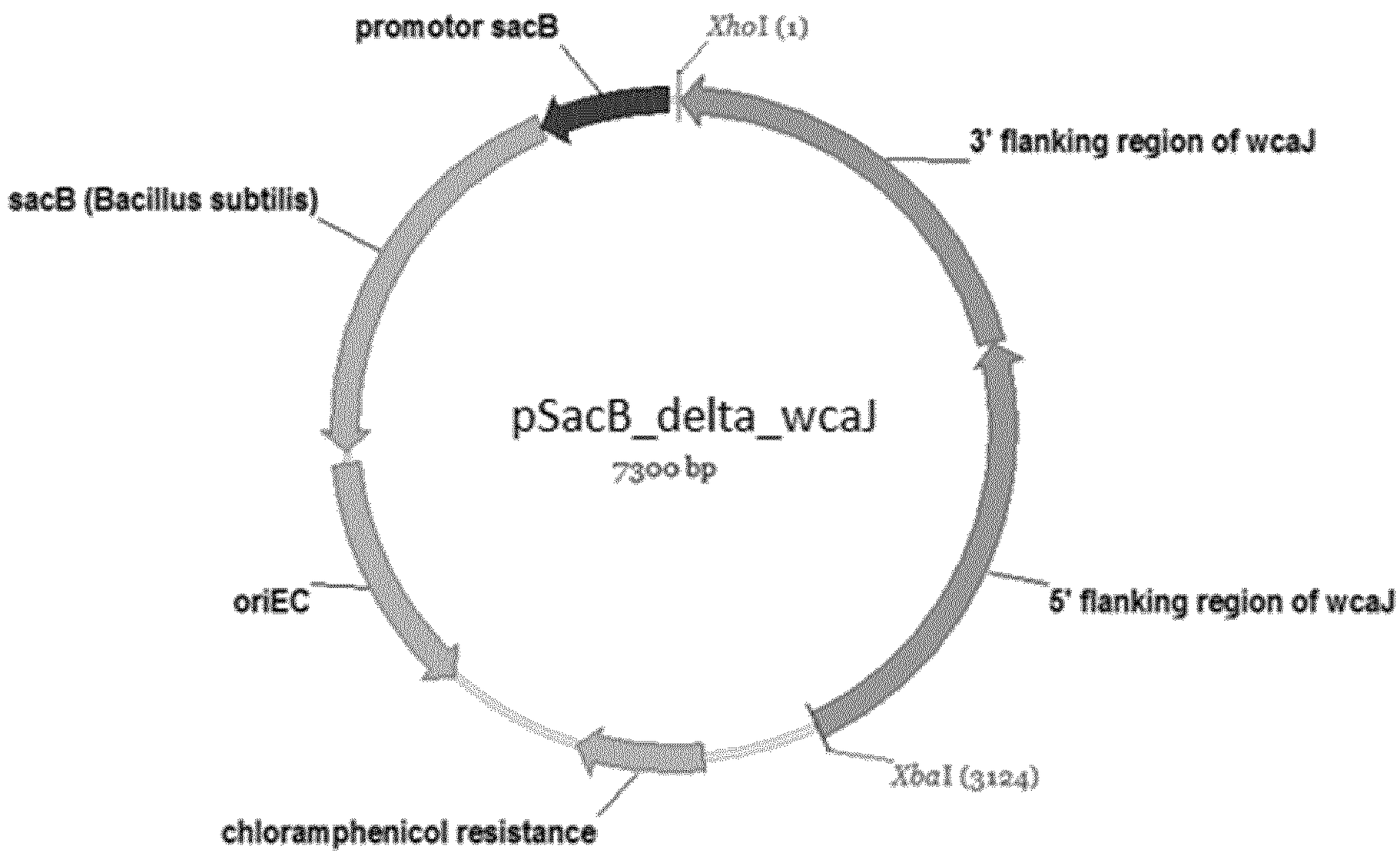

promotor sacB
XhoI (1)
3' flanking region of wcaJ
sacB (Bacillus subtilis)
pSacB_delta_wcaJ
7300 bp
oriEC
5' flanking region of wcaJ
XbaI (3124)
chloramphenicol resistance

Fig. 4

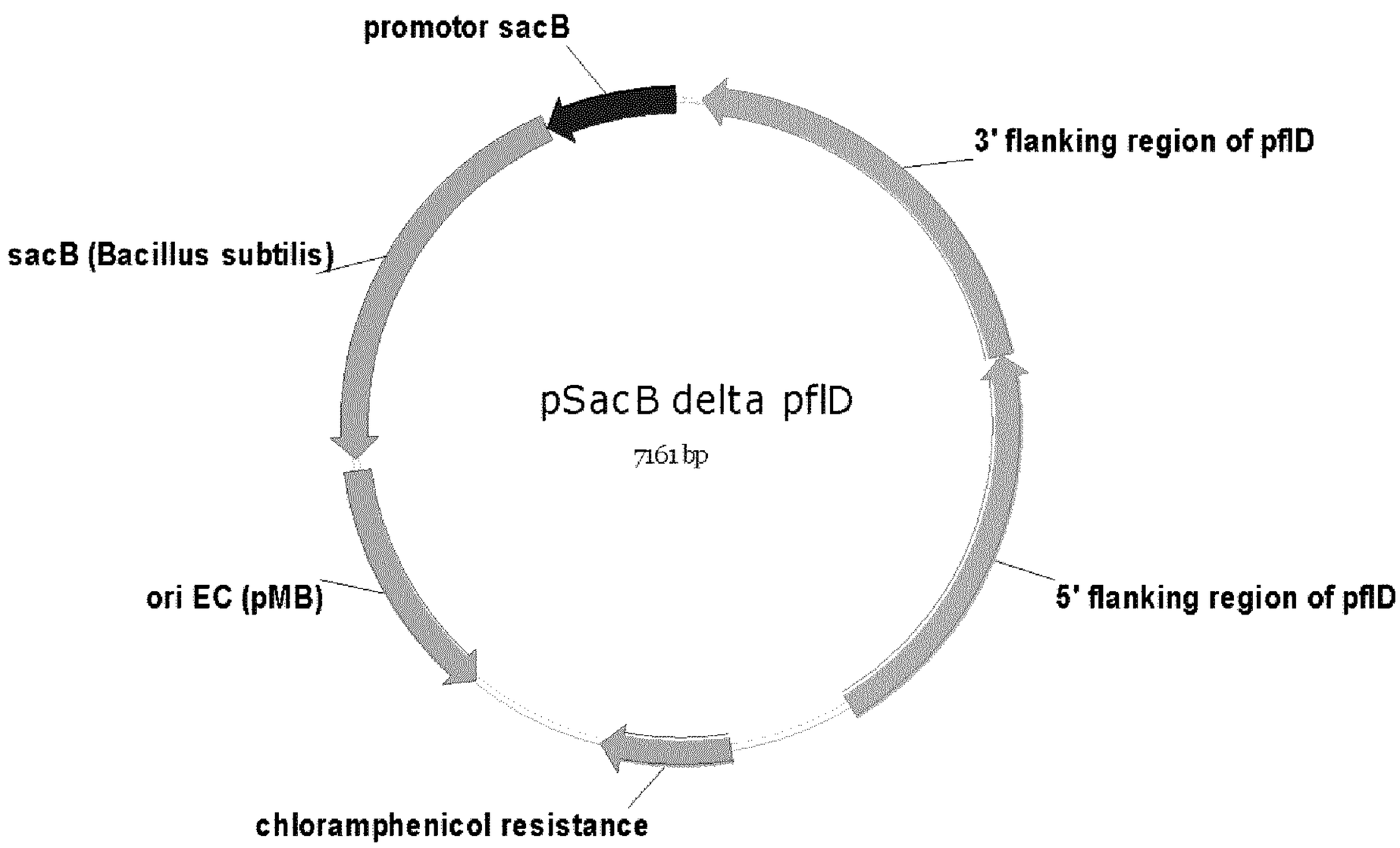

promotor sacB
3' flanking region of pflD
sacB (Bacillus subtilis)
pSacB delta pflD
7161 bp
ori EC (pMB)
5' flanking region of pflD
chloramphenicol resistance

Fig. 5

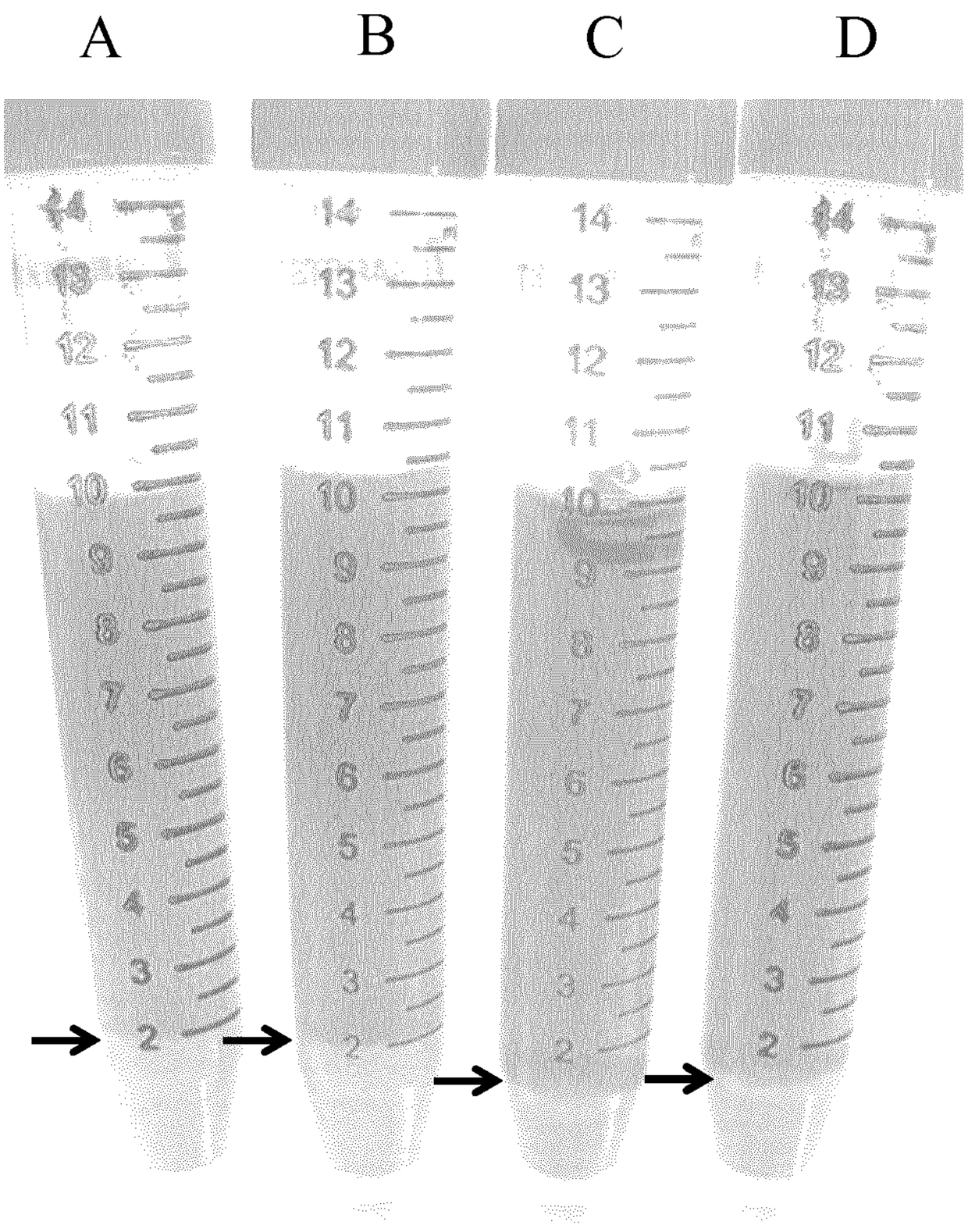
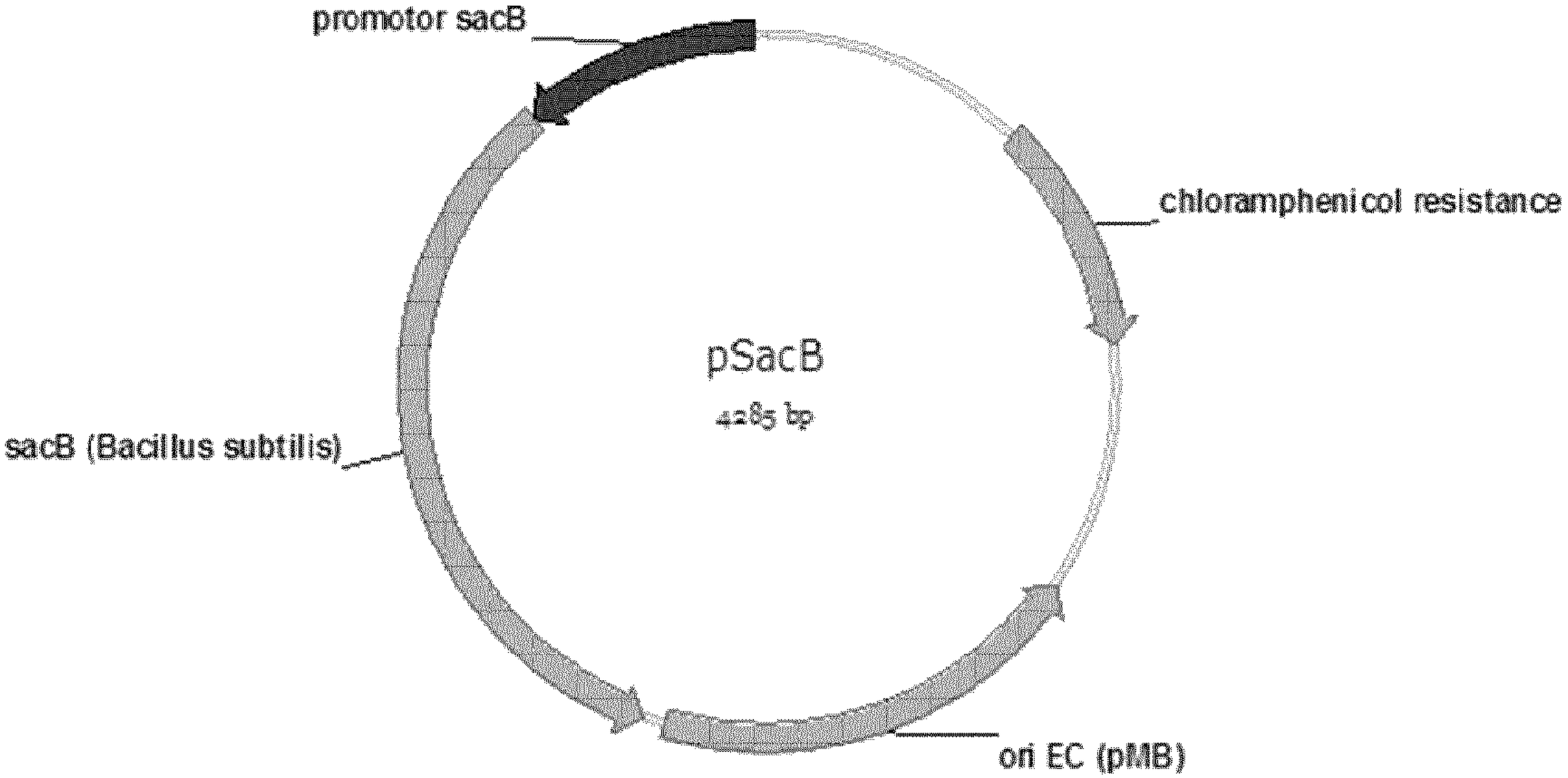


Fig. 6


promotor sacB
chloramphenicol resistance
pSacB
4285 bp
sacB (Bacillus subtilis)
ori EC (pMB)