amarchand mangaldas

Our Ref.: DR/AKV/sg/0034058.P3234IN00

November 12, 2014

E-FILING

The Controller of Patents
The Patent Office
New Delhi

Dear Sir,

Re: New Indian Patent Application based on PCT/IB2013/001071

International Filing Date:	April 25, 2013
Applicant:	Adisseo France S.A.S.
Title:	A Method of Production of 2,4-Dihydroxybutyric Acid
Priority:	International Patent Application No. PCT/IB2012/001123 dated April 26, 2012

Enclosed herewith is an application accompanied by a complete specification and supporting documents for grant of a patent under the Patents Act, 1970 by:

Adisseo France S.A.S., a company organized under the laws of France, of Immeuble Antony Parc II, 10, place du Général de Gaulle, 92160 Antony, France.

The requisite fee submitted in respect of making the application is as follows:

1.	On application for patent claiming a single priority	Rs. 8,000
2.	For additional sheets of specification	Rs. 1,00,000
3.	For additional claims	Rs. 24,000
	Total	**Rs. 1,32,000**

The applicant undertakes to submit an executed power of authority within one month from the filing date.

As required by Public Notice no. CG/Public Notice/PO/2012/15 dated July 2, 2012, the applicant has refrained from submitting documents already available with the International Bureau in connection with the corresponding PCT application. However, upon the Learned Controller's request, the applicant would submit any requested document (insofar as available) that is otherwise required to be furnished to the Patent Office by the International Bureau.

Contd...p/2

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amarchand mangaldas

The Controller is respectfully requested to take the application on record.

Yours sincerely,

Dev Robinson

Dev Robinson
IN/PA-367

Enc.:

i) Form 1;
ii) Complete specification (in conformation with the international application), No. of pages including sequence listings 155, No. of claims 25;
iii) Drawings, No. of sheets 3;
iv) Sequence listing;
v) Form 3;
vi) Form 5; and
vii) Abstract.

Herewith prescribed fee of Rs. 1,32,000/- through electronic transfer.

To Follow:

i) Power of authority; and
ii) Proof of right

FORM 1 THE PATENTS ACT 1970 (39 of 1970) & THE PATENTS RULES, 2003 APPLICATION FOR GRANT OF PATENT (*See* Sections 7, 54 & 135 and Rule 20(1)	(FOR OFFICE USE ONLY) Application No: Filing Date: Amount of Fee Paid: CBR No: Signature:

1. APPLICANT

Name	Nationality	Address
Adisseo France S.A.S.	France	Immeuble Antony Parc II 10, place du Général de Gaulle 92160 Antony France

2. INVENTORS

Name	Nationality	Address
WALTHER, Thomas	France	16 Impasse Julien Loupiac 31120 Lacroix-Falgarde France
DRESSAIRE, Clémentine	France	19 rue d'Eole 34340 Marseillan France
CORDIER, Hélène	France	Appt 115, Bat C 10 rue Claudius Rougenet 31500 Toulouse France
FRANCOIS, Jean Marie	France	21 rue Despiau 31100 Toulouse France

3. TITLE OF THE INVENTION: "A METHOD OF PRODUCTION OF 2,4-DIHYDROXYBUTYRIC ACID"

4. ADDRESS FOR CORRESPONDENCE OF APPLICANT/AUTHORIZED PATENT AGENT IN INDIA Amarchand & Mangaldas & Suresh A. Shroff & Co. Amarchand Towers 216, Okhla Industrial Estate Phase-III, New Delhi - 110020	Telephone No.: (91) (11) 26920500, 41590700 Fax No.: (91) (11) 26922900 Mobile No.: 91 98100 10435 E-mail: dev.robinson@amarchand.com

5. PRIORITY PARTICULARS OF THE APPLICATION(S) FILED IN CONVENTION COUNTRY

Country	Application Number	Filing Date	Name of the Applicant	Title of the Invention
International Application	PCT/IB2012/001123	April 26, 2012	Adisseo France S.A.S.	A Method of Production of 2,4-Dihydroxybutyric Acid

6. PARTICULARS FOR FILING PATENT COOPERATION TREATY (PCT) NATIONAL PHASE APPLICATION

International Application Number	International filing date as allotted by the receiving office
PCT/IB2013/001071	April 25, 2013

~~7. PARTICULARS FOR FILING DIVISIONAL APPLICATION~~

~~Original (first) application number~~	~~Date of filing of Original (first) application~~

~~8. PARTICULARS FOR FILING PATENT OF ADDITION~~

~~Main application/patent Number~~	~~Date of filing of main application~~

9. DECLARATIONS:

(i) Declaration by the inventor(s)

We, the above named inventors, are the true & first inventors for this invention and declare that the applicant herein is our assignee.

(a) Date _____

(b) Signature_____

(c) Name of Inventor: WALTHER, Thomas

(a) Date _____

(b) Signature_____
(c) Name of Inventor: DRESSAIRE, Clémentine

(a) Date _____

(b) Signature_____
(c) Name of Inventor: CORDIER, Hélène

(a) Date _____

(b) Signature_____
(c) Name of Inventor: FRANCOIS, Jean Marie

~~(ii) Declaration by the applicant in the convention country~~
~~I, the applicant in the convention country, declare that the applicant herein is my assignee.~~
~~(a) Date _____~~
~~(b) Signature_____~~
~~(c) Name:~~

(iii) Declaration by the applicant:
We, the applicant hereby declare that:-

- ❑ We are in possession of the above-mentioned invention. √
- ❑ The complete specification relating to the invention is filed with this application. √
- ❑ The invention as disclosed in the specification uses biological material from India and the necessary permission from the competent authority shall be submitted by us before the grant of patent to us. ×
- ❑ There is no lawful ground of objection to the grant of the patent to us. √
- ❑ We are the assignee of the true & first inventors. √
- ❑ The application, particulars of which are given in Para-5, was the first application in respect of our invention. √
- ❑ We claim priority from the above mentioned application and state that no application for protection in respect of the invention had been made before that date by us or by any person from which we derive title. √
- ❑ Our application in India is based on international application under Patent Cooperation Treaty (PCT) as mentioned in Para-6. √
- ❑ The application is divided out of our application, particulars of, which are given in Para-7 and pray that this application may be treated as deemed to have been filed on ___under sec.16 of the Act. ×
- ❑ The said invention is an improvement in or modification of the invention particulars of which are given in Para-8. ×

10. Following are the attachments with the application:

i) Complete specification (in conformation with the international application), No. of pages including sequence listings 155, No. of claims 25;
ii) Drawings, No. of sheets 3;
iii) Sequence listing;
iv) Form 3;
v) Form 5; and
vi) Abstract.

Herewith prescribed fee of Rs. 1,32,000/- through electronic transfer.

We hereby declare that to the best of our knowledge, information and belief the facts and matters stated herein are correct and we request that a patent may be granted to us for the said invention.

Dated this 12th day of November, 2014.

Adisseo France S.A.S.

Dev Robinson

(Dev Robinson)
of Amarchand & Mangaldas &
Suresh A. Shroff & Co.
Attorneys for the Applicant

The Controller of Patents
The Patent Office
New Delhi

FORM 2

THE PATENTS ACT 1970

(39 OF 1970)

&

THE PATENTS RULES, 2003

COMPLETE SPECIFICATION

(*See* section 10 and rule 13)

1. TITLE OF THE INVENTION :

"A METHOD OF PRODUCTION OF 2,4-DIHYDROXYBUTYRIC ACID"

2. APPLICANT:

(a)	NAME:	Adisseo France S.A.S.
(b)	NATIONALITY:	France
(c)	ADDRESS:	Immeuble Antony Parc II, 10, place du Général de Gaulle 92160 Antony, France

3. PREAMBLE TO THE DESCRIPTION

COMPLETE

The following specification particularly describes the invention and the manner in which it is to be performed.

A method of production of 2,4-dihydroxybutyric acid

The present invention relates to a novel method of production of 2,4-dihydroxybutyric acid from malate and/or glyoxylate and/or succinyl-CoA by the implementation of a synthetic pathway that converts malate and/or glyoxylate and/or succinyl-CoA into malylCoA, malylCoA into malate-4-semialdehyde, and then converting said malate-4-semialdehyde into 2,4-dihydroxybutyric acid (2,4-DHB).

The carboxylic acids cited within the present application are equally named under their salt (e.g. 2,4-dihydroxyburyrate) or acid forms (e.g. 2,4-dihydroxybutyric acid).

2,4-dihydroxybutyric acid (equally 2,4-DHB or DHB) is a compound of considerable economic interest. DHB can be readily converted into α-hydroxy-γ-butyrolactone in aqueous media by adjusting the appropriate pH. α-hydroxy-γ-butyrolactone is a prominent precursor for the production of the methionine substitute, 2-hydroxy-4-(methylthio)-butyrate (HMTB), (US 2009/318715) which has a large market in animal nutrition. At present, α-hydroxy-γ-butyrolactone is derived from γ-butyrolactone by a multi-stage process that implies halogenation of the γ-butyrolactone in position α, and subsequent substitution of the halogen atom by a hydroxyl group in alkaline medium (US 2009/318715).

From growing oil prices, the need for the production of DHB from renewable resources arises. Microorganisms are capable of transforming biomass-derived raw material, e.g. sugars or organic acids, into a large variety of different chemical compounds (Werpy & Petersen, 2004). With the growing body of biochemical and genomic information, it is possible to modify microorganisms such that they overproduce naturally occurring metabolic intermediates with high yield and productivity (Bailey, 1991). Optimization of production microorganisms often requires rational engineering of metabolic networks which ensures, among others, overexpression of enzymes required for the biosynthesis of the metabolite of interest, and alleviation of product feedback inhibition. Another possibility is the implementation of novel enzymatic systems that catalyze the production of a metabolite of interest.

Metabolic engineering approaches and enzymatic catalyses require detailed knowledge of the biochemistry and regulation of the metabolic pathway

leading to the metabolite of interest. In the case of 2,4-DHB production, this information is not available. Only few studies report the occurrence of 2,4-DHB in patients with succinic semialdehyde dehydrogenase deficiency (Shinka *et al.*, 2002) without, however, identifying enzymatic reactions implicated in DHB production. The zymotic or enzymatic production of 2,4-DHB, therefore, requires (i) the identification of a thermodynamically feasible pathway which transforms an accessible precursor into 2,4-DHB, (ii) the identification or construction of enzymes that are capable to catalyze individual reaction steps in the pathway and (iii) the functional expression of the pathway enzymes in an appropriate production organism.

The present invention has as an objective to satisfy these needs.

Accordingly, one object of the present invention is a method of producing 2,4-DHB comprising a first step of converting malate and/or glyoxylate and/or succinyl-CoA in malyl-CoA, a second step of converting malyl-CoA in malate-4-semialdehyde and, a third step of converting malate-4-semialdehyde in 2,4-DHB.

Accordingly, one object of the present invention is a method of producing 2,4-DHB which comprises a first reaction (see Figure 1), wherein malate is converted into malyl-CoA by the action of an enzyme which possesses malyl-CoA synthetase activity [1.1], and/or wherein succinyl-CoA is converted into malyl-CoA by the action of an enzyme having a succinyl-CoA:(L)-malate CoA transferase activity [1.2], and/or wherein glyoxylate is converted into malyl-CoA by the action of an enzyme which possesses malyl-CoA lyase activity [1.3]. In the second reaction [2], malyl-CoA is converted into malate-4-semialdehyde by the action of an enzyme which possesses malyl-CoA reductase activity. In the third reaction [3], malate-4-semialdehyde is converted into DHB by the action of an enzyme which possesses DHB dehydrogenase activity. More precisely, reaction [3] is catalysed by an enzyme bearing malate-4-semialdehyde reductase activity in the biosynthetic sense of the pathway.

Within another aspect of the invention, the first step of the method of producing 2,4-DHB involves an enzyme having malyl-CoA synthetase

(equally named malate thiokinase or malate-coenzyme A ligase (ADP forming), EC 6.2.1.9), succinyl-CoA:(L)-malate CoA transferase (EC 2.8.3.-), or malyl-CoA lyase (EC 4.1.3.24) activity that transforms malate, succinyl-CoA, or glyoxylate, respectively, into malyl-CoA.

Said enzymes have been identified in methylotrophic bacteria that employ the serine cycle for fixation of formaldehyde (Chistoserdova *et al.*, 2009; Smejkalová *et al.*, 2010; Vuilleumier *et al.*, 2009), in bacteria that rely on acetate assimilation pathways that are independent from the glyoxylate cycle and isocitrate lyase activity (Meister *et al.*, 2005), and in bacteria that employ the 3-hydroxypropionate CO2-fixation cycle for autotrophic growth (Zarzycki *et al.*, 2009).

Proteins sharing homology with the above enzymes are also another aspect of the invention such as functional variants or functional fragments.

Malyl-CoA synthetase consists of two subunits, MtkA and MtkB. Therefore, according to the invention, proteins comprising a malyl-CoA synthetase activity designate all polypeptides having at least 30 % of identity with the protein sequences of the malyl-CoA synthetase subunits MtkA and MtkB of M. *petroleiphilum* (YP 001022444 and YP 001022445) *Methylobacter extorquens* (YP002962851 and YP 002962852) or two subunits SucC and SucD of M. *capsulatus* (YP 114179 and YP 114180), preferentially at least 50 % and more preferentially 70 % of identity.

Malyl-CoA lyase is a homohexamer and found in bacteria that do not employ the glyoxylate cycle for acetate assimilation (Meister *et al.*, 2005). Therefore, according to the invention, proteins comprising a malyl-CoA lyase activity designate all polypeptides having at least 30 % of identity with the protein sequences of the malyl-CoA lyase, Mcl, of *Methylobacter extorquens*, *Rhodobacter capsulatus*, or *Streptomyces coelicolor*, preferentially at least 50 % and more preferentially 70 % of identity.

In a further aspect of the invention, the malyl-CoA lyase of the invention is represented by SEQ ID No. 1 or by any variant thereof.

Succinyl-CoA:(L)-malate CoA transferase consists of two subunits, SmtA and SmtB (Zarzycki *et al.*, 2009)(Friedmann *et al.*, 2006). Therefore, according to the invention, proteins having a succinyl-CoA:(L)-malate CoA transferase activity designate all polypeptides having at least 30 % of identity with the protein sequences of the succinyl-CoA:(L)-malate CoA transferase subunits SmtA and SmtB of *Chloroflexus aurantiacus (represented by SEQ ID No. 191 and SEQ ID No. 193* or encoded *by SEQ ID No. 192* and *SEQ ID No. 194)*, preferentially at least 50 % and more preferentially 70 % of identity.

More generally, within the meaning of the invention the identity between two protein sequences can be determined by methods well known by the skilled man in the art. Examples of such methods are the use of the CLUSTALW (Larkin *et al.*, 2007) software (http://www.ebi.ac.uk/Tools/msa/clustalw2/ with the default parameters indicated on the website) or the BLAST alignment program (http://blast.ncbi.nlm.nih.gov/Blast.cgi with the default parameters indicated on the website).

The term functional variant encompasses enzymes that may present substantial sequence modifications when compared to the sequences specifically described within the present application but that still retain the original enzymatic activity.

The term functional fragment, according to the invention, means that the sequence of the enzyme may comprise less amino acids than the original one but said truncated enzyme still retains the original enzymatic activity.

Improvement of said enzymes can be obtained by at least one mutation, said mutation(s) improving the activity and/or substrate affinity of the mutated enzyme for malate, succinyl-CoA, or glyoxylate respectively.

Within the present invention, the expression "improve the activity and/or substrate affinity" means that the enzyme before mutation was either

- unable to use the substrate, and/or
- synthesized the product of the reaction at a maximum specific rate at least three times lower, and/or

- had an affinity for malate, succinyl-CoA or glyoxylate, malyl-CoA or malate-4-semialdehyde that was at least two more preferably three times lower.

The malyl-CoA synthetase and the malyl-CoA lyase activities can be measured by the enzymatic tests described by (Smejkalová *et al.*, 2010) or (Meister *et al.*, 2005), respectively. The succinyl-CoA:(L)-malate CoA transferase activity can be measured as described by (Friedmann *et al.*, 2006)

Within a still further aspect, the second step of the method of producing 2,4-DHB according to the invention involves an enzyme having malyl-CoA reductase activity characterized in that it transforms malyl-CoA into malate-4-semialdehyde.

Said enzyme can be identified among enzymes having malonyl-CoA reductase, a succinyl-CoA reductase or reported 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, cinnamoyl-CoA reductase, or acetaldehyde dehydrogenase activity, or they can be obtained by modification of said enzymes.

Malonyl-CoA reductase (EC 1.2.1.75) and succinyl-CoA reductase (EC 1.2.1.76) were found in bacteria that possess a modified 3-hydroxypropionate cycle for carbon dioxide fixation (Alber *et al.*, 2006; Kockelkorn & Fuchs, 2009), and in bacteria that employ an anaerobic succinate degradation pathway (Seedorf *et al.*, 2008; Söhling & Gottschalk, 1993). HMG-CoA reductase (EC 1.1.1.38, EC 1.1.1.88) is part of the biosynthetic pathway of isoprenoids in eukaryotes and some bacteria. Cinnamoyl-CoA reductase (EC 1.2.1.44), is an enzyme implicated in lignin biosynthesis (Kawasaki *et al.*, 2006). Acetaldehyde dehydrogenase (EC 1.2.1.10) is found in a large variety of bacteria and catalyses the entry into the ethanol producing pathway or the detoxification of acetaldehyde.

Within a further aspect of the invention, the malyl-CoA reductase is represented by ID No. 7, or SEQ ID No.10 or by any functional variant thereof or any functional fragment thereof.

Therefore, according to the invention, proteins having a malonyl-CoA reductase activity designate all polypeptides having at least 30 % of identity with the protein sequences of the *Sulfolobus tokodaii* malonyl-CoA reductase, Mcr (SEQ ID No. 7). Preferentially they have at least 50 % and more preferentially 70 % of identity.

The malonyl-CoA reductase of *Chloroflexus auranthiacus* (SEQ ID No.189 encoded by SEQ ID No. 190) constitutes another aspect of the invention. Polypeptides having at least 30 % of identity with the protein sequences of *Chloroflexus auranthiacus are also part of the invention.* Preferentially they have at least 50 % and more preferentially 70 % of identity.

Therefore, according to the invention, proteins having a succinyl-CoA reductase activity designate all polypeptides having at least 30 % of identity with the protein sequences of the *Porphyromonas gingivalis* succinyl-CoA reductase, SucD (SEQ ID No. 10), or with the bifunctional *S. tokodaii* malonyl-CoA and succinyl-CoA reductase, Mcr (SEQ ID No. 7). Preferentially they have at least 50 % and more preferentially 70 % of identity.

The malyl-CoA reductase activity can be measured by the enzymatic test described in Example 2 (see "Enzymatic assay").

This enzyme activity can be improved by at least one mutation of an enzyme, said mutation(s) improving the activity and/or substrate affinity of the mutated enzyme for malyl-CoA or decreasing its activity on the natural substrate.

The present invention also encompasses modified malyl-CoA reductase having improved activities.

The malyl-CoA reductase according to the invention corresponds in a specific aspect to *Sulfolobus tokodaii* malonyl-CoA reductase comprising at least one mutation when compared to the wild type enzyme in at least one of the positions P111, L152, T154, L202, G203, D204, Y206, D207, K209, T210, T238,T239, D295, R318, wherein the naturally occurring amino acid in said positions is replaced by anyone of the other 19 naturally existing proteinogenic amino acids, that is by either alanine, arginine, asparagine, aspartic acid,

cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, or valine.

Within another aspect, the third step of the method of producing 2,4-DHB according to the invention involves a DHB dehydrogenase characterized in that it transforms malate-4-semialdehyde into 2,4-DHB, said enzyme bearing malate-4-semialdehyde reductase activity in the biosynthetic sense of the pathway.

Candidate DHB dehydrogenase enzymes that potentially already possess DHB dehydrogenase activity can be chosen from the class of beta-hydroxyacid dehydrogenases that act on C3, C4, or C5 compounds.

According to a still further aspect of the invention, said DHB dehydrogenase enzymes can be structurally and mechanistically related to β-hydroxyacid dehydrogenases such as tartronate semialdehyde reductases, succinate semialdehyde reductases, 4-hydroxybutyrate dehydrogenases, malonate semialdehyde reductases, methylbutyraldehyde reductases, zinc-type alcohol dehydrogenases, L-threonine-3-dehydrogenases, cinnamyl alcohol dehydrogenases, alcohol dehydrogenases, or homoserine dehydrogenases.

The present invention also deals with the use of a methylbutyraldehyde reductase, or of a succinic semialdehyde reductase (equally named as 4-hydroxybutyrate dehydrogenase), or of an alcohol dehydrogenase, to transform malate-4-semialdehyde in 2,4-DHB.

In another specific aspect of the invention, the DHB dehydrogenase corresponds to methylbutyraldehyde reductase (Ypr1) of *S. cerevisiae*, the succinic semialdehyde reductase of *M. sedula*, the 4-hydroxybutyrate dehydrogenase (4hbd) of *P. gingivalis*, or to the alcohol dehydrogenase (YqhD) of *Escherichia coli*.

In specific embodiments, said methylbutyraldehyde reductase is represented by SEQ ID No. 14, said succinic semialdehyde reductase is represented by SEQ ID No. 16, said 4-hydroxybutyrate dehydrogenase is

represented by SEQ ID No. 187, said alcohol dehydrogenase is represented by SEQ ID No. 185. The DHB dehydrogenase activity can be measured by the enzymatic test described in Example 3 (see "Enzymatic assay").

The affinity of DHB dehydrogenase for malate-4-semi aldehyde can be increased by at least one mutation of an enzyme, said mutation(s) increasing the activity and/or substrate affinity of the mutated enzyme for malate-4-semialdehyde, and/or reducing the activity or affinity for its natural substrate by at least factor 2.

The DHB dehydrogenase according to the invention corresponds in a specific aspect to *M. sedula* succinic semialdehyde reductase (SEQ ID No. 16) comprising at least one mutation when compared to the wild type enzyme in at least one of the positions S40, N43, H39 T49, F85, Q108, L281 and N305 wherein the naturally occurring amino acid in said positions is replaced by anyone of the other 19 naturally existing proteinogenic amino acids, that is by either alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, or valine.

As demonstrated in a non-exclusive example, the affinity of *M. sedula* succinic semialdehyde reductase for (L)-malate-4-semialdehyde was increased by introducing the double mutation H39R N43H by site-directed mutagenesis, as represented by SEQ ID No. 36. Simple mutants H39R (SEQ ID No. 32) and N43H (SEQ ID No. 34) are also encompassed by the present invention (Example 5).

. DHB dehydrogenase can be used to transform malate-4-semialdehyde into 2,4-DHB, which constitutes a further aspect of the invention.

The nucleic acid sequence of genes can be adapted to the codon usage of the host organism thereby increasing the production of the heterologously expressed proteins. This constitutes a further aspect of the invention.

The synthesis of a synthetic gene coding for *M. sedula* succinic semialdehyde reductase H39R N43H whose nucleotide sequence was

optimized for the expression of said enzyme in *E. coli* as represented by SEQ ID No. 38 is a further aspect of the invention.

In a still further aspect, the present invention also deals with nucleic acids, and more particularly with isolated nucleic acid sequences encoding malyl-CoA synthetase.

In a still further aspect, the present invention deals with isolated nucleic acid sequences encoding malyl-CoA lyase and more specifically by SEQ ID No.2.

In a still further aspect, the present invention deals with isolated nucleic acid sequences encoding malyl-CoA reductase and more specifically by SEQ ID No. 8, SEQ ID No.11, or SEQ ID No.190.

In a still further aspect, the present invention also deals with isolated nucleic acid sequences encoding a DHB dehydrogenase as described above.

In another aspect, said nucleic acid is represented by SEQ ID No. 15, SEQ ID No. 17, SEQ ID No. 37,SEQ ID No. 186 or SEQ ID No. 188.

In accordance with this invention, a "nucleic acid sequence" refers to a DNA or RNA molecule in single or double stranded form, preferably a DNA molecule. An "isolated DNA", as used herein, refers to a DNA which is not naturally-occurring or no longer in the natural environment wherein it was originally present, e.g., a DNA coding sequence associated with other regulatory elements in a chimeric gene, a DNA transferred into another host cell, or an artificial, synthetically-made DNA sequence having a different nucleotide sequence compared to any naturally-occurring DNA sequence.

The present invention also relates to a chimeric gene comprising, functionally linked to one another, at least one promoter which is functional in a host organism, a polynucleotide encoding anyone of the malyl-CoA synthetase or malyl-CoA lyase, malyl-CoA reductase, malonyl-CoA reductase, succinyl-CoA reductase or DHB dehydrogenase activities as defined according to the invention, and a terminator element that is functional in the same host organism. The various elements which a chimeric gene may contain are, firstly, elements regulating transcription, translation and maturation of proteins, such as a promoter, a sequence encoding a signal peptide or a transit peptide, or a

terminator element constituting a polyadenylation signal and, secondly, a polynucleotide encoding a protein. The expression "functionally linked to one another" means that said elements of the chimeric gene are linked to one another in such a way that the function of one of these elements is affected by that of another. By way of example, a promoter is functionally linked to a coding sequence when it is capable of affecting the expression of said coding sequence. The construction of the chimeric gene according to the invention and the assembly of its various elements can be carried out using techniques well known to those skilled in the art. The choice of the regulatory elements constituting the chimeric gene depends essentially on the host organism in which they must function, and those skilled in the art are capable of selecting regulatory elements which are functional in a given host organism. The term "functional" is intended to mean capable of functioning in a given host organism.

The promoters which the chimeric gene according to the invention may contain are either constitutive or inducible. By way of example, the promoters used for expression in bacteria may be chosen from the promoters mentioned below. For expression in *Escherichia coli* mention may be made of the lac, trp, lpp, phoA, recA, araBAD, prou, cst-I, tetA, cadA, nar, tac, trc, lpp-lac, Psyn, cspA, PL, PL-9G-50, PR-PL, T7, [lambda]PL-PT7, T3-lac, T5-lac, T4 gene 32, nprM-lac, VHb and the protein A promoters or else the Ptrp promoter (WO 99/64607). For expression in Gram-positive bacteria such as *Corynebacteria* or *Streptomyces*, mention may be made of the PtipA or PS1 and PS2 (FR91/09870) promoters or those described in application EP0629699A2. For expression in yeasts and fungi, mention may be made of the *K. lactis* PLAC4 promoters or the *K. lactis* Ppgk promoter (patent application FR 91/05294), the *Trichoderma* tef1 or cbh1 promoter (WO 94/04673), the *Penicillium* his, csl or apf promoter (WO 00/68401) and the *Aspergillus* gla promoter.

According to the invention, the chimeric gene may also comprise other regulatory sequences, which are located between the promoter and the coding sequence, such as transcription activators (enhancers).

As such, the chimeric gene of the invention comprises, in a specific embodiment at least, in the direction of transcription, functionally linked, a promoter regulatory sequence which is functional in a host organism, a nucleic

acid sequence encoding the malyl-CoA synthetase, and/or the succinyl-CoA:(L)- malate-CoA transferase, and/or the malyl-CoA lyase, the malyl-CoA reductase and the DHB dehydrogenase of the invention and a terminator regulatory sequence which is functional in said host organism.

The present invention also relates to a cloning and/or expression vector comprising a chimeric gene according to the invention or a nucleic acid sequence of the invention. The vector according to the invention is of use for transforming a host organism and expressing in this organism anyone of the malyl-CoA synthetase, and/or the succinyl-CoA:(L)- malate CoA transferase, and/or the malyl-CoA lyase, the malyl-CoA reductase and/or DHB dehydrogenase. This vector may be a plasmid, a cosmid, a bacteriophage or a virus. Preferentially, the transformation vector according to the invention is a plasmid. Generally, the main qualities of this vector should be an ability to maintain itself and to self-replicate in the cells of the host organism, in particular by virtue of the presence of an origin of replication, and to express anyone of the malyl-CoA synthetase, and/or the succinyl-CoA:(L)- malate CoA transferase and/or the malyl-CoA lyase, the malyl-CoA reductase and/or DHB dehydrogenase therein. For the purpose of stable transformation of a host organism, the vector may also integrate into the genome. The choice of such a vector, and also the techniques of insertion of the chimeric gene according to the invention into this vector and are part of the general knowledge of those skilled in the art. Advantageously, the vector used in the present invention also contains, in addition to the chimeric gene according to the invention, a chimeric gene encoding a selectable marker. This selectable marker makes it possible to select the host organisms which are effectively transformed, i.e. those which incorporated the vector. According to a particular embodiment of the invention, the host organism to be transformed is a bacterium, a yeast, a fungus. Among the selectable markers which can be used, mention may be made of markers containing genes for resistance to antibiotics, such as, for example, the hygromycin phosphotransferase gene. Other markers may be genes to complement an auxotrophy, such as the *pyrA, pyrB, pyrG, pyr4, arg4, argB* and *trpC* genes, the molybdopterin synthase gene or that of acetamidase. Mention may also be made of genes encoding readily identifiable enzymes such as the GUS enzyme, or genes encoding pigments or enzymes regulating the production of pigments in the transformed cells. Such selectable marker genes

are in particular described in patent applications WO 91/02071, WO 95/06128, WO 96/38567 and WO 97/04103.

The present invention also relates to transformed host organisms containing at least one chimeric gene according to the invention, either integrated into their genome or carried on an extra-chromosomal genetic element, for example a plasmid. In a more specific aspect of the invention, the transformed host organism comprises a nucleic acid of the invention encoding a polypeptide having malyl-CoA synthetase activity, and/or succinyl-CoA:(L)-malate-CoA transferase and/or malyl-CoA lyase activity or a chimeric gene comprising a nucleic acid encoding a polypeptide having malyl-CoA synthetase activity, and/or succinyl-CoA:(L)- malate CoA transferase and/or a malyl-CoA lyase activity or an expression vector comprising a nucleic acid encoding a polypeptide having malyl-CoA synthetase activity, and/or succinyl-CoA:(L)-malate CoA transferase and/or a malyl-CoA lyase activity, and/or a nucleic acid encoding a polypeptide having malyl-CoA reductase activity, or a chimeric gene comprising a nucleic acid encoding a polypeptide having malyl-CoA reductase activity or an expression vector comprising a nucleic acid encoding a polypeptide having malyl-CoA reductase activity, and/or a nucleic acid encoding a polypeptide having DHB dehydrogenase activity, a chimeric gene comprising a nucleic acid encoding a polypeptide having DHB dehydrogenase activity or an expression vector comprising a nucleic acid encoding a polypeptide having DHB dehydrogenase activity.

The activity of heterologously enzymes in the host organism is often limited by their poor solubility and the formation of inclusion bodies. Therefore, the present invention also relates to chimeric proteins in that a functional enzyme is physically fused to another protein or peptide (equally named fusion protein) in order to increase the activity of said enzyme upon expression in the host organism. Such fusion proteins are known in the art and are commonly selected among the following non-exclusive examples: maltose binding protein, Mbp, thioredoxin, ThrX, glutathione-S-transferase, Gst, transcription termination factor, NusA.

The term "host organism" is intended to mean any lower monocellular organism into which the chimeric gene(s), nucleic acid(s) or vector(s) according to the invention may be introduced in order to produce 2,4-DHB. Preferably, the host organism is a microorganism, in particular a fungus, for example of the *Penicillium*, *Aspergillus* and more particularly *Aspergillus flavus*, *Chrysosporium* or *Trichoderma* genus, a yeast, in particular of the *Saccharomycetaceae, Pichiaceae or Schizosaccharomycetaceae*, most preferentially *Saccharomyces cerevisiae*, *Schizosaccharomycespombe*, *Kluyveromyceslactis*, *Kluyveromyces marxianus*, or *Pichia jadinii, Pichia stipitis* or *Pichia pastoris*, a bacterium, preferentially selected among Enterobacteriaceae, Clostridiaceae, Bacillaceae, Streptomycetaceae, Streptococcaceae, Methylobacteriacae, and Corynebacteriaceae, most preferentially *Escherichia coli, Bacillus subtilis, Corynebacterium glutamicum, Clostridium acetobutylicum, Methylobacterium extorquens,* or *Lactococcus lactis.*

The host organism can be a host organism that naturally overproduces malate or succinate from sugars such as glucose or a host organism that was engineered to overproduce malate or succinate from sugars such as glucose and in which all potential membrane transporters that facilitate export of organic acids, such as malate, pyruvate, succinate, and fumarate have been deleted. The host organism can be an organism that was engineered to overproduce DHB and in which membrane transporters that facilitate export of organic acids such as malate, pyruvate, succinate, and fumarate have been deleted. Examples of permeases that facilitate export of malate and other organic acids are Mae1 from *Schizosaccharomyces pombe* (Camarasa *et al.*, 2001)(Grobler *et al.*, 1995), DctA from *Bacillus subtilis* (Groeneveld *et al.*, 2010), Dct 1-4 from *coli*, Jen1 from *S. cerevisiae* (Akita *et al.*, 2000). For an expert, it will be possible to identify candidate permeases in *E. coli* based on sequence identity. These constructions will serve to keep malate and other organic acids inside the cell to make them available for DHB production.

The expression "transformed host organism" is intended to mean a host organism which has incorporated into its genome, or in an extra chromosomal genetic element, for example a plasmid, at least one chimeric gene according to the invention, and consequently produces any one of malyl-

CoA synthetase, malyl-CoA lyase, malyl-CoA reductase and/or DHB dehydrogenase in cell interior or in a culture medium. To obtain the host organisms according to the invention, those skilled in the art may use one of the many known transformation methods.

One of these methods consists in bringing the cells of the host organisms to be transformed into contact with polyethylene glycol (PEG) and with the vectors according to the invention. Electroporation is another method, which consists in subjecting the cells to be transformed and the vectors of the invention to an electric field. Another method consists in directly injecting the vectors into the cells or the tissues by microinjection. The "biolistic" method may be used. It consists in bombarding cells or tissues with particles onto which the vectors of the invention are adsorbed (U.S. Pat. No. 4,945,050).

Several methods for transforming bacteria are described in the literature for *Escherichia coli* and other Gram-negative bacteria. Conjugation may also be used. For Gram-positive bacteria, electroporation may be used, and also protoplast transformation, in particular for bacteria of the *Streptomyces* genus.

Several methods for transforming fungi are also described in the literature. Protoplast transformation with PEG is described for *Aspergillus* in EP 0260762, and an adaptation of this method to the species *Penicillium funiculosum* is described in WO 00/36120. Transformation by restriction enzyme mediated integration, or REMI, is also known, as is protoplast transformation using bacteria of the *Agrobacterium* genus. Techniques for transforming yeasts are also described in the literature,

In a further aspect, the invention deals with a process of production of 2,4- DHB comprising the step of cultivating a transformed microorganism of the invention.

For the production of DHB various carbohydrates could be utilized individually or as a mixture such as glucose, fructose, sucrose, molasses, maltose, blackstrap molasses, starch hydrolysate (glucose, oligosaccharides), lactose, maltose, starch and starch hydrolysates, cellulose, cellulose hydrolysate, glycerol, acetate and certain hydrocarbons, oils and fats such as

soy bean oil, sunflower oil, groundnut oil and coconut oil as well as fatty acids such as e.g. palmitic acid, stearic acid and linoleic acid. Those substances may be used individually or as mixtures.

Various sources of nitrogen could be utilized individually or as mixtures for the commercial and pilot scale production, including inorganic compounds such as gaseous and aqueous ammonia, ammonium salts of inorganic or organic acids such as ammonium sulphate, ammonium nitrate, ammonium phosphate, ammonium chloride, ammonium acetate and ammonium carbonate. Alternatively, natural nitrogen containing organic materials like soybean-hydrolysate, soy protein HCl-hydrolysate (total nitrogen of about 7%), soy bean meal, soybean cake hydrolysate, corn steep liquor, casein hydrolysate, yeast extract, meat extract, malt extract, urea, peptones and amino acids may also be utilized

The production process can be carried out under aerobic, anaerobic, and oxygen limited conditions. It can be carried out as a fed-batch process or a batch process.

Said production of 2,4-DHB can be made by cultivating the host organism in media where malate (or another organic acid such as pyruvate, succinate, or fumarate) was added alone or together with another carbon source that ensures growth. Malate (and other organic acids) can be added either directly, or by designing a two-stage fermentation process where malate (or other organic acids) is produced in a first process stage by a malate-overproducing microorganism, and 2,4-DHB production is realised in the following stage by a host organism according to the invention.

Product separation and purification is very important factor enormously affecting overall process efficiency and product costs. Methods for product recovery commonly comprise the steps cell separation, as well as product purification, concentration and drying, respectively.

Cell separation

Ultra filtration and centrifugation can be used to separate cells from the fermentation medium. Cell separation from fermentation media is often complicated by high medium viscosity. Therefore, we can add additives such as mineral acids or alkali salts, or heating of the culture broth to optimize cell separation.

Product recovery

A variety of ion-exchange chromatographic methods can be applied for the separation of DHB either before or after biomass removal. They include the use of primary cation exchange resins that facilitate separation of products according to their isoelectric point. Typically, the resin is charged with the solution, and retained product is eluted separately following increase of pH (e.g. by adding ammonium hydroxide) in the eluent. Another possibility is the use of ion-exchange chromatography using fixed or simulated moving bed resins. Different chromatographic steps may have to be combined in order to attain adequate product purity. Those purification methods are more economical compared with a costly crystallization step, also providing additional advantages and flexibility regarding the form of final product.

Product concentration and drying

The purification process can also comprises a drying step which may involve any suitable drying means such as a spray granulator, spray dryer, drum dryer, rotary dryer, and tunnel dryer. Concentrated DHB solutions can be obtained by heating fermentation broths under reduced pressure by steam at 130°C using a multipurpose concentrator or thin film evaporator.

Efficient production of DHB can be ensured by optimizing carbon flux repartitioning in the metabolic network of the host organism and by ensuring sufficient NADPH and ATP supply for the three enzymes of the DHB pathway. Channeling of carbon flux into a desired metabolic pathway and supply of NAD(P)H cofactor is commonly facilitated by deleting or alleviating competing natural fermentative pathways. Nonexclusive examples are

- the optimization of malate production in *S. cerevisiae* by impeding the formation of ethanol (by the deletion of pyruvate decarboxylases (Zelle *et al.*, 2008)(Zelle *et al.*, 2010).
- the optimization of succinate or malate production in *E. coli* by impeding the formation of lactate (e.g. deletion of *ldhA*), the formation of acetate (e.g. deletion of *pta, ackA*), the formation of ethanol (e.g. deletion of adhE), the formation of formate (e.g. deletion of *pflB, focA*), the oxidation of pyruvate (e.g. deletion of *poxB*), the degradation of malate (deletion of *maeB* and *scfA*), the formation of succinate (e.g. deletion of *frdBC*), the formation of

methylglyoxal (deletion of *mgsA*) (Jantama *et al.*, 2008a)(Jantama *et al.*, 2008b)(Lin *et al.*, 2005)(Zhang *et al.*, 2011)(Sanchez *et al.*, 2005).

- the deletion of phosphoglucose isomerase, *pgi*, to channel carbon flux across the pentose phosphate pathway thereby increasing NADPH availability for biosynthetic reactions (Auriol *et al.*, 2011).

Another possibility to increase carbon flux and ATP supply for the production of organic acids is the engineering of the phosphoenolpyruvate (PEP)/pyruvate/oxaloacetate branch node (reviewed in (Sauer & Eikmanns, 2005)). Nonexclusive examples for metabolic engineering strategies that ensure the increase of carbon flux from phosphoenolpyruvate to oxaloacetate are:

- the optimization of malate production in *S. cerevisiae* by impeding the function of pyruvate kinase and increasing the activity of PEP carboxykinase (Zelle *et al.*, 2010).
- the optimization of succinate production in *E. coli* by increasing the activity of natural or heterologously expressed PEP carboxylase, PEP carboxykinase, or pyruvate carboxylase (Millard *et al.*, 1996)(Sanchez *et al.*, 2005)(Zhang *et al.*, 2011).

Another possibility to increase carbon flux and ATP supply for the production of organic acids in *E. coli* and other bacteria employing the PEP-consuming phosphotransferase system (PTS) for the initial phosphorylation step of glucose is the deletion of essential components of the PTS system (for example *ptsI* or *ptsG*) (Lin *et al.*, 2005)(Zhang *et al.*, 2009). To ensure further glucose uptake in mutants carrying deleterious mutations of the PTS system, the activity of alternative glucose uptake systems (e.g. GalP) has to be ensured.

Another possibility to increase carbon flux into the desired pathways for the production of organic acids is the engineering of the citric acid and glyoxylate cycle. Non-exclusive examples are

- the optimization of succinic acid production in *E. coli* by increasing the activity of isocitrate lyase (deletion of transcriptional repressor *iclR*) (Lin *et al.*, 2005)(Sanchez *et al.*, 2005)(Lin *et al.*, 2005; Sanchez *et al.*, 2005a).

- the optimization of succinic acid production by the deletion of isocitrate dehydrogenase, and/or succinate dehydrogenase (Lin et al., 2005).

Another possibility to increase the availability of malate, glyoxylate and acetyl-CoA, which are the substrates of the entry reactions into the DHB-producing pathways, is the attenuation of aspartate transaminase (*aspC, tyrB*), fumarase (*fumABC*) , fumarate reductase (*frdBC*), malate synthase (*aceB*) and glyoxylate reductase (*ghrAB*) enzymes.

In another metabolic setting it is possible to produce the 2,4-DHB precursor malate exclusively via the Krebs cycle and the glyoxylate shunt. This setting requires deletion of the cytosolic and membrane bound malate dehydrogenases, *mdh* and *mqo*, respectively. The approach largely avoids potential leakage of carbon flux into aspartate and its derivatives.

Another possibility to increase carbon flux into the desired pathways for the production of 2,4-DHB is the expression of appropriate pyruvate dehydrogenases and citrate synthases in the production organism. Natural pyruvate dehydrogenase and citrate synthase of *E. coli* are inhibited by high intracellular NADH concentrations rendering these enzymes less active under anaerobic conditions. In *E. coli*, the expression of a pyruvate dehydrogenase mutant that is insensitive to NADH resulted in the overproduction of acetyl-CoA under anaerobic conditions and modified carbon flux repartitioning between the fermentative end-products (acetate, lactate, ethanol, formate, and pyruvate) (Wang *et al.*, 2010). The heterologous expression of the *Bacillus subtilis* citrate synthase which is insensitive to NADH increased succinic acid production in engineered *E. coli* strains (Sanchez *et al.*, 2005). In combination with the above described mutations, the use of the appropriate pyruvate dehydrogenases and citrate synthases (NADH sensitive or insensitive) enables the tuning of carbon flux repartitioning between glyoxylate and citric acid cycle reactions and fermentative pathways under anaerobic and aerobic conditions.

Another possibility to increase carbon flux through the DHB pathway is the deletion of enzymatic reactions that may degrade the pathway intermediates malyl-CoA, or 4-malate semialdehyde. Candidate enzymes that may degrade malate semialdehyde are succinic semialdehyde

dehydrogenases (*sad, gabD*), and other dehydrogenases that are able to oxidize short and medium carbon chain molecules with terminal aldehyde groups. Furthermore, it is known that malyl-CoA may be degraded by citrate synthase.

Another possibility to increase 2,4-DHB productivity of the host organism is the deletion of metabolic reactions that degrade 2,4-DHB. 2,4-DHB is a competitive inhibitor of malic enzyme, thus, having comparatively high affinity for the active site of this enzyme (Rognstad & Katz, 1979). Therefore, 2,4-DHB may be recognized by other enzymes and potentially degraded. These enzymes can be identified and deleted from the host organism.

When 2,4-DHB production is based on addition of malate or other organic acids, the 2,4-DHB-producing microorganisms should functionally express a membrane transport protein that facilitates uptake of malate (or other organic acids such as pyruvate, succinate, etc).

The transformed host organisms of the invention may further contain an additional pathway of synthesizing 2,4-DHB, said host organism comprises at least one chimeric gene, either integrated into their genome or carried on an extra-chromosomal genetic element, for example a plasmid encoding a malate kinase or a chimeric gene comprising a nucleic acid encoding a malate kinase or an expression vector comprising a nucleic acid encoding a malate kinase, and/or a nucleic acid encoding a malate semialdehyde dehydrogenase, or a chimeric gene comprising a nucleic acid encoding a malate semialdehyde dehydrogenase or an expression vector comprising a nucleic acid encoding a malate semialdehyde dehydrogenase, and/or a nucleic acid encoding a DHB dehydrogenase, a chimeric gene comprising a nucleic acid encoding a DHB dehydrogenase or an expression vector comprising a nucleic acid encoding a DHB dehydrogenase. Said enzymes are described in the International patent application WO 2012/056318.

The following examples illustrate the invention. These examples are for purposes of illustration only and are not to be construed as limiting the scope of the invention in any manner.

Brief description of the figures

Figure 1: (i) Reaction scheme that describes the conversion of (L)-malate, succinyl-CoA, or glyoxylate into (L)-2,4-dihydroxybutyrate (2,4-DHB).

Figure 2: Figure shows (top graph) activity on malate semialdehyde, (middle graph) activity on succinic semialdehyde, (lower graph) changes of enzyme specificity compared to the wild-type enzyme expressed as the logarithm of the ratio of mutant activity on malate semialdehyde and succinic semialdehyde over the ratio of wild type activity on malate semialdehyde and succinic semialdehyde. (positive values indicate changes of specificity in favour of malate semialdehyde).

Figure 3: Chromatograms showing the presence of 2,4-DHB after incubation of 2 mM acetyl-CoA, 2 mM glyoxylate, and 2 mM NADPH, with different combinations of DHB pathway enzymes (Reaction 1: malyl-CoA lyase (150 µg/mL Me-Mcl), malyl-CoA reductase (100 µg/mL St-Mcr), and malate semialdehyde reductase (100 µg/mL Ms-SSAred H39R N43H); Reaction 2:same as reaction 1 but using 100 µg/mL Pg-SucD as malyl-CoA reductase; Control 1: same as reaction 1 but without malyl-CoA reductase; Control 2: same as reaction 1 but without malate semialdehyde reductase.)

Examples

Example 1: Demonstration of malyl-CoA lyase activity

Construction of plasmids containing wild-type genes coding for malyl-CoA lyase: The DNA sequences of the *mcl* genes coding for malyl-CoA lyase in *M. extorquens* (Arps *et al.*, 1993) and *Rhodobacter capsulatus* (Meister *et al.*, 2005)were optimized for the expression in *Escherichia coli* using the GENEius software (Eurofins). The optimized sequences were synthesized by Eurofins MWG OPERON® adding *Nhe*I and *EcoR*I restriction sites upstream of the start codon and downstream of the stop codon of *mcl*, respectively, which allowed direct cloning of the synthesized DNA fragments into the pET28a+ vector

(Novagen) using T4 DNA ligase (Biolabs). Ligation products were transformed into *E. coli* DH5α cells, amplified, and the plasmids pET28-Mex-mcl (expressing the malyl-CoA lyase from *M. extorquens*) and pET28-Rca-mcl (expressing the malyl-CoA lyase from *R. capsulatus*) were isolated using standard genetic protocols (Sambrook *et al.*, 1989). NCBI and Integrated Genomics references of the utilized *mcl* protein sequences, and the references for the corresponding natural and synthetic DNA sequences are listed in Table 1.

Organism	Protein	NCBI/Integrated Genomics accession number	Natural DNA sequence	Optimized DNA sequence
M. extorquens	Mcl SEQ ID No. 1	YP_002962854	SEQ ID No. 2	SEQ ID No. 3

Table 1: References to proteins from different organisms having annotated malyl-CoA lyase activity, and references to natural and optimized DNA sequences.

Expression of enzymes: E. coli BL21 (DE3) cells were transformed with the appropriate plasmids using standard genetic protocols (Sambrook *et al.*, 1989). Enzymes with an N-terminal hexa-His tag were expressed in 250 mL LB cultures that were inoculated from an overnight culture at OD_{600} of 0.1 and grown to OD_{600} of 0.6 before protein expression was induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to the culture medium. After 3 h of protein expression, cells were harvested by centrifugation at 13000 g for 10 min and the supernatant is discarded. Cell pellets were stored at – 20°C until further analysis. Growth and protein expression were carried out at 37 °C. Culture media contained 50µg/mL kanamycin.

Purification of enzymes: Frozen cell pellets of expression cultures were resuspended in 0.5 mL of breakage buffer (50 mM Hepes, 300mM NaCl, pH 7,5) and broken open by four successive rounds of sonication (sonication interval: 20 sec, power output: 30 %, sonicator: Bioblock Scientific, VibraCell™

72437). Cell debris were removed by centrifuging the crude extracts for 15 min at 4°C at 13000g and retaining the clear supernatant. RNA and DNA were removed from the extracts by adding 15 mg/mL streptomycin (Sigma), centrifuging the samples at 13000 g for 10 min at 4 °C and retaining the supernatant. Clear protein extract was incubated for 20 min at room temperature (1h at 4 °C) with 0.3 (0.75 mL) (bed volume) of Talon™ Cobalt affinity resin (Clontech). The suspension was centrifuged at 700 g in a table top centrifuge and supernatant was removed. The resin was washed with 10 bed volumes of wash buffer (50 mM Hepes, 300 mM NaCl, 15 mM Imidazole, pH 7,5) before proteins were eluted with 0.5 mL of elution buffer (50 mM Hepes, 300 mM NaCl, 200 mM Imidazole, pH 7,5). Purity of eluted enzymes was verified by SDS-PAGE analysis. Protein concentrations were estimated with the method of Bradford.

Enzymatic assays: Malyl-CoA lyase activity was assayed using a method adapted from (Meister *et al.*, 2005). Malyl-CoA synthesis by malyl-CoA lyase was coupled to the citrate synthase-catalyzed release of coenzyme A which was monitored by its spontaneous reaction with DTNB.

Reaction scheme

Malyl-CoA lyase:
acetyl-CoA + glyoxylate ->(L)-malyl-CoA

Citrate synthase:
(L)-malyl-CoA ->(L)-malate + Coenzyme A

Spontaneous :
coenzyme A + DTNB -> CoA-DTNB disulfide

The reaction mixture according to Assay 1 contained 50 mM MOPS/KOH (pH 7.5), 0.25 mM DTNB, 5 mM $MgCl_2$, 1 mM acetyl-CoA, 20 U/mL citrate synthetase (all products from Sigma), and appropriate amounts of purified malyl-CoA lyase or cell extract. Reactions were started by adding 10 mM glyoxylate. Enzymatic assays were carried out at 37 °C in 96-well flat

bottomed microtiter plates in a final volume of 250 µL. The reactions were followed by the characteristic absorption of DNTB at 412 nm ($\varepsilon_{DNTB+CoA}$ = 13.6 mM^{-1} cm^{-1}) in a microplate reader (BioRad 680XR).

Purified malyl-CoA lyase from *M. extorquens* characterized had a Vmax of 36 µmol/(min mg prot), and a Km on glyoxylate of 0.5 mM.

Example 2: Demonstration of malyl-CoA reductase activity

Construction of plasmids containing wild-type genes coding for malonyl-CoA reductase and succinyl-CoA reductase: The DNA sequence of the *mcr* gene coding for malyl-CoA reductase in *Sulfolobus tokodaii str 7* (Alber *et al.*, 2006) was optimized for the expression in *Escherichia coli* using the GENEius software (Eurofins). The optimized *mcr* sequence, and the natural DNA sequence of the *sucD* gene coding for succinyl-CoA reductase in *Porphyromonas gingivalis* W83 were synthesized by Eurofins MWG OPERON® adding *Nhe*I and *EcoR*I restriction sites upstream of the start codon and downstream of the stop codon of *mcr,* respectively, which allowed direct cloning of the synthesized DNA fragments into the pET28a+ vector (Novagen) using T4 DNA ligase (Biolabs). Ligation products were transformed into *E. coli* DH5α cells, amplified, and the plasmids, pET28-St-mcr (expressing the malonyl-CoA reductase from *S. tokodaii*), and pET28-Pgi-sucD (expressing the succinyl-CoA reductase from *P. gingivalis*), were isolated using standard genetic protocols (Sambrook *et al.*, 1989). NCBI references of the utilized *mcr* and *sucD* protein sequences, and the references for the corresponding natural and synthetic DNA sequences are listed in Table 2.

Organism	Protein	NCBI/Integrated Genomics accession number	Natural DNA sequence	Optimized DNA sequence
S. tokodaii	St-Mcr SEQ ID No. 7	NP_378167	SEQ ID No. 8	SEQ ID No. 9
P. gingivalis	Pg-SucD SEQ ID No. 10	AAQ65862	SEQ ID No. 11	

Table 2: References to proteins from different organisms having annotated malyl-CoA reductase or succinyl-CoA reductase activity, and references to natural and optimized DNA sequences.

Expression and purification of Pg-SucD was carried out as described in Example 1 using plasmid pET28-Pgi-sucD.

The St-*mcr* gene was amplified from plasmid pET28-St-mcr using primers 5'-TATAATGAGCTCGTTTAACTTTAAGAAGGAGATATACCATGATTCTGATGCGCCGT-3'(SEQ ID No. 12) and 5'-TATAATGGATCCCTCGAATTCTTACTTCTC-3' (SEQ ID No. 13) which added a SacI and a BamHI restriction site upstream of the start codon and downstream of the stop codon, respectively. The PCR fragment was ligated into the pACT3 expression vector using the SacI and BamHI restriction sites. The resulting plasmid pACT3-St-Mcr was transformed into strain *E. coli* MG1655. The resulting expression strain was cultivated on mineral medium at 37 °C. One liter mineral medium contained 20 g glucose, 18 g Na_2HPO_4 * 12 H_2O, 3 g KH_2PO_4, 0.5 gNaCl, 2 g NH_4Cl, 0.5 g $MgSO_4$ * 7 H_2O, 0.015 $CaCl_2$ * 2 H_2O, 1 mL of 0.06 mol/L $FeCl_3$ stock solution prepared in 100 times diluted concentrated HCl, 2 mL of 10 mM thiamine HCl stock solution, 20 g MOPS, 50 µg kanamycin sulphate (and 25 µg chloramphenicol when necessary), and 1 mL of trace element solution (containing per liter: 0.04 g Na_2EDTA * $2H_2O$, 0.18 g $CoCl_2$ * 6 H_2O, ZnSO4 * 7 H_2O, 0.04 g Na_2MoO4 * 2 H_2O, 0.01 g H_3BO_3, 0.12 g $MnSO_4$ * H_2O, 0.12 g $CuCl_2$ * H2O.). Medium pH was adjusted to 7 and medium was filter-sterilized.

When the exponentially growing culture reached an OD(600nm) of 0.6, 1 mM IPTG was added and cultures were incubated at 20 °C during 14 h before

harvesting the cells by centrifugation (13000 x g, 10 min). After discarding the supernatant cell pellets were stored at -20 °C.

To purify St-Mcr, frozen cell pellets of expression cultures were resuspended in 0.5 mL of breakage buffer (50 mM Hepes, 300mM NaCl, pH 7,5) and broken open by four successive rounds of sonication (sonication interval: 20 sec, power output: 30 %, sonicator: Bioblock Scientific, VibraCell™ 72437). Cell debris were removed by centrifuging the crude extracts for 15 min at 4°C at 13000 x g and retaining the clear supernatant. Native proteins of *E. coli* were removed by heat precipitation at 85 °c during 30 min followed by centrifugation at 13000 x g. Purity of the protein preparations was analysed by SDS-page analysis which showed only one band corresponding to the expected size of the St-Mcr protein.

Enzymatic assays: Malyl-CoA reductase activity was assayed in the reductive and in the oxidative sense of the reaction employing Assay 1 or Assay 2, respectively.

Assay 1 (reaction scheme):

Malyl-CoA lyase:

glyoxylate + acetyl-CoA -> malyl-CoA + acetate

Malyl-CoA reductase:
(L)-Malyl-CoA + NADPH -> (L)-Malate semialdehyde + Coenzyme A + NADP

Assay 2 (reaction scheme):

(L)-Malate semialdehyde + Coenzyme A + NADP -> (L)-Malyl-CoA + NADPH

The reaction mixture according to Assay 1 contained 50 mM MOPS/KOH (pH 7.5), 10 mM glyoxylate, 4 mM acetyl-CoA, 5 mM $MgCl_2$, 0.25 mM NADPH (all

products from Sigma), 5 U/mL of malyl-CoA lyase, and appropriate amounts of purified malyl-CoA reductase or cell extract. Reactions were started by adding glyoxylate. Enzymatic assays were carried out at 37 °C in 96-well flat bottomed microtiter plates in a final volume of 250 µL. The reactions were followed by the characteristic absorption of NADPH at 340 nm (ε_{NADPH} = 6.22 mM^{-1} cm^{-1}) in a microplate reader (BioRad 680XR).

The reaction mixture according to assay 2 contained 200 mM HEPES (pH 9), 5 mM $MgCl_2$, 1 mM NADP, 0.5 mM coenzyme A (all products from Sigma), and appropriate amounts of purified malyl-CoA reductase. Reactions were started by adding 5 mM (L)-malate semialdehyde. Enzymatic assays were carried out at 37°C in 96-well flat bottomed microtiter plates in a final volume of 250 µL. The reactions were followed by the characteristic absorption of NADPH at 340 nm (ε_{NADPH} = 6.22 mM^{-1} cm^{-1}) in a microplate reader (BioRad 680XR). Unstable malate semialdehyde was produced freshly prior to the enzymatic tests by the deprotection of the stable malate semialdehyde derivative 2-[(4S)-2,2-dimethyl-5-oxo-1,3-dioxolan-4-yl]acetaldehyde(DMODA) (provided by Activation®). Malate semialdehyde was obtained by dissolving appropriate amounts of DMODA in 2 M hydrochloric acid, short heating of the suspension to boiling temperature, and leaving the hot suspension for 15 min at room temperature. The released acetone was evaporated at 35 °C and 50 mbar in a rotary evaporator. The pH of the malate semialdehyde solution was fixed at 3.5 using sodium bicarbonate.

Results listed in Tables 3 and 4 demonstrate malyl-CoA reductase activity for malonyl-CoA reductase, Mcr, of *S. tokodaii* and succinyl-CoA reductase, SucD, of *P. gingivalis*.

Substrate	Malonyl-CoA		Succinyl-CoA		Malyl-CoA	
Enzyme	Vmax [μmol/(min mg)]	Km [mM]	Vmax [μmol/(min mg)]	Km [mM]	Vmax [μmol/(min mg)]	Km [mM]
St-Mcr	0.67 ± 0.15	nd	0.98 ±0.17	0.2	0.24 ±0.045	nd
Pg-SucD	nd	nd	1	1	0.025	nd

Table 3: Kinetic parameters for the reductive sense of reaction (malonyl-CoA reductase and succinyl-CoA reductase activities were estimated by directly adding the substrates malonyl-CoA or succinyl-CoA to the reaction mixture).

Substrate	Succinic semialdehyde		Malate semialdehyde	
Enzyme	Vmax [μmol/(min mg)]	Km [mM]	Vmax [μmol/(min mg)]	Km [mM]
St-Mcr	1.7	1.15	0.1	0.25
Pg-SucD	4	nd	0.007	nd

Table 4: Kinetic parameters for the oxidative sense of reaction

Example 3: Demonstration of DHB dehydrogenase activity

To identify a suitable 2,4 DHB dehydrogenase, beta-hydroxyacid dehydrogenases from different biological sources were tested for their ability to reduce malate semialdehyde. Among the tested enzymes were the methylbutyraldehyde reductase from *Saccharomyces cerevisiae*, Ypr1 (Ford & Ellis, 2002)(SEQ ID No.14),the 4-hydroxybutyrate dehydrogenase, 4hbdh, of P. gingivalis (SEQ ID No.187), the alcohol dehydrogenase, YqhD, of E; coli (SEQ ID no. 185), and the succinic semialdehyde reductase, Ms-Ssr, from *Metallosphaera sedula* (Kockelkorn& Fuchs, 2009) (SEQ ID No. 16). The

genes *YPR1, 4hbdh, yqhD,* and *Ms-SSR* were amplified using primers listed in Table 5 and cloned into vector pET28 (restriction enzymes see Table 5) yielding plasmids pET28-Sce-YPR1, pET28-Pgi-4hbdh, pET28-Eco-yqhd and pET28-Mse-SSR, respectively. The proteins were expressed and purified as described in Example 1.

Enzyme	**Accession No**	**Primer 5'-3'**	**Restriction enzymes**
YPR1	GI:6320576	TATAATGCTAGCATGCCTGCTACGTTAAAGAA (SEQ ID No. 18) TATAATGAGCTCTCATTGGAAAATTGGGAAGG (SEQ ID No.18)	*Nhe*I *Sac*I
YqhD	GI:16130909	TATAATGAATTCTTAGCGGGCGGCTTCGTATATACGGCGGCTGACA (SEQ ID No. 20) TATCGTGCTAGCATGAACAACTTTAATCTGCACA (SEQ ID No .21)	*EcoRI* *NheI*
4hbdh	GI:188994588	TATAATGGATCCTTAGTAGAGTCTTCTGTAG (SEQ ID No.22) TATAATCATATGCAACTTTTCAAACTC (SEQ ID No.23)	*BamHI* *NdeI*
Ms-SSR	GI:146304190	TATAATGCTAGCATGAAAGCTGCAGTACTTCA (SEQ ID No. 24) TATAATGAATTCTTACGGGATTATGAGACTTC (SEQ ID No.25)	*Nhe*I *Eco*RI

Table 5 Primers and restriction enzymes used to clone candidate beta-hydroxyacid dehydrogenases

Test for malate semialdehyde reductase activity:

Reaction scheme:

(L)-Malate semialdehyde + NAD(P)H → (L)-2,4-dihydroxybutyric acid + NAD(P)

The assay mixture contained 200 mM Hepes (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 0,24 mM NADH or NADPH, and appropriate amounts of purified enzyme or cell extract. Reactions were started by adding 10 mM (L)-malate semialdehyde (malate semialdehyde was prepared freshly for each test, see Example 3). Enzymatic assays were carried out at 30 °C in 96-well flat bottomed microtiter plates in a final volume of 250 µL. The reactions were followed by the characteristic absorption of NAD(P)H at 340 nm (ε_{NADPH} = 6.22 mM^{-1} cm^{-1}) in a microplate reader (BioRad 680XR). Results are listed in Table 6.

Enzyme	Origin	Reported function	Activity on malate semialdehyde (cofactor NADH) [µmol/(min*mg_prot)]	Activity on malate semialdehyde (cofactor NADPH) [µmol/(min*mg_prot)]
Ms-SSR (SEQ ID No.16)	*M. sedula*	Succinic semialdehyde reductase	**4.9**	**4.9**
YqhD (SEQ ID No 185)	*E. coli*	Alcohol dehydrogenase	nd	**1.2**
4hbdh (SEQ ID No 187)	*P. gingivalis*	4-hydroxybutyrate dehydrogenase	33	**nd**
YPR1 (SEQ ID No. 14)	*S. cerevisiae*	Methylbutyraldehyde reductase	nd	**0.19**

Table 6: Reducing activity of selected beta-hydroxyacid dehydrogenases on malate semialdehyde (Results represent the average of at least two independent experiments).

The succinic semialdehyde dehydrogenase from *M. sedula* and the methylbutyraldehyde reductase from *S. cerevisiae* have malate semialdehyde reductase activity. The Km of Ms-SSR for malate semialdehyde was 4 mM.

Example 4: Rational construction of an improved malyl-CoA reductase enzyme

Site-directed mutagenesis was carried out using the oligonucleotide pairs listed in Table 7 and the pET28-Sto-mcr plasmid as the template. Point mutations to change the amino acid sequences were introduced by PCR (Phusion 1U, HF buffer 20 % (v/v), dNTPs 2.5 mM, direct and reverse primers 1 µM each, template plasmid 200 ng, water). When possible, plasmids created by PCR contained new restriction sites (introduced using silent mutations) in addition to the functional mutation to facilitate identification of mutated clones. The PCR products were digested by *DpnI* at 37 °C for 2 x 2h to remove template DNA, and transformed into NEB DH5-α competent *E. coli* cells (NEB). The mutated plasmids were identified by restriction site analysis and verified to carry the desired mutations by DNA sequencing.

Mutation	**Primer 5'-3'**
Tyr206	Forward CATTCTGCCTTTAGGGGACGGCNNKGACGCCAAAAC G (SEQ ID No. 26) Revers CGTTTTGGCGTCMNNGCCGTCCCCTAAAGGCAGAAT G (SEQ ID No . 27)

Table 7: Primer pairs used to mutate the *mcr* gene of S. tokodaii.

The impact of the genetic modifications of St-Mcr was tested in the oxidative sense of the reaction using Assay 3 described in Example 2. Figure 3 shows that replacing the natural Tyr206 by other amino acids decreases the activity on the natural substrate, succinic semialdehyde, causing at the same

time an increased or at least constant activity on malate semialdehyde. Thus, replacing Tyr206 by appropriate amino acid residues provides a selective advantage regarding the specificity of Mcr for the DHB pathway intermediate.

Preferred amino acid residues in position 206 are therefore phenylalanine, histidine, isoleucine, lysine, methionine, glycine, asparagine, proline, arginine, glutamine, leucine, serine, tryptophane, and threonine.

The protein wherein the Tyrosine 206 is replaced by a Proline residu is represented by SEQ ID No. 202.

Example 5: Rational construction of an improved DHB dehydrogenase

Site-directed mutagenesis was carried out using the oligonucleotide pairs listed in Table 6 and the pET28-Mse-SSR plasmid as the template. Point mutations to change the amino acid sequences were introduced by PCR (Phusion 1U, HF buffer 20 % (v/v), dNTPs 2.5 mM, direct and reverse primers 1 µM each, template plasmid 200 ng, water). When possible, plasmids created by PCR contained new restriction sites (introduced using silent mutations) in addition to the functional mutation to facilitate identification of mutated clones. The PCR products were digested by *DpnI* at 37 °C for 2 x 2h to remove template DNA, and transformed into NEB DH5-α competent *E. coli* cells (NEB). The mutated plasmids were identified by restriction site analysis and verified to carry the desired mutations by DNA sequencing. Table 8 summarizes kinetic parameters of the mutants. The results demonstrate that the double mutant Ms-SSR H39R N43H (SEQ ID No.38) has improved affinity for malate semialdehyde when compared to the wild type enzyme.

Mutation	Primer 5'-3'	Restriction enzymes
H39R	gtcaaggcaaccggtctctgtcgctccgacgtcaatg (SEQ ID No. 28) cattgacgtcggagcgacagagaccggttgccttgac (SEQ ID No.29)	*Nhe*I
N43H	ggctctgtcactccgacgtacatgtctttgaggggaaaac (SEQ ID No. 30) gttttcccctcaaagacatgtacgtcggagtgacagagcc (SEQ ID No.31)	*Nhe*I

Table 8: Primer pairs used to mutate *M. sedula* succinic semialdehyde reductase (Ms-SSR)

Mutant	Maximum activity [μmol/(min* mg_{prot})]	Km [mmol/L]
Wild type (SEQ ID No. 16)	4.9	4
H39R (SEQ ID No.32)	1.7	1
N43H (SEQ ID No.34)	4.3	5
H39R N43H (SEQ ID No. 36)	4.7	1

Table 9: Summary of kinetic parameters of *M. sedula* succinic semialdehyde reductase (Ms-SSR) mutants (Results represent the average of at least two independent experiments).

The corresponding nucleic sequences are represented by SEQ ID No. 17, SEQ ID No. 33, SEQ ID No. 35 and SEQ ID No. 37.

The coding sequence of *M. sedula* succinic semialdehyde reductase including the mutations H39R and N43H was optimized for maximum expression in *E. coli*, using the GeneOptimizer® software. The synthetic gene was produced by GeneArt® Gene Synthesis (Invitrogen Life Technologie). *Nhe*I and *EcoR*I restriction sites were introduced upstream of the start codon and downstream of the stop codon, respectively, allowing direct cloning into pET28a+ (Novagen).

The resulting pET28-Mse-DHB-Dh-H39R_N43H-opt plasmid was isolated and shown by DNA sequencing to contain the full-length *M. sedula SSR H39R N43H* gene having the correct sequence (SEQ ID No.38).

Example 6: Demonstration of in vitro production of DHB by the synthetic malyl-CoA pathway

The enzymes malyl-CoA lyase (Me-Mcl), malyl-CoA reductase (St-Mcr or Pg-SucD), and DHB dehydrogenase (Ms-SSA-red H39N N43H) were expressed and purified as described in Examples 1, 2, and 3.

Production of DHB by the pathway comprising malyl-CoA lyase, malyl-CoA reductase, and DHB dehydrogenase was demonstrated *in vitro* by adding 2 mM glyoxylate to a reaction mixture that contained 50 mM Hepes (pH 7.5), 2 mM acetyl-CoA, 2 mM NADPH, 100 µg/mL DHB dehydrogenase, 150 µg/mL

malyl-CoA lyase,and 100 µg/mL malyl-CoA reductase (which was either St-Mcr (reaction 1), or Pg-SucD (reaction 2)).

Control reactions contained all components but were lacking either DHB dehydrogenase (Control 1) or malyl-CoA reductase (Control 2). After 120 min of incubation at 37 °C the DHB content in the reaction mixture was analysed by gas chromatography [GCMS-QP2010 Ultra Shimadzu; equipped with a FID detector (FID-2010 Plus Shimadzu); autosampler AOC20s (Shimadzu) ; splitless injector AOC20i (Shimadzu) (230 °C); column: Zebron ZB-FFAP, 30 m × 0.25 mm, d_f 0.25 µm; and liner: Tapered focus Liner5 x 95 x 3.4 mm (SGE). Carrier gas was hydrogen at a total flow rate of 25 mL/min. Flame ionization was carried out using an air-hydrogen mixture (flow rates were 300 mL/min and 30 mL/min, respectively). Detector temperature was 240 °C. Injected sample volume was 1 µL. Temperature program is provided in Table 10.

Chromatograms showing presence of DHB in the reactions containing all pathway enzymes and absence of DHB in samples containing only two out of three pathway enzymes are shown in Figure 2.

Column temperature [°C]	Hold [min]	Gradient [°C/min]	Runtime [min]
90	0	0	0
115	1.8	30	2.63
170	1	4	17.38
230	3	50	21.58

Table 10: Temperature program for GC analysis of reaction mixtures

Example 7: Construction of optimized DHB producer strains

Construction of a plasmid for simultaneous expression of malyl-CoA synthetase, malyl-CoA reductase, and DHB-dehydrogenase:

The coding sequence of the malyl-CoA lyase from *M. extorquens*, *Me-mcl*, was amplified from plasmid pET28-Mex-mcl using the high fidelity polymerase Phusion (Fermentas) and the forward and reverse primers 5'-TCACACAGGAAACAGAATTCGAGCTCGGTAATGTCGTTTACCCTGATTCAG CAAGCGACT-3' (SEQ ID No. 39) and 5'-GGTATATCTCCTTCTTAAAGTTAAACTTATTTGCCGCCCATTGCATCCGCTT TCTG-3' (SEQ ID No. 40) which contained restriction sites for SacI upstream of the start codon (underlined). The coding sequence of the malonyl-CoA reductase from *S. tokodaii*, *St-mcr*, was amplified from plasmid pET28-Sto-mcr using the forward and reverse primers 5'-GTTTAACTTTAAGAAGGAGATATACCATGATTCTGATGCGCCGTACCCTGA AAGCG-3' (SEQ ID No. 41) and 5'-GGTATATCTCCTTCTTAAAGTTAAACTTACTTCTCGATGTAGCCTTTCTCCA CGAG-3' (SEQ ID No. 42) which contained restriction sites for BamHI downstream of the stop codon. The plasmid pET28-Mse-DHB-Dh-H39R_N43H-opt (Example 5) was used as the template to amplify the optimized coding sequence of the succinic semialdehyde reductase H39R N43H from *M. sedula* using the forward and reverse primers 5'-GTTTAACTTTAAGAAGGAGATATACCATGAAAGCAGCAGTTCTGCATACCT ATAAAGAACCGCTGAGCAT-3' (SEQ ID No. 43) and 5'-ATGCCTGCAGGTCGACTCTAGAGGATCCTTACGGAATAATCAGGCTACGA ATTGCTTC-3' (SEQ ID No. 44) that introduced a *BamHI* restriction site downstream of the stop codon (underlined).

The forward primers for *St-mcr* and the succinic semialdehyde reductase H39R N43H from *M. sedula* contained a rbs motif. The three genes were simultaneously cloned into the pACT3 expression vector by homologous recombination using the In-Fusion cloning kit (Clontech).

The resulting and pACT3-MCL-DHB (SEQ ID No. 45) plasmid was isolated and shown by DNA sequencing to have the correct sequence.

Construction of a plasmid for simultaneous expression of malyl-CoA synthetase, malyl-CoA reductase, and DHB-dehydrogenase:

The DNA sequences coding for the two protein subunits of malyl-CoA synthetase, mtkA (YP_00296285) and mtkB (YP_002962852), from *Methylobacterium extorquens* AM1 were optimized for the expression in *Escherichia coli* using the GENEius software (Eurofins). The optimized DNA sequences of the subunit were physically linked by the DNA sequence naturally occurring between the mtkA and mtkB genes in *M. Extorquens* genome (CGAACGGGGGAGGAATCACGCC, SEQ ID No. 46). The resulting DNA fragment, 'mtkA gene - linker DNA - mtkB gene', was synthesized by Eurofins MWG OPERON® and subcloned into pET28b expression vector using NheI and EcoRI restriction enzymes. The resulting DNA plasmid pET28-Mex-mtkAB (SEQ ID No. 47) was used to simultaneously amplify the two codons optimized genes encoding malyl-CoA synthetase from *M. extorquens*, *Me-mtkA and Me-mtkB* using the high fidelity polymerase Phusion (Fermentas) and the forward and reverse primers 5'-CAGGAAACAGAATTCGAGCTCGGTAATGGATGTGCACGAATATCAGGCGAAAGAACTGCT-3' (SEQ ID No. 48) and 5'-TACGGCGCATCAGAATCATtacgccgcacgtgctaacacatcggcaac-3' (SEQ ID No. 49) which contained restriction sites for SacI upstream of the start codon (underlined). The coding sequence of the malonyl-CoA reductase from *S. tokodaii*, *St-mcr*, was amplified from plasmid pET28-Sto-mcr using the forward and reverse primers 5'-GGCGTAATGATTCTGATGCGCCGTACCCTGAAAGCG-3' (SEQ ID No. 50) and 5'-CTGCTGCTTTCATTACTTCTCGATGTAGCCTTTCTCCACGAG-3' (SEQ ID No. 51) which contained restriction sites for BamHI downstream of the stop codon. The plasmid pET28-Mse-DHB-Dh-H39R_N43H-opt (Example 5) was used as the template to amplify the optimized coding sequence of the succinic semialdehyde reductase H39R N43H from *M. sedula* using the forward and reverse primers 5'-TACATCGAGAAGTAATGAAAGCAGCAGTTCTGCATACCTATAAAGAAC-3' (SEQ ID No. 52) and 5'-

CCTGCAGGTCGACTCTAGAGGATCCTTACGGAATAATCAGGCTACGAATT GCTTCAC-3' (SEQ ID No. 53) that introduced a *BamHI* restriction site downstream of the stop codon (underlined).

The three genes were simultaneously cloned into the pEXT20 expression vector by homologous recombination using the In-Fusion cloning kit (Clonetch).

The resulting pEXT20-MCS-DHB (SEQ ID No.54) plasmid was isolated and shown by DNA sequencing to have the correct sequence.

Construction of plasmids for overexpression of phosphoenolpyruvate (PEP) carboxykinase, PEP carboxylase, pyruvate kinase, pyruvate carboxylase, isocitrate lyase enzymes and the galactose symporter permease:

The plasmid pACT3-pck harbouring the PEP carboxykinase encoding *pck* gene of *E. coli* was constructed by amplifying the *pck* coding sequence using genomic DNA from *E. coli* MG1655 as the template and the forward and reverse primers, respectively, $^{5'}$TATAATCCCGGGATGCGCGTTAACAATGGTTTGACC$^{3'}$ (SEQ ID No. 56 and $^{5'}$TATAATTCTAGATTACAGTTTCGGACCAGCCG$^{3'}$ (SEQ ID No. 57). The DNA fragment was digested with *Xma*I and *Xba*I, ligated into the corresponding sites of the pACT3 expression vector (Dykxhoorn *et al.*, 1996) using T4 DNA ligase (Biolabs), and transformed into *E. coli* DH5α cells. The transformants were selected on solid LB medium containing chloramphenicol (25 µg/mL). The resulting plasmid was isolated and correct insertion of the *pck* gene was verified by sequencing. Plasmids pACT3-aceA, pACT3-ppc, pACT3-galP, pACT3-pck and pACT3-pyc harbouring, respectively, *aceA*, *ppc*, *galP*, or *pykA* (all *E. coli*) or *pck* from *Lactococcus lactis* were constructed analogously using the primers listed in Table 11.

Gene	Primer	Linker	Sequence
Ec_pck	*Ec_pck_clon_for*	*Xma*I	tataatcccgggatgcgcgttaacaatggtttgacc (SEQ ID No.57)

	Ec_pck_clon_rev	*XbaI*	tataattctagattacagtttcggaccagccg (SEQ ID No.58)
Ec_ppc	*Ec_ppc_clon_for*	*XmaI*	tataatcccgggatgaacgaacaatattcc (SEQ ID No.59)
	Ec_ppc_clon_rev	*XbaI*	tataattctagattagccggtattacgcat (SEQ ID No.60)
Ec_pykA	*Ec_pykA_clon_for*	*XmaI*	tataatcccgggatgtccagaaggcttcgcagaaca (SEQ ID No.61)
	Ec_pykA_clon_rev	*XbaI*	tataattctagattactctaccgttaaaatac (SEQ ID No.62)
Ec_aceA	*Ec_aceA_clon_for*	*XmaI*	tataatcccgggatgaaaacccgtacacaacaaatt (SEQ ID No63)
	Ec_aceA_clon_rev	*XbaI*	tataattctagattagaactgcgattcttcag (SEQ ID No.64)
Ll_pycA	*Ll_pycA_clon_for*	*XmaI*	tataatcccgggatgaaaaaactactcgtcgccaat (SEQ ID No.65)
	Ll_pycA_clon_rev	*XbaI*	tataattctagattaattaatttcgattaaca (SEQ ID No.66)
Ec_galP	*Ec_galP_clon_for*	*XmaI*	tataatcccgggatgcctgacgctaaaaaacaggggcggt (SEQ ID No.67)
	Ec_galP_clon_rev	*XbaI*	tataattctagattaatcgtgagcgcctatttc (SEQ ID No.68)

Table 11: Primers used for construction of plasmids for gene overexpression. Restriction sites used for cloning into pACT3 are underlined

It is understood that removal of the *lacI* gene from the backbone of the above described plasmids along with the genomic deletion of *lacI* in the host strain may render protein expression from above described plasmids constitutive.

Construction of strains with optimized carbon flux repartitioning for DHB production

Several genes were disrupted in *E. coli* strain MG1655 in order to optimise carbon flux repartitioning and cofactor supply for DHB production. Gene deletions were carried out using either the lambda red recombinase method according to Datsenko *et al.*(Datsenko & Wanner, 2000), or the phage transduction method adapted from Miller(Miller, 1992).

Protocol for introduction of gene deletions using the lambda red recombinase method: the deletion cassettes were prepared by PCR using high fidelity polymerase Phusion™ (Finnzymes), and the FRT-flanked kanamycin resistance gene (kan) of plasmid pKD4 as the template (Datsenko & Wanner,

2000). Sense primers contained sequences corresponding to the 5'-end of each targeted gene (underlined) followed by 20 bp corresponding to the FRT-kan-FRT cassette of pKD4. Anti-sense primers contained sequences corresponding to the 3'-end region of each targeted gene (underlined) followed by 20 bp corresponding to the cassette. The primers are described in Table 12. PCR products were digested with *Dpn*I and purified prior to transformation.

E. coli MG1655 strain was rendered electro-competent by growing the cells to an OD_{600} of 0.6 in LB liquid medium at 37 °C, concentrating the cells 100-fold, and washing them twice with ice-cold 10 % glycerol. The cells were transformed with plasmid pKD46 (Datsenko & Wanner, 2000) by electroporation (2.5 kV, 200 Ω, 25 µF, in 2 mm gap cuvettes). Transformants were selected at 30 °C on ampicillin (100 µg/mL) LB solid medium.

Disruption cassettes were transformed into electro-competent *E. coli* strains harbouring the lambda Red recombinase-expressing plasmid pKD46. The cells were grown at 30 °C in liquid SOB medium containing ampicillin (100 µg/mL). The lambda red recombinase system was induced by adding 10 mM arabinose when OD_{600} of the cultures reached 0.1. Cells were further grown to an OD_{600} of 0.6 before they were harvested by centrifugation, washed twice with ice-cold 10 % glycerol, and transformed with the disruption cassette by electroporation. After an overnight phenotypic expression at 30 °C in LB liquid medium, cells were plated on solid LB medium containing 25 µg/mL kanamycin. Transformants were selected after cultivation at 30 °C.

The gene replacement was verified by colony PCR using Crimson Taq polymerase (NEB). A first reaction was carried out with the flanking *locus*-specific primers (see Table 12) to verify simultaneous loss of the parental fragment and gain of the new mutant specific fragment. Two additional reactions were done by using one *locus*-specific primer together with one of the corresponding primers k1rev, or k2for (see Table 12) that align within the FRT-kanamycin resistance cassette (sense locus primer/k1rev and k2for/reverse locus primer).

The resistance gene (FRT-kan-FRT) was subsequently excised from the chromosome using the FLP recombinase-harbouring plasmid pCP20 (Cherepanov & Wackernagel, 1995) leaving a scar region containing one FRT site. pCP20 is an ampicillin and CmR plasmid that shows temperature-sensitive replication and thermal induction of FLP recombinase synthesis. Kanamycin resistant mutants were transformed with pCP20, and ampicillin-resistant

transformants were selected at 30 °C. Transformants were then grown on solid LB medium at 37 °C and tested for loss of all antibiotic resistances. Excision of the FRT-kanamycin cassette was analysed by colony PCR using crimson Taq polymerase and the flanking locus-specific primers (Table 12). Multiple deletions were obtained by repeating the above described steps.

Gene	Primer	Sequence
ldhA	*Δ_ldhA_for*	gaaggttgcgcctacactaagcatagttgttgatgagtgtaggctggagctgcttc (SEQ ID No.69)
	Δ_ldhA_rev	ttaaaccagttcgttcgggcaggtttcgccttttcatgggaattagccatggtcc SEQ ID No.70)
adhE	*Δ_adhE_for*	atggctgttactaatgtcgctgaacttaacgcactcgtagagcgtgtgtaggctggagctgcttc (SEQ ID No.71)
	Δ_adhE_rev	ttaagcggatttttcgcttttttctcagctttagccggagcagccatatgaatatcctccttag (SEQ ID No.72)
ackA	*Δ_ackA_for*	atgtcgagtaagttagtactggttctgaactgcggtagttcttcagtgtaggctggagctgcttc (SEQ ID No.73)
	Δ_ackA_rev	tcaggcagtcaggcggctcgcgtcttgcgcgataaccagttcttccatatgaatatcctccttag (SEQ ID No.74)
focA-pflB	*Δ_focA-pflB_for*	ttactccgtatttgcataaaaaccatgcgagttacgggcctataagtgtaggctggagctgcttc (SEQ ID No.75)
	Δ_focA-pflB_rev	atagattgagtgaaggtacgagtaataacgtcctgctgctgttctcatatgaatatcctccttag (SEQ ID No.76)
pta	*Δ_pta_for*	gtgtcccgtattattatgctgatccctaccggaaccagcgtcggtgtgtaggctggagctgcttc (SEQ ID No.77)
	Δ_pta_rev	ttactgctgctgtgcagactgaatcgcagtcagcgcgatggtgtacatatgaatatcctccttag (SEQ ID No.78)
poxB	*Δ_poxB_for*	atgaaacaaacggttgcagcttatatcgccaaaacactcgaatcggtgtaggctggagctgcttc (SEQ ID No.79)
	Δ_poxB_rev	ttaccttagccagtttgttttcgccagttcgatcacttcatcacccatatgaatatcctccttag (SEQ ID No.80)
sad	*Δ_sad_for*	atgaccattactccggcaactcatgcaatttcgataaatcctgccgtgtaggctggagctgcttc (SEQ ID No.81)
	Δ_sad_rev	tcagatccggtctttccacaccgtctggatattacagaattcgtgcatatgaatatcctccttag (SEQ ID No.82)
gabD	*Δ_gabD_for*	atgaaacttaacgacagtaacttattccgccagcaggcgttgattgtgtaggctggagctgcttc (SEQ ID No.83)
	Δ_gabD_rev	ttaaagaccgatgcacatatatttgatttctaagtaatcttcgatcatatgaatatcctccttag (SEQ ID No.847)
gadA	*Δ_gadA_for*	atggaccagaagctgttaacggatttccgctcagaactactcgatgtgtaggctggagctgcttc (SEQ ID No.85)
	Δ_gadA_rev	tcaggtgtgtttaaagctgttctgctgggcaataccctgcagtttcatatgaatatcctccttag (SEQ ID No.86)

gadB	*Δ_gadB_for*	atggataagaagcaagtaacggatttaaggtcggaactactcgatgtgtaggctggagctgcttc (SEQ ID No.87)
	Δ_gadB_rev	tcaggtatgtttaaagctgttctgttgggcaatacctgcagtttcatatgaatatcctccttag (SEQ ID No.88)
gadC	*Δ_gadC_for*	atggctacatcagtacagacaggtaaagctaagcagctcacattagtgtaggctggagctgcttc (SEQ ID No.89)
	Δ_gadC_rev	ttagtgtttcttgtcattcatcacaatatagtgtggtgaacgtgccatatgaatatcctccttag (SEQ ID No.90)
sfcA	*Δ_sfcA_for*	atggaaccaaaaacaaaaaaacagcgttcgctttatatcccttacgtgtaggctggagctgcttc (SEQ ID No.91)
	Δ_sfcA_rev	ttagatggaggtacggcggtagtcgcggtattcggcttgccagaacatatgaatatcctccttag (SEQ ID No.92)
maeB	*Δ_maeB_for*	atggatgaccagttaaaacaaagtgcacttgatttccatgaatttgtgtaggctggagctgcttc (SEQ ID No.93)
	Δ_maeB_rev	ttacagcggttgggtttgcgcttctaccacggccagcgccaccatcatatgaatatcctccttag (SEQ ID No.94)
ppc	*Δ_ppc_for*	atgaacgaacaatattccgcattgcgtagtaatgtcagtatgctcgtgtaggctggagctgcttc (SEQ ID No.95)
	Δ_ppc_rev	ttagccggtattacgcatacctgccgcaatcccggcaatagtgaccatatgaatatcctccttag (SEQ ID No.96)
pykA	*Δ_pykA_for*	atgtccagaaggcttcgcagaacaaaaatcgttaccacgttaggcgtgtaggctggagctgcttc (SEQ ID No.97)
	Δ_pykA_rev	ttactctaccgttaaaatacgcgtggtattagtagaacccacggtcatatgaatatcctccttag (SEQ ID No.98)
pykF	*Δ_pykF_for*	atgaaaaagaccaaaattgtttgcaccatcggaccgaaaaccgaagtgtaggctggagctgcttc (SEQ ID No.99)
	Δ_pykF_rev	ttacaggacgtgaacagatgcggtgttagtagtgccgctcggtaccatatgaatatcctccttag (SEQ ID No.100)
mgsA	*Δ_mgsA_for*	atggaactgacgactcgcactttacctgcgcggaaacatattgcggtgtaggctggagctgcttc (SEQ ID No.101)
	Δ_mgsA_rev	ttacttcagacggtccgcgagataacgctgataatcggggatcagcatatgaatatcctccttag (SEQ ID No.102)
iclR	*Δ_iclR_for*	atggtcgcacccattcccgcgaaacgcggcagaaaacccgccgttgtgtaggctggagctgcttc (SEQ ID No.103)
	Δ_iclR_rev	tcagcgcattccaccgtacgccagcgtcacttccttcgccgctttcatatgaatatcctccttag (SEQ ID No.104)
icd	*Δ_icd_for*	atggaaagtaaagtagttgttccggcacaaggcaagaagatcaccgtgtaggctggagctgcttc (SEQ ID No.105)
	Δ_icd_rev	ttacatgttttcgatgatcgcgtcaccaaactctgaacatttcagcatatgaatatcctccttag (SEQ ID No.106)
sucA	*Δ_sucA_for*	atgcagaacagcgctttgaaagcctggttggactcttcttacctcgtgtaggctggagctgcttc (SEQ ID No.107)
	Δ_sucA_rev	ttattcgacgttcagcgcgtcattaaccagatcttgttgctgtttcatatgaatatcctccttag (SEQ ID No.108)

sucB	*Δ_sucB_for*	atgagtagcgtagatattctggtccctgacctgcctgaatccgtagtgtaggctggagctgcttc (SEQ ID No.109)
	Δ_sucB_rev	ctacacgtccagcagcagacgcgtcggatcttccagcaactctttcatatgaatatcctccttag (SEQ ID No.110)
frdA	*Δ_frdA_for*	gtgcaaacctttcaagccgatcttgccattgtaggcgccggtggcgtgtaggctggagctgcttc (SEQ ID No.111)
	Δ_frdA_rev	tcagccattcgccttctccttcttattggctgcttccgccttatccatatgaatatcctccttag (SEQ ID No.112)
frdB	*Δ_frdB_for*	atggctgagatgaaaaacctgaaaattgaggtggtgcgctataacgtgtaggctggagctgcttc (SEQ ID No.113)
	Δ_frdB_rev	ttagcgtggtttcagggtcgcgataagaaagtctttcgaactttccatatgaatatcctccttag (SEQ ID No.114)
frdC	*Δ_frdC_for*	atgacgactaaacgtaaaccgtatgtacggccaatgacgtccaccgtgtaggctggagctgcttc (SEQ ID No.115)
	Δ_frdC_rev	ttaccagtacagggcaacaaacaggattacgatggtggcaaccaccatatgaatatcctccttag (SEQ ID No.116)
frdD	*Δ_frdD_for*	atgattaatccaaatccaaagcgttctgacgaaccggtattctgggtgtaggctggagctgcttc (SEQ ID No.117)
	Δ_frdD_rev	ttagattgtaacgacaccaatcagcgtgacaactgtcaggatagccatatgaatatcctccttag (SEQ ID No.118)
ptsI	*Δ_ptsI_for*	atgatttcaggcattttagcatccccgggtatcgctttcggtaaagtgtaggctggagctgcttc (SEQ ID No.119)
	Δ_ptsI_rev	ttagcagattgttttttcttcaatgaacttgttaaccagcgtcatcatatgaatatcctccttag (SEQ ID No.120)
ptsG	*Δ_ptsG_for*	atgtttaagaatgcatttgctaacctgcaaaaggtcggtaaatcggtgtaggctggagctgcttc (SEQ ID No.121)
	Δ_ptsG_rev	ttagtggttacggatgtactcatccatctcggttttcaggttatccatatgaatatcctccttag (SEQ ID No.122)
lacI	*Δ_lacI_for*	gtgaaaccagtaacgttatacgatgtcgcagagtatgccggtgtcgtgtaggctggagctgcttc(SEQ ID. No.123)
	Δ_lacI_rev	tcactgcccgctttccagtcgggaaacctgtcgtgccagctgcatcatatgaatatcctcctag(SEQ ID. No.124)
lldD	*Δ_lldD_for*	atgattatttccgcagccagcgattatcgcgccgcagcgcaacgcgtgtaggctggagctgcttc (SEQ ID. No.125)
	Δ_lldD_rev	ctatgccgcattccctttcgccatgggagccagtgccgcaggcaacatatgaatatcctccttag (SEQ ID. No.126)
pgi	*Δ_pgi_for*	atgaaaaacatcaatccaacgcagaccgctgcctggcaggcactagtgtaggctggagctgcttc (SEQ ID. No.127)
	Δ_pgi_rev	ttaaccgcgccacgctttatagcggttaatcagaccattggtcgacatatgaatatcctccttag (SEQ ID. No.128)

Table 12: Primers used for gene disruptions. Sequences homologous to target genes are underlined

5

Deleted gene	Sequence (5' - 3')

	Forward primer	Reverse primer
K2 for / k1 rev	cggtgccctgaatgaactgc (SEQ ID No.129)	cagtcatagccgaatagcct (SEQ ID No.130)
ldhA	atacgtgtcccgagcggtag (SEQ ID No.131)	tacacatcccgccatcagca (SEQ ID No.132)
adhE	gaagtaaacgggaaaatcaa (SEQ ID No.133)	agaagtggcataagaaaacg (SEQ ID No.134)
ackA	ccattggctgaaaattacgc (SEQ ID No.135)	gttccattgcacggatcacg (SEQ ID No.136)
focA_pflB	atgccgtagaagccgccagt (SEQ ID No.137)	tgttggtgcgcagctcgaag (SEQ ID No.138)
pta	gcaaatctggtttcatcaac (SEQ ID No.139)	tcccttgcacaaaacaaagt (SEQ ID No.140)
poxB	ggatttggttctcgcataat (SEQ ID No.141)	agcattaacggtagggtcgt (SEQ ID No.142)
sad	gctgattctcgcgaataaac (SEQ ID No.143)	aaaaacgttcttgcgcgtct (SEQ ID No.144)
gabD	tctgtttgtcaccaccccgc (SEQ ID No.145)	aagccagcacctggaagcag (SEQ ID No.146)
gadA	aagagctgccgcaggaggat (SEQ ID No.147)	gccgccctcttaagtcaaat (SEQ ID No.148)
gadB	ggattttagcaatattcgct (SEQ ID No.149)	cctaatagcaggaagaagac (SEQ ID No.150)
gadC	gctgaactgttgctggaaga (SEQ ID No.151)	ggcgtgcttttacaactaca (SEQ ID No.152)
sfcA	tagtaaataacccaaccggc (SEQ ID No.153)	tcagtgagcgcagtgtttta (SEQ ID No.154)
maeB	attaatggtgagagtttgga (SEQ ID No.155)	tgcttttttttattattcgc (SEQ ID No.156)
ppc	gctttataaaagacgacgaa (SEQ ID No.157)	gtaacgacaattccttaagg (SEQ ID No.158)
pykA	tttatatgcccatggtttct (SEQ ID No.159)	atctgttagaggcggatgat (SEQ ID No.160)
pykF	ctggaacgttaaatctttga (SEQ ID No.161)	ccagtttagtagctttcatt (SEQ ID No.162)
iclR	gatttgttcaacattaactcatcgg (SEQ ID No.163)	tgcgattaacagacaccctt (SEQ ID No.164)
mgsA	tctcaggtgctcacagaaca (SEQ ID No.165)	tatggaagaggcgctactgc (SEQ ID No.166)
icd	cgacctgctgcataaacacc (SEQ ID No.167)	tgaacgctaaggtgattgca (SEQ ID No.168)
sucA	acgtagacaagagctcgcaa (SEQ ID No.169)	catcacgtacgactgcgtcg (SEQ ID No.170)
sucB	tgcaactttgtgctgagcaa (SEQ ID No.171)	tatcgcttccgggcattgtc (SEQ ID No.172)
frdA	aaatcgatctcgtcaaatttcagac (SEQ ID No.173)	aggaaccacaaatcgccata (SEQ ID No.174)
frdB	gacgtgaagattactacgct (SEQ ID No.175)	agttcaatgctgaaccacac (SEQ ID No.176)
frdC	tagccgcgaccacggtaagaaggag (SEQ ID No.177)	cagcgcatcacccggaaaca SEQ ID No.178)
frdD	atcgtgatcattaacctgat (SEQ ID No.179)	ttaccctgataaattaccgc (SEQ ID No.180)
lacI	gaatctggtgtatatggcga (SEQ ID No.181)	tcttcgctattacgccagct (SEQ ID No.182)
lldD	cgtcagcggatgtatctggt (SEQ ID No.183)	gcggaatttctggttcgtaa (SEQ ID No.184)
pgi	ttgtcaacgatggggtcatg (SEQ ID No.195)	aaaaatgccgacataacgtc (SEQ ID No.196)
ptsG	ccatccgttgaatgagtttt (SEQ ID No.197)	tggtgttaactggcaaaatc (SEQ ID No.198)
ptsI	gtgacttccaacggcaaaag (SEQ ID No.199)	ccgttggtttgatagcaata (SEQ ID No.200)

Table 13: Primer pairs used for verification of gene disruptions

Protocol for introduction of gene deletions using the phage transduction method: strains carrying the desired single deletions were obtained from the Keio collection (Baba *et al.*, 2006). Phage lysates of single deletion mutants were prepared by inoculating 10 mL of LB medium containing 50 µg/mL kanamycin, 2 g/L glucose, and 5 mM $CaCl_2$ with 100 µL of overnight precultures. Following an incubation of 1 h at 37 °C, 200 µL of phage lysate prepared from the wild-type MG1655 strain were added, and cultures were incubated for another 2-3 h until cell lysis had completed. After addition of 200 µL chloroform, cell preparations were first vigorously vortexed and then centrifuged for 10 min at 4500 x g. The clear lysate was recovered and stored at 4 °C.

The receptor strain was prepared for phage transduction by an overnight cultivation at 37 °C in LB medium. A volume of 1.5 mL of the preculture was centrifuged at 1500 x g for 10 min. The supernatant was discarded and the cell pellet was resuspended in 600 µL of a solution containing 10 mM $MgSO_4$ and 5 mM $CaCl_2$. The transduction was carried out by mixing 100 µL of the solution containing the receptor strain with 100 µL of lysate and incubating this mixture at 30 °C for 30 min. Thereafter, 100 µL of a 1M sodium citrate solution were added followed by vigorous vortexing. After addition of 1 mL LB medium, the cell suspension was incubated at 37 °C for 1 h before spreading the cells on LB agar dishes containing 50 µg/mL kanamycin. Clones able to grow in presence of the antibiotic were confirmed by colony PCR to contain the desired deletion using the primers listed in Table 13. After the introduction of each gene deletion, the antibiotic marker was removed as described above following the method of (Cherepanov & Wackernagel, 1995)

The plasmids co-expressing malyl-CoA synthetase, malyl-CoA reductase, and DHB dehydrogenase (pEXT20-MCS-DHB or pACT3-MCS-DHB); or plasmids co-expressing malyl-CoA lyase, malyl-CoA reductase, and

DHB dehydrogenase(pEXT20-MCL-DHB or pACT3-MCL-DHB);or the empty control plasmids (pEXT20 or pACT3) were transformed alone or together with one of the plasmids pACT3-aceA, pACT3-ppc, pACT3-galP, pACT3-pck or pACT3-pyc into the optimized host strains. Transformants containing both a plasmid expressing the DHB-pathway enzymes, and a plasmid expressing an anaplerotic enzyme were selected on solid LB medium containing chloramphenicol (25 μg/mL) and kanamycin (50 μg/mL). Non-exclusive examples of constructed strains are listed in Table 14.

Strain	**Relevant Genotype**
MG1655	Wild-type
ECE50	pEXT20-MCS-DHB
ECE51	pACT3-MCS-DHB
ECE52	pEXT20-MCL-DHB
ECE53	pACT3-MCL-DHB
ECE54	ΔldhA ΔadhE Δpta-ack ΔpflB pEXT20-MCS-DHB
ECE55	ΔldhA ΔadhE Δpta-ack ΔpflB pACT3-MCS-DHB
ECE56	ΔldhA ΔadhE Δpta-ack ΔpflB pACT3-MCS-DHB, pACT3-ppc
ECE57	ΔldhA ΔadhE Δpta-ack ΔpflB ΔpoxB pEXT20-MCS-DHB
ECE58	ΔldhA ΔadhE Δpta-ack ΔpflB ΔpoxB pACT3-MCS-DHB
ECE59	ΔldhA ΔadhE Δpta-ack ΔpflB ΔpoxB pEXT20-MCS-DHB, pACT3-ppc
ECE60	ΔldhA ΔadhE Δpta-ack ΔpflB ΔpoxB ΔmaeB ΔsfcA pEXT20-MCS-DHB
ECE61	ΔldhA ΔadhE Δpta-ack ΔpflB ΔpoxB ΔmaeB ΔsfcA pACT3-MCS-DHB
ECE62	ΔldhA ΔadhE Δpta-ack ΔpflB ΔpoxB ΔmaeB ΔsfcA pEXT20-MCS-DHB, pACT3-ppc
ECE63	ΔldhA ΔadhE Δpta-ack ΔpflB ΔpoxB ΔmaeB ΔsfcA Δpts pEXT20-MCS-DHB
ECE64	ΔldhA ΔadhE Δpta-ack ΔpflB ΔpoxB ΔmaeB ΔsfcAΔpts pACT3-MCS-DHB
ECE65	ΔldhA ΔadhE Δpta-ack ΔpflB ΔpoxB ΔmaeB ΔsfcAΔpts pEXT20-MCS-DHB, pACT3-ppc
ECE66	ΔldhA ΔadhE Δpta-ack ΔpflB ΔpoxB ΔmaeB ΔsfcA Δpts ΔfrdBC pEXT20-MCS-DHB
ECE67	ΔldhA ΔadhE Δpta-ack ΔpflB ΔpoxB ΔmaeB ΔsfcA Δpts ΔfrdBC pACT3-MCS-DHB
ECE68	ΔldhA ΔadhE Δpta-ack ΔpflB ΔpoxB ΔmaeB ΔsfcA Δpts ΔfrdBC pEXT20-MCS-DHB, pACT3-ppc
ECE69	Δpta ΔiclR ΔaceB

	pACT3-MCL-DHB
ECE70	Δpta ΔiclR ΔaceB
ECE71	Δpta ΔiclR ΔaceB ΔadhE pACT3-MCL-DHB
ECE72	Δpta ΔiclR ΔaceB ΔadhE
ECE73	ΔldhA ΔadhE Δpta-ack ΔpoxB ΔmaeB ΔsfcA Δmdh Δmqo ΔiclR ΔaceB pEXT20-MCS-DHB, pACT3- MCL-DHB
ECE74	ΔldhA ΔadhE Δpta-ack ΔpoxB ΔmaeB ΔsfcA Δmdh Δmqo ΔiclR ΔaceB Δpts pEXT20-MCS-DHB, pACT3- MCL-DHB
ECE75	ΔldhA ΔadhE Δpta-ack ΔpoxB ΔmaeB ΔsfcA Δmdh Δmqo ΔiclR ΔaceB Δpts Δpgi pEXT20-MCS-DHB, pACT3- MCL-DHB
ECE76	ΔldhA ΔadhE Δpta-ack ΔpoxB ΔmaeB ΔsfcA Δmdh Δmqo ΔiclR ΔaceB Δpgi pEXT20-MCS-DHB, pACT3- MCL-DHB
ECE77	ΔldhA ΔadhE Δpta-ack ΔpoxB ΔmaeB ΔsfcA Δmdh Δmqo ΔiclR ΔaceB ΔghrAB pEXT20-MCS-DHB, pACT3- MCL-DHB
ECE79	ΔldhA ΔadhE Δpta-ack ΔpflB ΔpoxB ΔmaeB ΔsfcA aspC pEXT20-MCS-DHB, pACT3-ppc
ECE80	ΔldhA ΔadhE Δpta-ack ΔpflB ΔpoxB ΔmaeB ΔsfcA ΔaspC ΔiclR ΔaceB pEXT20-MCS-DHB, pACT3-ppc
ECE81	ΔldhA ΔadhE Δpta-ack ΔpflB ΔpoxB ΔmaeB ΔsfcA ΔaspC ΔiclR ΔaceB ΔghrAB pEXT20-MCS-DHB, pACT3-ppc

Table 14: Examples of strains constructed for DHB production

Example 8: Demonstration of zymotic production of DHB by the synthetic malyl-CoA pathway

Strains and cultivation conditions: Experiments were carried out using strain ECE69 which expressed the DHB pathway from plasmid pACT3-MCL-DHB represented by SEQ ID No. 203 (the wild-type Mcr enzyme was replaced by the Mcr Tyr206Pro mutant in this experiment) and the isogenic control strain ECE70 containing the empty plasmid pACT3. All cultivations were carried out at 37 °C on an Infors rotary shaker running at 170 rpm. Overnight cultures (3 mL medium in test tube) were inoculated from glycerol stocks and used to adjust an initial OD_{600} of 0.05 in 100 mL growth cultures cultivated in 500 mL shake flasks. IPTG was added at a concentration of 1 mmol/L when OD_{600} of the growth cultures reached 1. The composition of the growth mineral medium is provided in Example 2.

*Estimation of DHB concentration by LC-MS/MS analyses:*DHB was quantified using LC-MS: Liquid anion exchange chromatography was performed on an ICS-3000 system from Dionex (Sunnyvale, USA) equipped with an automatic eluent (KOH) generator system (RFIC, Dionex), and an autosampler (AS50, Dionex) holding the samples at 4 °C. Analytes were separated on an IonPac AS11 HC (250 x 2 mm, Dionex) column protected by an AG11 HC (50 x 2 mm, Dionex) pre-column. Column temperature was held at 25 °C, flow rate was fixed at 0.25 mL/min, and analytes were eluted applying the KOH gradient described earlier (Groussac E, Ortiz M & Francois J (2000) Improved protocols for quantitative determination of metabolites from biological samples using high performance ionic-exchange chromatography with conductimetric and pulsed amperometric detection. *Enzyme.Microb. Technol.***26**, 715-723). Injected sample volume was 15 µL. For background reduction, an ASRS ultra II (2 mm, external water mode, 75 mA) anion suppressor was used. Analytes were quantified a mass-sensitive detector (MSQ Plus, Thermo) running in ESI mode (split was 1/3, nitrogen pressure was 90 psi, capillary voltage was 3.5 kV, probe temperature was 450 °C).

Results: After 24 h of cultivation the supernatant of strains ECE69 and ECE70 contained 0.05 mM DHB and 0 mM DHB, respectively, demonstrating DHB production via the synthetic pathway.

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We claim:

1. Method for the preparation of 2,4-dihydroxybutyric acid (2,4-DHB) comprising the successive steps of :
   a) a first step of converting malate, and/or succinyl-CoA and/or glyoxylate into malyl-CoA,
   b) a second step of converting malyl-CoA previously obtained into malate-4-semialdehyde,
   c) a third step of converting malate-4-semialdehyde into 2,4-DHB using a DHB dehydrogenase.

2. The method of claim 1 wherein step a) is catalyzed by an enzyme having malyl-CoA synthetase, succinyl-CoA: (L)-Malate-CoA transferase and/or malyl-CoA lyase activity, respectively.

3. The method of claim 2 wherein the enzyme is represented by by any one of SEQ ID No. 1, SEQ ID No. 193 and SEQ ID No.195 or any variant or fragment thereof.

4. The method of claim 2 or 3 wherein the enzyme is encoded by any one of the nucleic acid defined in SEQ ID No. 2, SEQ ID No. 194 and SEQ ID No. 196 or any variant or fragment thereof.

5. The method of claim 1 or 2 wherein step b) is catalyzed by an enzyme having malyl-CoA reductase activity.

6. The method according to claim 5 in which the malate semialdehyde dehydrogenase is obtainable by at least one

mutation of an enzyme said mutation(s) improving the activity and/or substrate affinity of the mutated enzyme for malyl-CoA

7. The method of claim 6 wherein the malyl-CoA reductase comprises at least one mutation when compared to the wild type enzyme in at least one of the positions P111, L152, T154, L202, G203, D204, Y206, D207, K209, T210, T238,T239, D295, R318, wherein the naturally occurring amino acid in said positions is replaced by anyone of the other 19 naturally existing proteinogenic amino acids, that is by either alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, or valine.

8. The method of claim 7 wherein the malyl-CoA reductase is represented by SEQ ID No. 202.

9. The method of claim 8 wherein the enzyme is encoded by the nucleic acid of SEQ ID No. 201.

10. The method of claim 5 wherein the enzyme is a malonyl-CoA reductase, or a succinyl-CoA reductase.

11. The method of claim 10 wherein the enzyme is obtained by modification of an enzyme having malonyl-CoA reductase or succinyl-CoA reductase activity.

12. The method of claim 10 or 11 wherein the enzyme is represented by any one of SEQ ID No. 7, SEQ ID No. 8, or SEQ ID No.191 or any variant or fragment thereof.

13. The method of claim 12 wherein the enzyme is encoded by any one of the nucleic acid defined in SEQ ID No. 8, SEQ ID No. 10, or SEQ ID No. 192 or any variant or fragment thereof.

14. The method of anyone of claim 1 to 13 wherein step c) is catalyzed by an enzyme having DHB dehydrogenase activity.

15. The method of claim 14 wherein the enzyme is a methylbutyraldehyde reductase, a succinic semialdehyde reductase, a 4-hydroxybutyrate dehydrogenase, or an alcohol dehydrogenase.

16. The method of claim 15 wherein the enzyme is represented by SEQ ID No. 14, SEQ ID No. 16, SEQ ID No. 32, SEQ ID No. 34, SEQ ID No. 36, SEQ ID No. 187 or SEQ ID No. 189 or any variant thereof.

17. The method of claim 16 the enzyme is encoded by any one of the nucleic acid defined in SEQ ID No. 15, SEQ ID No. 17, SEQ ID No. 33, SEQ ID No. 35, SEQ ID No. 37, SEQ ID No. 188, SEQ ID No. 190 or any variant or fragment thereof.

18. The method according to any one of claims 1 to 17 wherein, steps a), b) and c) are performed by a modified microorganism heterogeously expressing at least one of the enzymes performing enzymatic activities described in steps a), b) or c).

19. The method according to any one of claims 1 to 17 wherein steps a), b) and c) are performed within the same microorganism.

20. A modified microorganism for an improved production of 2,4-DHB wherein said microorganism expresses the genes coding for the enzmes having the enzymatic activitites necessary for the catalysis of steps a), b) and c) as defined in any one of claims 1 to 17.

21. The microorganism of claim 20 being a bacterium, preferentially selected among Enterobacteriaceae, Clostridiaceae, Bacillaceae, Streptomycetaceae, Streptococcaceae,

Methylobacteriacae, and Corynebacteriaceae, most preferentially *Escherichia coli, Bacillus subtilis, Corynebacterium glutamicum, Clostridium acetobutylicum, Methylobacterium extorquens,* or *Lactococcus lactis*, or a yeast preferentially selected among Saccharomycetaceae, Pichiaceae, and Schizosaccharomycetaceae, most preferentially *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kluyveromyces lactis, Kluyveromyces marxianus, Pichia jadinii, Pichia stipitis,* or *Pichia pastoris*.

22. The microorganism of claim 20 or 21 wherein the expression of at least of one the enzymatic activities chosen among phosphoenolpyruvate carboxylase, phosphoenolpyruvate carboxykinase, pyruvate carboxylase, isocitrate lyase, pyruvate carboxylase, and hexose symporter permease is increased, and/or at least one of the enzymatic activities chosen among lactate dehydrogenase, alcohol dehydrogenase, acetate kinase, phosphate acetyltransferase, pyruvate oxidase, isocitrate lyase, fumarate reductase, fumarase, 2-oxoglutarate dehydrogenase, pyruvate kinase, malic enzyme, phosphoglucose isomerase, phosphoenolpyruvate carboxylase, phosphoenolpyruvate carboxykinase, pyruvate-formate lyase, succinic semialdehyde dehydrogenase, sugar-transporting phosphotransferase, aspartate aminotransferase, glyoxylate reductase, malate synthase, or methylglyoxal synthase is decreased.

23. The microorganisms according to claim 22 being *Escherichia coli* overexpressing at least one of the genes chosen among *ppc, pck, aceA, galP* all *E. coli*; *pycA* from *L lactis*, and/or having at least one of the genes deleted chosen among *ldhA, adhE, ackA, pta, poxB, focA, pflB, sad, gabABC, sfcA, maeB, ppc, pykA, pykF, mgsA, frdABCD, sucAB, ptsI, ptsG, pgi, fumABC, aldA, lldD, iclR, aceB, aspC, ghrAB*.

24. A method of production of 2,4-DHB comprising the steps of

- culturing the modified microorganism of anyone of claims 15 to 18 in an appropriate culture medium,
- recovering 2,4-DHB from the culture medium.

25. The method of claim 24 wherein the 2,4-DHB is further purified.

Dated this 12th day of November, 2014.

Adisseo France S.A.S.

Dev Robinson

(Dev Robinson)
of Amarchand & Mangaldas &
Suresh A. Shroff & Co.
Attorneys for the Applicant

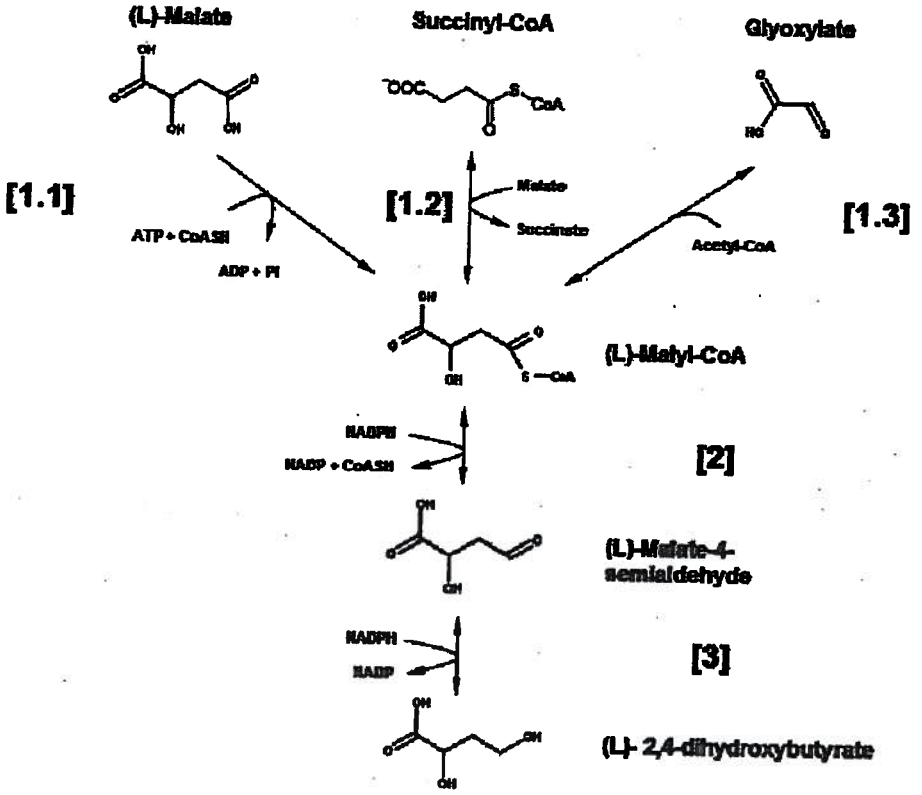

FIGURE 1

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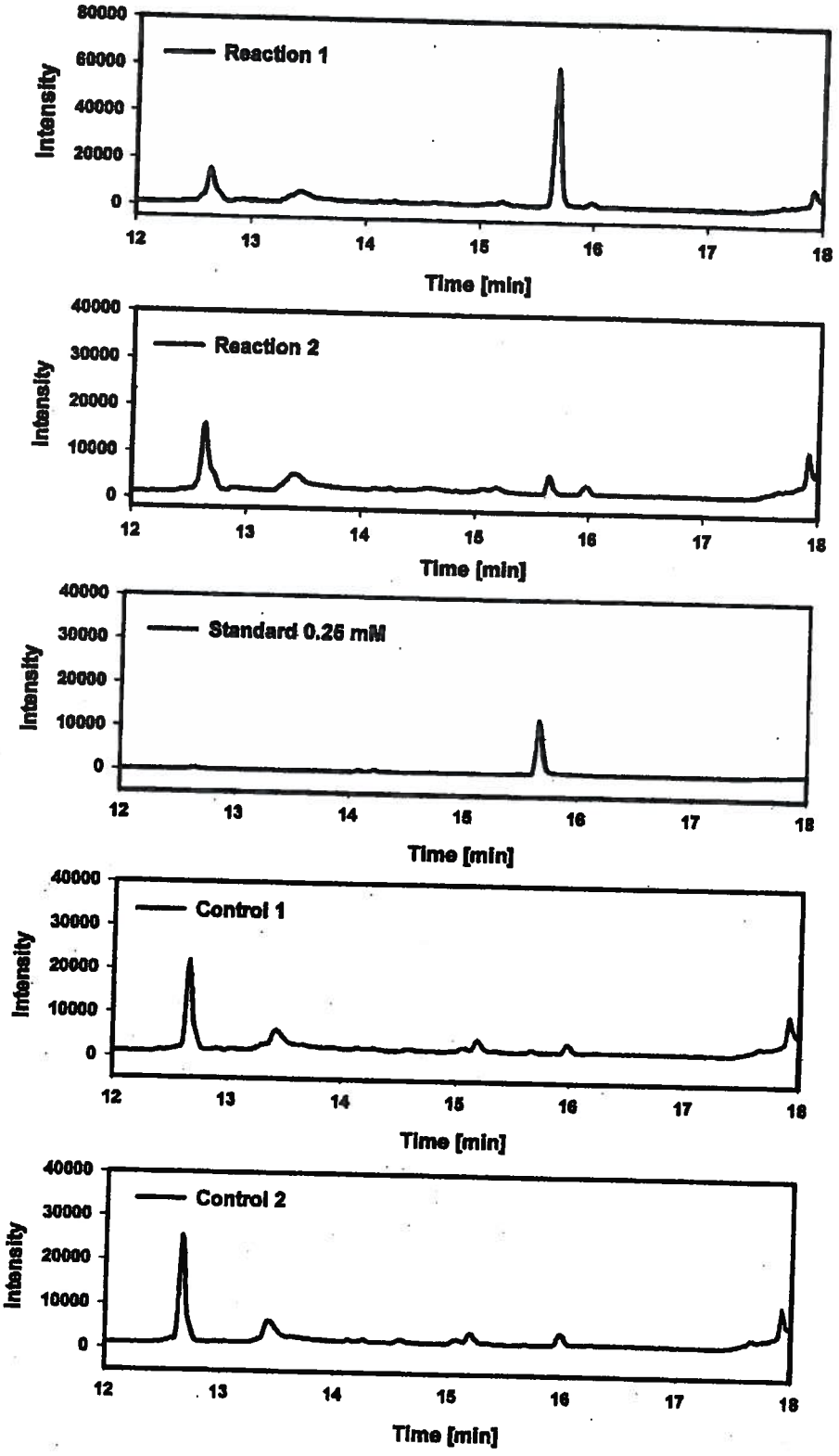
SURESH A. SHROFF & CO.
ATTORNEYS FOR THE APPLICANT



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OF AMARCHAND & MANGALDAS &
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ATTORNEYS FOR THE APPLICANT

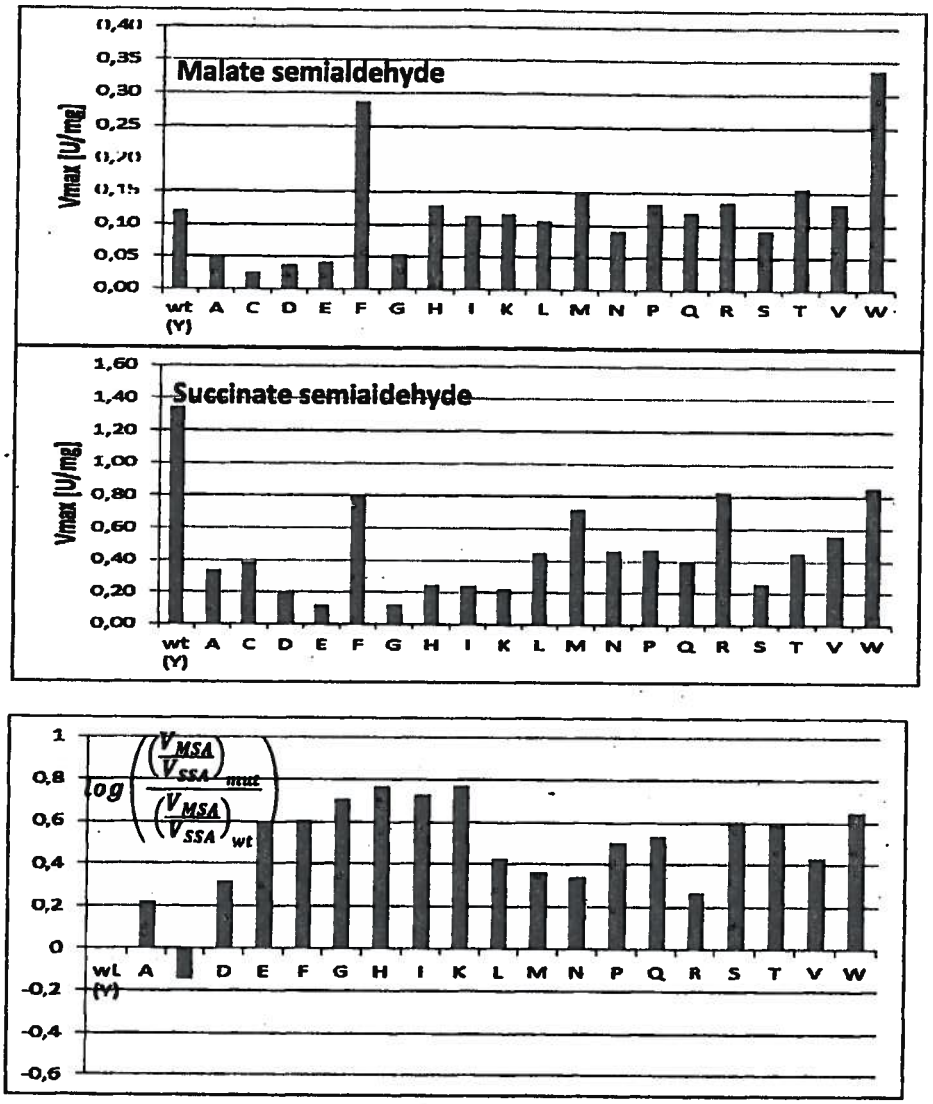

FIGURE 3

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SURESH A. SHROFF & CO.
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ABSTRACT

A METHOD OF PRODUCTION OF 2,4-DIHYDROXYBUTYRIC ACID

The present invention deals with a method for the preparation of 2,4-dihydroxybutyric acid (2,4-DHB) comprising the successive steps of converting malate, succinyl-CoA and/or glyoxylate into malyl-CoA, converting malyl-CoA previously obtained into malate-4-semialdehyde, and converting malate-4-semialdehyde into 2,4-DHB using a DHB dehydrogenase.

SEQUENCE LISTING

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<120> A method of production of 2,4-dihydroxybutyric acid

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<151> 2012-04-26

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cgcattgcaa ccattcatgg ccactatgag gtgttgtacg tgtcgtttaa ggaagaaaca 780

gcagcggaga aggtcaaaga aacgctggaa aactttcgcg gtgaacctca ggatctcaaa 840

ctgccgacag cgccctcgaa accgatcatt gtgatgaacg aagatactcg cccacaggta 900

tatttcgatc gttgggcggg cgacattccg ggcatgagtg tcgttgttgg ccgtctgaaa 960

caggtcaaca aacgcatgat tcgtctggta tcgcttatcc acaacaccgt acgtggtgct 1020

gcgggcggtg ggattttagc tgcggagttg ctcgtggaga aaggctacat cgagaagtaa 1080

<210> 10
<211> 451
<212> PRT
<213> Porphyromonas gingivalis

<400> 10

Met Glu Ile Lys Glu Met Val Ser Leu Ala Arg Lys Ala Gln Lys Glu
1 5 10 15

Tyr Gln Ala Thr His Asn Gln Glu Ala Val Asp Asn Ile Cys Arg Ala
20 25 30

Ala Ala Lys Val Ile Tyr Glu Asn Ala Ala Ile Leu Ala Arg Glu Ala
35 40 45

Val Asp Glu Thr Gly Met Gly Val Tyr Glu His Lys Val Ala Lys Asn
50 55 60

Gln Gly Lys Ser Lys Gly Val Trp Tyr Asn Leu His Asn Lys Lys Ser
65 70 75 80

Ile Gly Ile Leu Asn Ile Asp Glu Arg Thr Gly Met Ile Glu Ile Ala
85 90 95

Lys Pro Ile Gly Val Val Gly Ala Val Thr Pro Thr Thr Asn Pro Ile
100 105 110

Val Thr Pro Met Ser Asn Ile Ile Phe Ala Leu Lys Thr Cys Asn Ala
115 120 125

Ile Ile Ile Ala Pro His Pro Arg Ser Lys Lys Cys Ser Ala His Ala
130 135 140

Val Arg Leu Ile Lys Glu Ala Ile Ala Pro Phe Asn Val Pro Glu Gly
145 150 155 160

Met Val Gln Ile Ile Glu Glu Pro Ser Ile Glu Lys Thr Gln Glu Leu
165 170 175

Met Gly Ala Val Asp Val Val Val Ala Thr Gly Gly Met Gly Met Val
180 185 190

Lys Ser Ala Tyr Ser Ser Gly Lys Pro Ser Phe Gly Val Gly Ala Gly
195 200 205

Asn Val Gln Val Ile Val Asp Ser Asn Ile Asp Phe Glu Ala Ala Ala
210 215 220

Glu Lys Ile Ile Thr Gly Arg Ala Phe Asp Asn Gly Ile Ile Cys Ser
225 230 235 240

Gly Glu Gln Ser Ile Ile Tyr Asn Glu Ala Asp Lys Glu Ala Val Phe
245 250 255

Thr Ala Phe Arg Asn His Gly Ala Tyr Phe Cys Asp Glu Ala Glu Gly
260 265 270

Asp Arg Ala Arg Ala Ala Ile Phe Glu Asn Gly Ala Ile Ala Lys Asp
275 280 285

Val Val Gly Gln Ser Val Ala Phe Ile Ala Lys Lys Ala Asn Ile Asn
290 295 300

Ile Pro Glu Gly Thr Arg Ile Leu Val Val Glu Ala Arg Gly Val Gly
305 310 315 320

Ala Glu Asp Val Ile Cys Lys Glu Lys Met Cys Pro Val Met Cys Ala
325 330 335

Leu Ser Tyr Lys His Phe Glu Glu Gly Val Glu Ile Ala Arg Thr Asn
340 345 350

Leu Ala Asn Glu Gly Asn Gly His Thr Cys Ala Ile His Ser Asn Asn
355 360 365

Gln Ala His Ile Ile Leu Ala Gly Ser Glu Leu Thr Val Ser Arg Ile
370 375 380

Val Val Asn Ala Pro Ser Ala Thr Thr Ala Gly Gly His Ile Gln Asn
385 390 395 400

Gly Leu Ala Val Thr Asn Thr Leu Gly Cys Gly Ser Trp Gly Asn Asn
405 410 415

Ser Ile Ser Glu Asn Phe Thr Tyr Lys His Leu Leu Asn Ile Ser Arg
420 425 430

Ile Ala Pro Leu Asn Ser Ser Ile His Ile Pro Asp Asp Lys Glu Ile
435 440 445

Trp Glu Leu
450

<210> 11
<211> 1356
<212> DNA

<213> Porphyromonas gingivalis

<400> 11
atggaaatca aagaaatggt gagccttgca cgcaaggctc agaaggagta tcaagctacc 60

cataaccaag aagcagttga caacatttgc cgagctgcag caaaagttat ttatgaaaat 120

gcagctattc tggctcgcga agcagtagac gaaaccggca tgggcgttta cgaacacaaa 180

gtggccaaga atcaaggcaa atccaaaggt gtttggtaca acctccacaa taaaaaatcg 240

attggtatcc tcaatataga cgagcgtacc ggtatgatcg agattgcaaa gcctatcgga 300

gttgtaggag ccgtaacgcc gacgaccaac ccgatcgtta ctccgatgag caatatcatc 360

tttgctctta agacctgcaa tgccatcatt attgcccccc accccagatc caaaaaatgc 420

tctgcacacg cagttcgtct gatcaaagaa gctatcgctc cgttcaacgt accggaaggt 480

atggttcaga tcatcgaaga acccagcatc gagaagacgc aggaactcat gggcgccgta 540

gacgtagtag ttgctacggg tggtatgggc atggtgaagt ctgcatattc ttcaggaaag 600

ccttctttcg gtgttggagc cggtaacgtt caggtgatcg tggatagcaa catcgatttc 660

gaagctgctg cagaaaaaat catcaccggt cgtgctttcg acaacggtat catctgctca 720

ggcgaacaga gcatcatcta caacgaggct gacaaggaag cagttttcac agcattccgc 780

aaccacggtg catatttctg tgacgaagcc gaaggagatc gggctcgtgc agctatcttc 840

gaaaatggag ccatcgcgaa agatgtagta ggtcagagcg ttgccttcat tgccaagaaa 900

gcaaacatca atatccccga gggtacccgt attctcgttg ttgaagctcg cggcgtagga 960

gcagaagacg ttatctgtaa ggaaaagatg tgtcccgtaa tgtgcgccct cagctacaag 1020

cacttcgaag aaggtgtaga aatcgcacgt acgaacctcg ccaacgaagg taacggccac 1080

acctgtgcta tccactccaa caatcaggca cacatcatcc tcgcaggatc agagctgacg 1140

gtatctcgta tcgtagtgaa tgctccgagt gccactacag caggcggtca catccaaaac 1200

ggtcttgccg taaccaatac gctcggatgc ggatcatggg gtaataactc tatctccgag 1260

aacttcactt acaagcacct cctcaacatt tcacgcatcg caccgttgaa ttcaagcatt 1320

cacatccccg atgacaaaga aatctgggaa ctctaa 1356

<210> 12
<211> 56
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 12
tataatgagc tcgtttaact ttaagaagga gatataccat gattctgatg cgccgt 56

<210> 13
<211> 30
<212> DNA
<213> Artificial Sequence

<220>

<223> Primer for amplification

<400> 13
tataatggat ccctcgaatt cttacttctc 30

<210> 14
<211> 312
<212> PRT
<213> Saccharomyces cerevisiae

<400> 14

Met Pro Ala Thr Leu Lys Asn Ser Ser Ala Thr Leu Lys Leu Asn Thr
1 5 10 15

Gly Ala Ser Ile Pro Val Leu Gly Phe Gly Thr Trp Arg Ser Val Asp
20 25 30

Asn Asn Gly Tyr His Ser Val Ile Ala Ala Leu Lys Ala Gly Tyr Arg
35 40 45

His Ile Asp Ala Ala Ala Ile Tyr Leu Asn Glu Glu Glu Val Gly Arg
50 55 60

Ala Ile Lys Asp Ser Gly Val Pro Arg Glu Glu Ile Phe Ile Thr Thr
65 70 75 80

Lys Leu Trp Gly Thr Glu Gln Arg Asp Pro Glu Ala Ala Leu Asn Lys
85 90 95

Ser Leu Lys Arg Leu Gly Leu Asp Tyr Val Asp Leu Tyr Leu Met His
100 105 110

Trp Pro Val Pro Leu Lys Thr Asp Arg Val Thr Asp Gly Asn Val Leu
115 120 125

Cys Ile Pro Thr Leu Glu Asp Gly Thr Val Asp Ile Asp Thr Lys Glu
130 135 140

Trp Asn Phe Ile Lys Thr Trp Glu Leu Met Gln Glu Leu Pro Lys Thr
145 150 155 160

Gly Lys Thr Lys Ala Val Gly Val Ser Asn Phe Ser Ile Asn Asn Ile
165 170 175

Lys Glu Leu Leu Glu Ser Pro Asn Asn Lys Val Val Pro Ala Thr Asn
180 185 190

Gln Ile Glu Ile His Pro Leu Leu Pro Gln Asp Glu Leu Ile Ala Phe
195 200 205

Cys Lys Glu Lys Gly Ile Val Val Glu Ala Tyr Ser Pro Phe Gly Ser
210 215 220

Ala Asn Ala Pro Leu Leu Lys Glu Gln Ala Ile Ile Asp Met Ala Lys
225 230 235 240

Lys His Gly Val Glu Pro Ala Gln Leu Ile Ile Ser Trp Ser Ile Gln
245 250 255

Arg Gly Tyr Val Val Leu Ala Lys Ser Val Asn Pro Glu Arg Ile Val
260 265 270

Ser Asn Phe Lys Ile Phe Thr Leu Pro Glu Asp Asp Phe Lys Thr Ile
275 280 285

Ser Asn Leu Ser Lys Val His Gly Thr Lys Arg Val Val Asp Met Lys
290 295 300

Trp Gly Ser Phe Pro Ile Phe Gln
305 310

<210> 15
<211> 939
<212> DNA
<213> Saccharomyces cerevisiae

<400> 15
atgcctgcta cgttaaagaa ttcttctgct acattaaaac taaatactgg tgcctccatt 60

ccagtgttgg gtttcggcac ttggcgttcc gttgacaata acggttacca ttctgtaatt 120

gcagctttga aagctggata cagacacatt gatgctgcgg ctatctattt gaatgaagaa 180

gaagttggca gggctattaa agattccgga gtccctcgtg aggaaatttt tattactact 240

aagctttggg gtacggaaca acgtgatccg gaagctgctc taaacaagtc tttgaaaaga 300

ctaggcttgg attatgttga cctatatctg atgcattggc cagtgccttt gaaaaccgac 360

agagttactg atggtaacgt tctgtgcatt ccaacattag aagatggcac tgttgacatc 420

gatactaagg aatggaattt tatcaagacg tgggagttga tgcaagagtt gccaaagacg 480

ggcaaaacta aagccgttgg tgtctctaat ttttctatta acaacattaa agaattatta 540

gaatctccaa ataacaaggt ggtaccagct actaatcaaa ttgaaattca tccattgcta 600

ccacaagacg aattgattgc cttttgtaag gaaaagggta ttgttgttga agcctactca 660

ccatttggga gtgctaatgc tcctttacta aaagagcaag caattattga tatggctaaa 720

aagcacggcg ttgagccagc acagcttatt atcagttgga gtattcaaag aggctacgtt 780

gttctggcca aatcggttaa tcctgaaaga attgtatcca attttaagat tttcactctg 840

cctgaggatg atttcaagac tattagtaac ctatccaaag tgcatggtac aaagagagtc 900

gttgatatga agtggggatc cttcccaatt ttccaatga 939

<210> 16
<211> 360
<212> PRT
<213> Metallosphaera sedula

<400> 16

Met Lys Ala Ala Val Leu His Thr Tyr Lys Glu Pro Leu Ser Ile Glu
1 5 10 15

Asp Val Asn Ile Ser Gln Pro Lys Ala Gly Glu Val Lys Ile Lys Val
20 25 30

Lys Ala Thr Gly Leu Cys His Ser Asp Val Asn Val Phe Glu Gly Lys
35 40 45

Thr Pro Val Pro Pro Pro Val Val Ala Gly His Glu Ile Ser Gly Ile
50 55 60

Val Glu Glu Val Gly Pro Gly Val Thr Arg Val Lys Pro Gly Asp Arg
65 70 75 80

Val Ile Ser Ala Phe Ile His Pro Cys Gly Lys Cys Gly Asn Cys Val
85 90 95

Ala Gly Lys Glu Asn Leu Cys Glu Thr Phe Ser Gln Val Arg Leu Lys
100 105 110

Gly Val Met Pro Asp Gly Thr Ser Arg Leu Ser Lys Asp Gly Lys Glu
115 120 125

Ile Arg Thr Phe Leu Gly Gly Gly Phe Ala Glu Tyr Ala Ile Val Gly
130 135 140

Glu Asn Ala Leu Thr Arg Val Pro Glu Asp Met Asp Leu Glu Lys Val
145 150 155 160

Ala Val Leu Gly Cys Ala Gly Leu Thr Gly Tyr Gly Ala Ile Ser Ser
165 170 175

Ser Lys Ile Glu Pro Gly Asp Thr Val Ala Val Ile Gly Val Gly Gly
180 185 190

Val Gly Leu Ser Thr Ile Gln Leu Leu Arg Ala Ser Gly Ala Gly Arg
195 200 205

Ile Ile Ala Val Gly Thr Lys Lys Trp Lys Leu Asp Arg Ala Met Glu
210 215 220

Leu Gly Ala Thr Asp Val Val Asn Ser Lys Glu Ile Asp Pro Val Lys
225 230 235 240

Ala Ile Lys Glu Ile Thr Gly Gly Gly Pro Gln Val Val Ile Glu Ala
245 250 255

Gly Gly Asn Glu Asp Thr Ile His Met Ala Leu Asp Ser Val Arg Ile

260 265 270

Gly Gly Lys Val Val Leu Val Gly Leu Pro Pro Ala Thr Ala Met Ile
275 280 285

Pro Ile Arg Val Ala Ser Ile Val Arg Gly Gly Ile Glu Val Val Gly
290 295 300

Asn Tyr Gly Gly Arg Pro Arg Val Asp Met Pro Lys Leu Leu Glu Leu
305 310 315 320

Val Arg Gln Gly Arg Tyr Asp Pro Ser Arg Leu Val Thr Gly Arg Phe
325 330 335

Arg Leu Glu Glu Ile Asn Glu Ala Val Lys Met Leu Glu Glu Gly Glu
340 345 350

Ala Ile Arg Ser Leu Ile Ile Pro
355 360

<210> 17
<211> 1083
<212> DNA
<213> Metallosphaera sedula

<400> 17
atgaaagctg cagtacttca tacgtataag gaaccgctgt ccattgagga cgtgaatatc 60

tcccaaccta aggctgggga agtcaagatc aaggtcaagg caaccgggct ctgtcactcc 120

gacgtcaatg tctttgaggg gaaaacccca gttcctcccc cagtggttgc tggacacgaa 180

atatcaggga ttgtggagga agtgggacct ggggtgacca gggttaaacc aggtgatagg 240

gtgatttcag cgtttattca cccctgtggt aaatgcggta actgcgttgc aggaaaggag 300

aatctgtgtg agaccttctc ccaggtcaga ctcaagggag taatgccaga tggaacgtca 360

aggctgtcaa aggacggaaa ggagataagg actttccttg gaggcggttt cgcggagtac 420

gccattgtgg gagagaacgc gctaaccagg gttccagagg acatggacct agagaaggta 480

gctgtcctag gttgtgctgg gttaacaggg tacggtgcca tatcatcatc caagattgag 540

cctggagaca ctgtggccgt gataggcgta ggaggagtgg gtttgtccac aatacaactc 600

ctaagggcct cgggtgccgg gaggataatc gccgtgggaa cgaaaaagtg gaaacttgac 660

agggccatgg agctaggtgc aactgacgtg gtaaactcga aggagataga tcccgtcaaa 720

gcaataaagg agatcacggg tggagggcca caggtggtga tagaggctgg aggaaatgag 780

gatacgattc atatggcgct ggattcagtt agaattggag gaaaggtggt tctggtaggg 840

ttacctccag caacggccat gatacccatc agggtagcgt caatagttag gggaggcata 900

gaggttgtgg ggaattacgg aggaagacct agggttgata tgcccaagct tctcgagcta 960

gtgaggcagg gaagatacga tccgtctagg cttgtgacgg gtagattcag gttggaggaa 1020

ataaatgagg cagtcaaaat gcttgaggaa ggagaggcca taagaagtct cataatcccg 1080

taa 1083

<210> 18
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 18
tataatgcta gcatgcctgc tacgttaaag aa 32

<210> 19
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 19
tataatgagc tctcattgga aaattgggaa gg 32

<210> 20
<211> 46
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 20
tataatgaat tcttagcggg cggcttcgta tatacggcgg ctgaca 46

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

<220>
<223> Primer for amplification

<400> 21
tatcgtgcta gcatgaacaa ctttaatctg caca 34

<210> 22
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 22
tataatcata tgcaactttt caaactc 27

<210> 23
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 23
tataatggat ccttagtaga gtcttctgta g 31

<210> 24
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 24
tataatgcta gcatgaaagc tgcagtactt ca 32

<210> 25
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 25
tataatgaat tcttacggga ttatgagact tc 32

<210> 26
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<220>
<221> misc_feature
<222> (23)..(24)
<223> n is a, c, g, or t

<400> 26
cattctgcct ttaggggacg gcnnkgacgc caaaacg 37

<210> 27
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<220>
<221> misc_feature
<222> (14)..(15)
<223> n is a, c, g, or t

<400> 27
cgttttggcg tcmnngccgt cccctaaagg cagaatg 37

<210> 28
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 28
gtcaaggcaa ccggtctctg tcgctccgac gtcaatg 37

<210> 29
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 29
cattgacgtc ggagcgacag agaccggttg ccttgac 37

<210> 30
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 30
ggctctgtca ctccgacgta catgtctttg aggggaaaac 40

<210> 31
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 31
gttttcccct caaagacatg tacgtcggag tgacagagcc 40

<210> 32
<211> 360
<212> PRT
<213> Metallosphaera sedula

<400> 32

Met Lys Ala Ala Val Leu His Thr Tyr Lys Glu Pro Leu Ser Ile Glu
1 5 10 15

Asp Val Asn Ile Ser Gln Pro Lys Ala Gly Glu Val Lys Ile Lys Val
20 25 30

Lys Ala Thr Gly Leu Cys Arg Ser Asp Val Asn Val Phe Glu Gly Lys
35 40 45

Thr Pro Val Pro Pro Pro Val Val Ala Gly His Glu Ile Ser Gly Ile

50 55 60

Val Glu Glu Val Gly Pro Gly Val Thr Arg Val Lys Pro Gly Asp Arg
65 70 75 80

Val Ile Ser Ala Phe Ile His Pro Cys Gly Lys Cys Gly Asn Cys Val
85 90 95

Ala Gly Lys Glu Asn Leu Cys Glu Thr Phe Ser Gln Val Arg Leu Lys
100 105 110

Gly Val Met Pro Asp Gly Thr Ser Arg Leu Ser Lys Asp Gly Lys Glu
115 120 125

Ile Arg Thr Phe Leu Gly Gly Gly Phe Ala Glu Tyr Ala Ile Val Gly
130 135 140

Glu Asn Ala Leu Thr Arg Val Pro Glu Asp Met Asp Leu Glu Lys Val
145 150 155 160

Ala Val Leu Gly Cys Ala Gly Leu Thr Gly Tyr Gly Ala Ile Ser Ser
165 170 175

Ser Lys Ile Glu Pro Gly Asp Thr Val Ala Val Ile Gly Val Gly Gly
180 185 190

Val Gly Leu Ser Thr Ile Gln Leu Leu Arg Ala Ser Gly Ala Gly Arg
195 200 205

Ile Ile Ala Val Gly Thr Lys Lys Trp Lys Leu Asp Arg Ala Met Glu
210 215 220

Leu Gly Ala Thr Asp Val Val Asn Ser Lys Glu Ile Asp Pro Val Lys
225 230 235 240

Ala Ile Lys Glu Ile Thr Gly Gly Gly Pro Gln Val Val Ile Glu Ala
245 250 255

Gly Gly Asn Glu Asp Thr Ile His Met Ala Leu Asp Ser Val Arg Ile
260 265 270

Gly Gly Lys Val Val Leu Val Gly Leu Pro Pro Ala Thr Ala Met Ile
275 280 285

Pro Ile Arg Val Ala Ser Ile Val Arg Gly Gly Ile Glu Val Val Gly
290 295 300

Asn Tyr Gly Gly Arg Pro Arg Val Asp Met Pro Lys Leu Leu Glu Leu
305 310 315 320

Val Arg Gln Gly Arg Tyr Asp Pro Ser Arg Leu Val Thr Gly Arg Phe

325 330 335

Arg Leu Glu Glu Ile Asn Glu Ala Val Lys Met Leu Glu Glu Gly Glu
340 345 350

Ala Ile Arg Ser Leu Ile Ile Pro
355 360

<210> 33
<211> 1083
<212> DNA
<213> Metallosphaera sedula

<400> 33
atgaaagctg cagtacttca tacgtataag gaaccgctgt ccattgagga cgtgaatatc 60
tcccaaccta aggctgggga agtcaagatc aaggtcaagg caaccggtct ctgtcgctcc 120
gacgtcaatg tctttgaggg gaaaacccca gttcctcccc cagtggttgc tggacacgaa 180
atatcaggga ttgtggagga agtgggacct ggggtgacca gggttaaacc aggtgatagg 240
gtgatttcag cgtttattca cccctgtggt aaatgcggta actgcgttgc aggaaaggag 300
aatctgtgtg agaccttctc ccaggtcaga ctcaagggag taatgccaga tggaacgtca 360
aggctgtcaa aggacggaaa ggagataagg actttccttg gaggcggttt cgcggagtac 420
gccattgtgg gagagaacgc gctaaccagg gttccagagg acatggacct agagaaggta 480
gctgtcctag gttgtgctgg gttaacaggg tacggtgcca tatcatcatc caagattgag 540
cctggagaca ctgtggccgt gataggcgta ggaggagtgg gtttgtccac aatacaactc 600
ctaagggcct cgggtgccgg gaggataatc gccgtgggaa cgaaaaagtg gaaacttgac 660
agggccatgg agctaggtgc aactgacgtg gtaaactcga aggagataga tcccgtcaaa 720
gcaataaagg agatcacggg tggagggcca caggtggtga tagaggctgg aggaaatgag 780
gatacgattc atatggcgct ggattcagtt agaattggag gaaaggtggt tctggtaggg 840
ttacctccag caacggccat gatacccatc agggtagcgt caatagttag gggaggcata 900
gaggttgtgg ggaattacgg aggaagacct agggttgata tgcccaagct tctcgagcta 960
gtgaggcagg gaagatacga tccgtctagg cttgtgacgg gtagattcag gttggaggaa 1020
ataaatgagg cagtcaaaat gcttgaggaa ggagaggcca taagaagtct cataatcccg 1080
taa 1083

<210> 34
<211> 360
<212> PRT
<213> Metallosphaera sedula

<400> 34

Met Lys Ala Ala Val Leu His Thr Tyr Lys Glu Pro Leu Ser Ile Glu
1 5 10 15

Asp Val Asn Ile Ser Gln Pro Lys Ala Gly Glu Val Lys Ile Lys Val

20 25 30

Lys Ala Thr Gly Leu Cys His Ser Asp Val His Val Phe Glu Gly Lys
35 40 45

Thr Pro Val Pro Pro Pro Val Val Ala Gly His Glu Ile Ser Gly Ile
50 55 60

Val Glu Glu Val Gly Pro Gly Val Thr Arg Val Lys Pro Gly Asp Arg
65 70 75 80

Val Ile Ser Ala Phe Ile His Pro Cys Gly Lys Cys Gly Asn Cys Val
85 90 95

Ala Gly Lys Glu Asn Leu Cys Glu Thr Phe Ser Gln Val Arg Leu Lys
100 105 110

Gly Val Met Pro Asp Gly Thr Ser Arg Leu Ser Lys Asp Gly Lys Glu
115 120 125

Ile Arg Thr Phe Leu Gly Gly Gly Phe Ala Glu Tyr Ala Ile Val Gly
130 135 140

Glu Asn Ala Leu Thr Arg Val Pro Glu Asp Met Asp Leu Glu Lys Val
145 150 155 160

Ala Val Leu Gly Cys Ala Gly Leu Thr Gly Tyr Gly Ala Ile Ser Ser
165 170 175

Ser Lys Ile Glu Pro Gly Asp Thr Val Ala Val Ile Gly Val Gly Gly
180 185 190

Val Gly Leu Ser Thr Ile Gln Leu Leu Arg Ala Ser Gly Ala Gly Arg
195 200 205

Ile Ile Ala Val Gly Thr Lys Lys Trp Lys Leu Asp Arg Ala Met Glu
210 215 220

Leu Gly Ala Thr Asp Val Val Asn Ser Lys Glu Ile Asp Pro Val Lys
225 230 235 240

Ala Ile Lys Glu Ile Thr Gly Gly Gly Pro Gln Val Val Ile Glu Ala
245 250 255

Gly Gly Asn Glu Asp Thr Ile His Met Ala Leu Asp Ser Val Arg Ile
260 265 270

Gly Gly Lys Val Val Leu Val Gly Leu Pro Pro Ala Thr Ala Met Ile
275 280 285

Pro Ile Arg Val Ala Ser Ile Val Arg Gly Gly Ile Glu Val Val Gly

290 295 300

Asn Tyr Gly Gly Arg Pro Arg Val Asp Met Pro Lys Leu Leu Glu Leu
305 310 315 320

Val Arg Gln Gly Arg Tyr Asp Pro Ser Arg Leu Val Thr Gly Arg Phe
325 330 335

Arg Leu Glu Glu Ile Asn Glu Ala Val Lys Met Leu Glu Glu Gly Glu
340 345 350

Ala Ile Arg Ser Leu Ile Ile Pro
355 360

<210> 35
<211> 1083
<212> DNA
<213> Metallosphaera sedula

<400> 35
atgaaagctg cagtacttca tacgtataag gaaccgctgt ccattgagga cgtgaatatc 60
tcccaaccta aggctgggga agtcaagatc aaggtcaagg caaccgggct ctgtcactcc 120
gacgtacatg tctttgaggg gaaaacccca gttcctcccc cagtggttgc tggacacgaa 180
atatcaggga ttgtggagga agtgggacct ggggtgacca gggttaaacc aggtgatagg 240
gtgatttcag cgtttattca cccctgtggt aaatgcggta actgcgttgc aggaaaggag 300
aatctgtgtg agaccttctc ccaggtcaga ctcaagggag taatgccaga tggaacgtca 360
aggctgtcaa aggacggaaa ggagataagg actttccttg gaggcggttt cgcggagtac 420
gccattgtgg gagagaacgc gctaaccagg gttccagagg acatggacct agagaaggta 480
gctgtcctag gttgtgctgg gttaacaggg tacggtgcca tatcatcatc caagattgag 540
cctggagaca ctgtggccgt gataggcgta ggaggagtgg gtttgtccac aatacaactc 600
ctaagggcct cgggtgccgg gaggataatc gccgtgggaa cgaaaaagtg gaaacttgac 660
agggccatgg agctaggtgc aactgacgtg gtaaactcga aggagataga tcccgtcaaa 720
gcaataaagg agatcacggg tggagggcca caggtggtga tagaggctgg aggaaatgag 780
gatacgattc atatggcgct ggattcagtt agaattggag gaaaggtggt tctggtaggg 840
ttacctccag caacggccat gatacccatc agggtagcgt caatagttag gggaggcata 900
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Thr Pro Val Pro Pro Pro Val Val Ala Gly His Glu Ile Ser Gly Ile
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Val Glu Glu Val Gly Pro Gly Val Thr Arg Val Lys Pro Gly Asp Arg
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Glu Asn Ala Leu Thr Arg Val Pro Glu Asp Met Asp Leu Glu Lys Val
145 150 155 160

Ala Val Leu Gly Cys Ala Gly Leu Thr Gly Tyr Gly Ala Ile Ser Ser
165 170 175

Ser Lys Ile Glu Pro Gly Asp Thr Val Ala Val Ile Gly Val Gly Gly
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195 200 205

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210 215 220

Leu Gly Ala Thr Asp Val Val Asn Ser Lys Glu Ile Asp Pro Val Lys
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260 265 270

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Val Arg Gln Gly Arg Tyr Asp Pro Ser Arg Leu Val Thr Gly Arg Phe
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taa 1083

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cttag 65

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gcttc 65

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cttag 65

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gcttc 65

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<210> 95
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gcttc 65

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gcttc 65

<210> 98
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cttag 65

<210> 99
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<210> 101
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gcttc 65

<210> 102
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<210> 103
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gcttc 65

<210> 104
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cttag 65

<210> 107
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gcttc 65

<210> 108
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gcttc 65

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gcttc 65

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<210> 113
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cttag 65

<210> 125
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<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 125
atgattattt ccgcagccag cgattatcgc gccgcagcgc aacgcgtgta ggctggagct 60

gcttc 65

<210> 126
<211> 65
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 126
ctatgccgca ttcccctttcg ccatgggagc cagtgccgca ggcaacatat gaatatcctc 60

cttag 65

<210> 127
<211> 65
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 127
atgaaaaaca tcaatccaac gcagaccgct gcctggcagg cactagtgta ggctggagct 60

gcttc 65

<210> 128
<211> 65
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 128
ttaaccgcgc cacgctttat agcggttaat cagaccattg gtcgacatat gaatatcctc 60

cttag 65

<210> 129
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<220>
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<400> 129
cggtgccctg aatgaactgc 20

<210> 130
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<220>
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<400> 130
cagtcatagc cgaatagcct 20

<210> 131
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<400> 131
atacgtgtcc cgagcggtag 20

<210> 132
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<220>
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<400> 132
tacacatccc gccatcagca 20

<210> 133
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gaagtaaacg ggaaaatcaa 20

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<400> 134
agaagtggca taagaaaacg 20

<210> 135
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<400> 135
ccattggctg aaaattacgc 20

<210> 136
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<220>
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<400> 136
gttccattgc acggatcacg 20

<210> 137
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<220>
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atgccgtaga agccgccagt 20

<210> 138
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<400> 138
tgttggtgcg cagctcgaag 20

<210> 139
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<220>
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<400> 139
gcaaatctgg tttcatcaac 20

<210> 140
<211> 20
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<220>
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<400> 140
tcccttgcac aaaacaaagt 20

<210> 141
<211> 20
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<220>
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<400> 141
ggatttggtt ctcgcataat 20

<210> 142
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<220>
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<400> 142
agcattaacg gtagggtcgt 20

<210> 143
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<400> 143
gctgattctc gcgaataaac 20

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<400> 144
aaaaacgttc ttgcgcgtct 20

<210> 145
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<400> 145
tctgtttgtc accaccccgc 20

<210> 146
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aagccagcac ctggaagcag 20

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aagagctgcc gcaggaggat 20

<210> 148
<211> 20
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<400> 148
gccgccctct taagtcaaat 20

<210> 149
<211> 20
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<220>
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<400> 149
ggattttagc aatattcgct 20

<210> 150
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<220>
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<400> 150
cctaatagca ggaagaagac 20

<210> 151
<211> 20
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<220>
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<400> 151
gctgaactgt tgctggaaga 20

<210> 152
<211> 20
<212> DNA
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<220>
<223> Primer for amplification

<400> 152
ggcgtgcttt tacaactaca 20

<210> 153
<211> 20
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<220>
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<400> 153
tagtaaataa cccaaccggc 20

<210> 154
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<220>
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<400> 154
tcagtgagcg cagtgtttta 20

<210> 155
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<220>
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<400> 155
attaatggtg agagtttgga 20

<210> 156
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<220>
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<400> 156
tgctttttt tattattcgc 20

<210> 157
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<220>
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<400> 157
gctttataaa agacgacgaa 20

<210> 158
<211> 20
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<220>
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<400> 158
gtaacgacaa ttccttaagg 20

<210> 159
<211> 20
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<220>
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<400> 159
tttatatgcc catggtttct 20

<210> 160
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 160
atctgttaga ggcggatgat 20

<210> 161
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 161
ctggaacgtt aaatctttga 20

<210> 162
<211> 20
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<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 162
ccagtttagt agctttcatt 20

<210> 163
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 163
gatttgttca acattaactc atcgg 25

<210> 164
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 164
tgcgattaac agacaccctt 20

<210> 165
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 165
tctcaggtgc tcacagaaca 20

<210> 166
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 166
tatggaagag gcgctactgc 20

<210> 167
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 167
cgacctgctg cataaacacc 20

<210> 168
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 168
tgaacgctaa ggtgattgca 20

<210> 169
<211> 20
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<220>
<223> Primer for amplification

<400> 169
acgtagacaa gagctcgcaa 20

<210> 170
<211> 20
<212> DNA

<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 170
catcacgtac gactgcgtcg 20

<210> 171
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 171
tgcaactttg tgctgagca 19

<210> 172
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 172
tatcgcttcc gggcattgtc 20

<210> 173
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 173
aaatcgatct cgtcaaattt cagac 25

<210> 174
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 174
aggaaccaca aatcgccata 20

<210> 175
<211> 20
<212> DNA
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<220>
<223> Primer for amplification

<400> 175
gacgtgaaga ttactacgct 20

<210> 176
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 176
agttcaatgc tgaaccacac 20

<210> 177
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 177
tagccgcgac cacggtaaga aggag 25

<210> 178
<211> 20
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<220>
<223> Primer for amplification

<400> 178
cagcgcatca cccggaaaca 20

<210> 179
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 179
atcgtgatca ttaacctgat 20

<210> 180
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 180
ttaccctgat aaattaccgc 20

<210> 181
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 181
gaatctggtg tatatggcga 20

<210> 182
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 182
tcttcgctat tacgccagct 20

<210> 183
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 183
cgtcagcgga tgtatctggt 20

<210> 184
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer for amplification

<400> 184
gcggaatttc tggttcgtaa 20

<210> 185
<211> 387
<212> PRT
<213> Escherichia coli

<400> 185

Met Asn Asn Phe Asn Leu His Thr Pro Thr Arg Ile Leu Phe Gly Lys
1 5 10 15

Gly Ala Ile Ala Gly Leu Arg Glu Gln Ile Pro His Asp Ala Arg Val
20 25 30

Leu Ile Thr Tyr Gly Gly Gly Ser Val Lys Lys Thr Gly Val Leu Asp
35 40 45

Gln Val Leu Asp Ala Leu Lys Gly Met Asp Val Leu Glu Phe Gly Gly
50 55 60

Ile Glu Pro Asn Pro Ala Tyr Glu Thr Leu Met Asn Ala Val Lys Leu
65 70 75 80

Val Arg Glu Gln Lys Val Thr Phe Leu Leu Ala Val Gly Gly Gly Ser
85 90 95

Val Leu Asp Gly Thr Lys Phe Ile Ala Ala Ala Ala Asn Tyr Pro Glu
100 105 110

Asn Ile Asp Pro Trp His Ile Leu Gln Thr Gly Gly Lys Glu Ile Lys
115 120 125

Ser Ala Ile Pro Met Gly Cys Val Leu Thr Leu Pro Ala Thr Gly Ser
130 135 140

Glu Ser Asn Ala Gly Ala Val Ile Ser Arg Lys Thr Thr Gly Asp Lys
145 150 155 160

Gln Ala Phe His Ser Ala His Val Gln Pro Val Phe Ala Val Leu Asp
165 170 175

Pro Val Tyr Thr Tyr Thr Leu Pro Pro Arg Gln Val Ala Asn Gly Val
180 185 190

Val Asp Ala Phe Val His Thr Val Glu Gln Tyr Val Thr Lys Pro Val
195 200 205

Asp Ala Lys Ile Gln Asp Arg Phe Ala Glu Gly Ile Leu Leu Thr Leu
210 215 220

Ile Glu Asp Gly Pro Lys Ala Leu Lys Glu Pro Glu Asn Tyr Asp Val
225 230 235 240

Arg Ala Asn Val Met Trp Ala Ala Thr Gln Ala Leu Asn Gly Leu Ile
245 250 255

Gly Ala Gly Val Pro Gln Asp Trp Ala Thr His Met Leu Gly His Glu
260 265 270

Leu Thr Ala Met His Gly Leu Asp His Ala Gln Thr Leu Ala Ile Val
275 280 285

Leu Pro Ala Leu Trp Asn Glu Lys Arg Asp Thr Lys Arg Ala Lys Leu
290 295 300

Leu Gln Tyr Ala Glu Arg Val Trp Asn Ile Thr Glu Gly Ser Asp Asp
305 310 315 320

Glu Arg Ile Asp Ala Ala Ile Ala Ala Thr Arg Asn Phe Phe Glu Gln
325 330 335

Leu Gly Val Pro Thr His Leu Ser Asp Tyr Gly Leu Asp Gly Ser Ser
340 345 350

Ile Pro Ala Leu Leu Lys Lys Leu Glu Glu His Gly Met Thr Gln Leu
355 360 365

Gly Glu Asn His Asp Ile Thr Leu Asp Val Ser Arg Arg Ile Tyr Glu
370 375 380

Ala Ala Arg
385

<210> 186
<211> 1164
<212> DNA
<213> Escherichia coli

<400> 186
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ggtttacgcg aacaaattcc tcacgatgct cgcgtattga ttacctacgg cggcggcagc 120

gtgaaaaaaa ccggcgttct cgatcaagtt ctggatgccc tgaaaggcat ggacgtgctg 180

gaatttggcg gtattgagcc aaacccggct tatgaaacgc tgatgaacgc cgtgaaactg 240

gttcgcgaac agaaagtgac tttcctgctg gcggttggcg gcggttctgt actggacggc 300

accaaattta tcgccgcagc ggctaactat ccggaaaata tcgatccgtg gcacattctg 360

caaacgggcg gtaaagagat taaaagcgcc atcccgatgg gctgtgtgct gacgctgcca 420

gcaaccggtt cagaatccaa cgcaggcgcg gtgatctccc gtaaaaccac aggcgacaag 480

caggcgttcc attctgccca tgttcagccg gtatttgccg tgctcgatcc ggtttatacc 540

tacaccctgc cgccgcgtca ggtggctaac ggcgtagtgg acgcctttgt acacaccgtg 600

gaacagtatg ttaccaaacc ggttgatgcc aaaattcagg accgtttcgc agaaggcatt 660

ttgctgacgc taatcgaaga tggtccgaaa gccctgaaag agccagaaaa ctacgatgtg 720

cgcgccaacg tcatgtgggc ggcgactcag gcgctgaacg gtttgattgg cgctggcgta 780

ccgcaggact gggcaacgca tatgctgggc cacgaactga ctgcgatgca cggtctggat 840

cacgcgcaaa cactggctat cgtcctgcct gcactgtgga atgaaaaacg cgataccaag 900

cgcgctaagc tgctgcaata tgctgaacgc gtctggaaca tcactgaagg ttccgatgat 960

gagcgtattg acgccgcgat tgccgcaacc cgcaatttct ttgagcaatt aggcgtgccg 1020

acccacctct ccgactacgg tctggacggc agctccatcc cggctttgct gaaaaaactg 1080

gaagagcacg gcatgaccca actgggcgaa aatcatgaca ttacgttgga tgtcagccgc 1140

cgtatatacg aagccgcccg ctaa 1164

<210> 187
<211> 371
<212> PRT
<213> Porphyromonas gingivalis

<400> 187

Met Gln Leu Phe Lys Leu Lys Ser Val Thr His His Phe Asp Thr Phe
1 5 10 15

Ala Glu Phe Ala Lys Glu Phe Cys Leu Gly Glu Arg Asp Leu Val Ile
20 25 30

Thr Asn Glu Phe Ile Tyr Glu Pro Tyr Met Lys Ala Cys Gln Leu Pro
35 40 45

Cys His Phe Val Met Gln Glu Lys Tyr Gly Gln Gly Glu Pro Ser Asp
50 55 60

Glu Met Met Asn Asn Ile Leu Ala Asp Ile Arg Asn Ile Gln Phe Asp
65 70 75 80

Arg Val Ile Gly Ile Gly Gly Gly Thr Val Ile Asp Ile Ser Lys Leu
85 90 95

Phe Val Leu Lys Gly Leu Asn Asp Val Leu Asp Ala Phe Asp Arg Lys
100 105 110

Ile Pro Leu Ile Lys Glu Lys Glu Leu Ile Ile Val Pro Thr Thr Cys
115 120 125

Gly Thr Gly Ser Glu Val Thr Asn Ile Ser Ile Ala Glu Ile Lys Ser
130 135 140

Arg His Thr Lys Met Gly Leu Ala Asp Asp Ala Ile Val Ala Asp His
145 150 155 160

Ala Ile Ile Ile Pro Glu Leu Leu Lys Ser Leu Pro Phe His Phe Tyr
165 170 175

Ala Cys Ser Ala Ile Asp Ala Leu Ile His Ala Ile Glu Ser Tyr Val
180 185 190

Ser Pro Lys Ala Ser Pro Tyr Ser Arg Leu Phe Ser Glu Ala Ala Trp
195 200 205

Asp Ile Ile Leu Glu Val Phe Lys Lys Ile Ala Glu His Gly Pro Glu
210 215 220

Tyr Arg Phe Glu Lys Leu Gly Glu Met Ile Met Ala Ser Asn Tyr Ala
225 230 235 240

Gly Ile Ala Phe Gly Asn Ala Gly Val Gly Ala Val His Ala Leu Ser
245 250 255

Tyr Pro Leu Gly Gly Asn Tyr His Val Pro His Gly Glu Ala Asn Tyr
260 265 270

Gln Phe Phe Thr Glu Val Phe Lys Val Tyr Gln Lys Lys Asn Pro Phe
275 280 285

Gly Tyr Ile Val Glu Leu Asn Trp Lys Leu Ser Lys Ile Leu Asn Cys
290 295 300

Gln Pro Glu Tyr Val Tyr Pro Lys Leu Asp Glu Leu Leu Gly Cys Leu
305 310 315 320

Leu Thr Lys Lys Pro Leu His Glu Tyr Gly Met Lys Asp Glu Glu Val
325 330 335

Arg Gly Phe Ala Glu Ser Val Leu Lys Thr Gln Gln Arg Leu Leu Ala
340 345 350

Asn Asn Tyr Val Glu Leu Thr Val Asp Glu Ile Glu Gly Ile Tyr Arg
355 360 365

Arg Leu Tyr
370

<210> 188
<211> 1116
<212> DNA
<213> Porphyromonas gingivalis

<400> 188
atgcaacttt tcaaactcaa gagtgtaaca tcatcactttg acacttttgc agaatttgcc 60

aaggaattct gtcttggaga acgcgacttg gtaattacca acgagttcat ctatgaaccg 120

tatatgaagg catgccagct cccctgccat tttgttatgc aggagaaata tgggcaaggc 180

gagccttctg acgaaatgat gaataacatc ttggcagaca tccgtaatat ccagttcgac 240

cgcgtaatcg gtatcggagg aggtacggtt attgacatct ctaaactttt cgttctgaaa 300

ggattaaatg atgtactcga tgcattcgac cgcaaaatac ctcttatcaa agagaaagaa 360

ctgatcattg tgcccacaac atgcggaacg ggtagcgagg tgacgaacat ttctatcgca 420

gaaatcaaaa gccgtcacac caaaatggga ttggctgacg atgccattgt tgcagaccat 480

gccatcatca tacctgaact tctgaagagc ttgcctttcc acttctacgc atgcagtgca 540

atcgatgctc ttatccatgc catcgagtca tacgtatctc ctaaagccag tccatattct 600

cgtctgttca gtgaggcggc ttgggacatt atcctggaag tattcaagaa aatcgccgaa 660

cacggccctg aataccgctt cgaaaagctg ggagaaatga tcatggccag caactatgcc 720

ggtatagcct tcggaaatgc aggagtagga gccgtccacg cactatccta cccgttggga 780

ggcaactatc acgtgccgca tggagaagca aactatcagt tcttcacaga ggtattcaaa 840

gtataccaaa agaagaatcc tttcggctat atagtcgaac tcaactggaa gctctccaag 900

atactgaact gccagcccga atacgtatat ccgaagctgg atgaacttct cggatgcctt 960

cttaccaaga aacctttgca cgaatacggc atgaaggacg aagaggtaag aggctttgcg 1020

gaatcagtgc ttaagacaca gcaaagattg ctcgccaaca actacgtaga gcttactgta 1080

gatgagatcg aaggtatcta cagaagactc tactaa 1116

<210> 189
<211> 1220
<212> PRT
<213> Chloroflexus aurantiacus

<400> 189

Met Ser Gly Thr Gly Arg Leu Ala Gly Lys Ile Ala Leu Ile Thr Gly
1 5 10 15

Gly Ala Gly Asn Ile Gly Ser Glu Leu Thr Arg Arg Phe Leu Ala Glu
20 25 30

Gly Ala Thr Val Ile Ile Ser Gly Arg Asn Arg Ala Lys Leu Thr Ala
35 40 45

Leu Ala Glu Arg Met Gln Ala Glu Ala Gly Val Pro Ala Lys Arg Ile
50 55 60

Asp Leu Glu Val Met Asp Gly Ser Asp Pro Val Ala Val Arg Ala Gly
65 70 75 80

Ile Glu Ala Ile Val Ala Arg His Gly Gln Ile Asp Ile Leu Val Asn
85 90 95

Asn Ala Gly Ser Ala Gly Ala Gln Arg Arg Leu Ala Glu Ile Pro Leu
100 105 110

Thr Glu Ala Glu Leu Gly Pro Gly Ala Glu Glu Thr Leu His Ala Ser
115 120 125

Ile Ala Asn Leu Leu Gly Met Gly Trp His Leu Met Arg Ile Ala Ala
130 135 140

Pro His Met Pro Val Gly Ser Ala Val Ile Asn Val Ser Thr Ile Phe
145 150 155 160

Ser Arg Ala Glu Tyr Tyr Gly Arg Ile Pro Tyr Val Thr Pro Lys Ala
165 170 175

Ala Leu Asn Ala Leu Ser Gln Leu Ala Ala Arg Glu Leu Gly Ala Arg
180 185 190

Gly Ile Arg Val Asn Thr Ile Phe Pro Gly Pro Ile Glu Ser Asp Arg
195 200 205

Ile Arg Thr Val Phe Gln Arg Met Asp Gln Leu Lys Gly Arg Pro Glu
210 215 220

Gly Asp Thr Ala His His Phe Leu Asn Thr Met Arg Leu Cys Arg Ala
225 230 235 240

Asn Asp Gln Gly Ala Leu Glu Arg Arg Phe Pro Ser Val Gly Asp Val
245 250 255

Ala Asp Ala Ala Val Phe Leu Ala Ser Ala Glu Ser Ala Ala Leu Ser
260 265 270

Gly Glu Thr Ile Glu Val Thr His Gly Met Glu Leu Pro Ala Cys Ser
275 280 285

Glu Thr Ser Leu Leu Ala Arg Thr Asp Leu Arg Thr Ile Asp Ala Ser
290 295 300

Gly Arg Thr Thr Leu Ile Cys Ala Gly Asp Gln Ile Glu Glu Val Met
305 310 315 320

Ala Leu Thr Gly Met Leu Arg Thr Cys Gly Ser Glu Val Ile Ile Gly
325 330 335

Phe Arg Ser Ala Ala Ala Leu Ala Gln Phe Glu Gln Ala Val Asn Glu
340 345 350

Ser Arg Arg Leu Ala Gly Ala Asp Phe Thr Pro Pro Ile Ala Leu Pro
355 360 365

Leu Asp Pro Arg Asp Pro Ala Thr Ile Asp Ala Val Phe Asp Trp Gly
370 375 380

Ala Gly Glu Asn Thr Gly Gly Ile His Ala Ala Val Ile Leu Pro Ala
385 390 395 400

Thr Ser His Glu Pro Ala Pro Cys Val Ile Glu Val Asp Asp Glu Arg
405 410 415

Val Leu Asn Phe Leu Ala Asp Glu Ile Thr Gly Thr Ile Val Ile Ala
420 425 430

Ser Arg Leu Ala Arg Tyr Trp Gln Ser Gln Arg Leu Thr Pro Gly Ala
435 440 445

Arg Ala Arg Gly Pro Arg Val Ile Phe Leu Ser Asn Gly Ala Asp Gln
450 455 460

Asn Gly Asn Val Tyr Gly Arg Ile Gln Ser Ala Ala Ile Gly Gln Leu
465 470 475 480

Ile Arg Val Trp Arg His Glu Ala Glu Leu Asp Tyr Gln Arg Ala Ser
485 490 495

Ala Ala Gly Asp His Val Leu Pro Pro Val Trp Ala Asn Gln Ile Val
500 505 510

Arg Phe Ala Asn Arg Ser Leu Glu Gly Leu Glu Phe Ala Cys Ala Trp
515 520 525

Thr Ala Gln Leu Leu His Ser Gln Arg His Ile Asn Glu Ile Thr Leu
530 535 540

Asn Ile Pro Ala Asn Ile Ser Ala Thr Thr Gly Ala Arg Ser Ala Ser
545 550 555 560

Val Gly Trp Ala Glu Ser Leu Ile Gly Leu His Leu Gly Lys Val Ala
565 570 575

Leu Ile Thr Gly Gly Ser Ala Gly Ile Gly Gly Gln Ile Gly Arg Leu
580 585 590

Leu Ala Leu Ser Gly Ala Arg Val Met Leu Ala Ala Arg Asp Arg His
595 600 605

Lys Leu Glu Gln Met Gln Ala Met Ile Gln Ser Glu Leu Ala Glu Val
610 615 620

Gly Tyr Thr Asp Val Glu Asp Arg Val His Ile Ala Pro Gly Cys Asp
625 630 635 640

Val Ser Ser Glu Ala Gln Leu Ala Asp Leu Val Glu Arg Thr Leu Ser
645 650 655

Ala Phe Gly Thr Val Asp Tyr Leu Ile Asn Asn Ala Gly Ile Ala Gly
660 665 670

Val Glu Glu Met Val Ile Asp Met Pro Val Glu Gly Trp Arg His Thr
675 680 685

Leu Phe Ala Asn Leu Ile Ser Asn Tyr Ser Leu Met Arg Lys Leu Ala
690 695 700

Pro Leu Met Lys Lys Gln Gly Ser Gly Tyr Ile Leu Asn Val Ser Ser
705 710 715 720

Tyr Phe Gly Gly Glu Lys Asp Ala Ala Ile Pro Tyr Pro Asn Arg Ala
725 730 735

Asp Tyr Ala Val Ser Lys Ala Gly Gln Arg Ala Met Ala Glu Val Phe
740 745 750

Ala Arg Phe Leu Gly Pro Glu Ile Gln Ile Asn Ala Ile Ala Pro Gly
755 760 765

Pro Val Glu Gly Asp Arg Leu Arg Gly Thr Gly Glu Arg Pro Gly Leu
770 775 780

Phe Ala Arg Arg Ala Arg Leu Ile Leu Glu Asn Lys Arg Leu Asn Glu
785 790 795 800

Leu His Ala Ala Leu Ile Ala Ala Ala Arg Thr Asp Glu Arg Ser Met
805 810 815

His Glu Leu Val Glu Leu Leu Leu Pro Asn Asp Val Ala Ala Leu Glu
820 825 830

Gln Asn Pro Ala Ala Pro Thr Ala Leu Arg Glu Leu Ala Arg Arg Phe
835 840 845

Arg Ser Glu Gly Asp Pro Ala Ala Ser Ser Ser Ser Ala Leu Leu Asn
850 855 860

Arg Ser Ile Ala Ala Lys Leu Leu Ala Arg Leu His Asn Gly Gly Tyr
865 870 875 880

Val Leu Pro Ala Asp Ile Phe Ala Asn Leu Pro Asn Pro Pro Asp Pro
885 890 895

Phe Phe Thr Arg Ala Gln Ile Asp Arg Glu Ala Arg Lys Val Arg Asp
900 905 910

Gly Ile Met Gly Met Leu Tyr Leu Gln Arg Met Pro Thr Glu Phe Asp
915 920 925

Val Ala Met Ala Thr Val Tyr Tyr Leu Ala Asp Arg Asn Val Ser Gly
930 935 940

Glu Thr Phe His Pro Ser Gly Gly Leu Arg Tyr Glu Arg Thr Pro Thr
945 950 955 960

Gly Gly Glu Leu Phe Gly Leu Pro Ser Pro Glu Arg Leu Ala Glu Leu
965 970 975

Val Gly Ser Thr Val Tyr Leu Ile Gly Glu His Leu Thr Glu His Leu
980 985 990

Asn Leu Leu Ala Arg Ala Tyr Leu Glu Arg Tyr Gly Ala Arg Gln Val
995 1000 1005

Val Met Ile Val Glu Thr Glu Thr Gly Ala Glu Thr Met Arg Arg
1010 1015 1020

Leu Leu His Asp His Val Glu Ala Gly Arg Leu Met Thr Ile Val
1025 1030 1035

Ala Gly Asp Gln Ile Glu Ala Ala Ile Asp Gln Ala Ile Thr Arg
1040 1045 1050

Tyr Gly Arg Pro Gly Pro Val Val Cys Thr Pro Phe Arg Pro Leu
1055 1060 1065

Pro Thr Val Pro Leu Val Gly Arg Lys Asp Ser Asp Trp Ser Thr
1070 1075 1080

Val Leu Ser Glu Ala Glu Phe Ala Glu Leu Cys Glu His Gln Leu
1085 1090 1095

Thr His His Phe Arg Val Ala Arg Lys Ile Ala Leu Ser Asp Gly
1100 1105 1110

Ala Ser Leu Ala Leu Val Thr Pro Glu Thr Thr Ala Thr Ser Thr
1115 1120 1125

Thr Glu Gln Phe Ala Leu Ala Asn Phe Ile Lys Thr Thr Leu His
1130 1135 1140

Ala Phe Thr Ala Thr Ile Gly Val Glu Ser Glu Arg Thr Ala Gln
1145 1150 1155

Arg Ile Leu Ile Asn Gln Val Asp Leu Thr Arg Arg Ala Arg Ala
1160 1165 1170

Glu Glu Pro Arg Asp Pro His Glu Arg Gln Gln Glu Leu Glu Arg
1175 1180 1185

Phe Ile Glu Ala Val Leu Leu Val Thr Ala Pro Leu Pro Pro Glu
1190 1195 1200

Ala Asp Thr Arg Tyr Ala Gly Arg Ile His Arg Gly Arg Ala Ile
1205 1210 1215

Thr Val
1220

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<213> Chloroflexus aurantiacus

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cggaatcggg cgaagttgac cgcactggcc gaacggatgc aggcagaggc aggagtgccg 180

gcaaagcgca tcgatctcga agtcatggat gggagtgatc cggtcgcggt acgtgccggt 240

atcgaagcga ttgtggcccg tcacggccag atcgacattc tggtcaacaa tgcaggaagt 300

gccggtgccc agcgtcgtct ggccgagatt ccactcactg aagctgaatt aggccctggc 360

gccgaagaga cgcttcatgc cagcatcgcc aatttacttg gtatgggatg gcatctgatg 420

cgtattgcgg cacctcatat gccggtagga agtgcggtca tcaatgtctc gaccatcttt 480

tcacgggctg agtactacgg gcggattccg tatgtcaccc ctaaagctgc tcttaatgct 540

ctatctcaac ttgctgcgcg tgagttaggt gcacgtggca tccgcgttaa tacgatcttt 600

cccggcccga ttgaaagtga tcgcatccgt acagtgttcc agcgtatgga tcagctcaag 660

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aacgaccagg gcgcgcttga acgtcggttc ccctccgtcg gtgatgtggc agacgccgct 780

gtctttctgg ccagtgccga atccgccgct ctctccggtg agacgattga ggttacgcac 840

ggaatggagt tgccggcctg cagtgagacc agcctgctgg cccgtactga tctgcgcacg 900

attgatgcca gtggccgcac gacgctcatc tgcgccggcg accagattga agaggtgatg 960

gcgctcaccg gtatgttgcg tacctgtggg agtgaagtga tcatcggctt ccgttcggct 1020

gcggcgctgg cccagttcga gcaggcagtc aatgagagtc ggcggctggc cggcgcagac 1080

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aaactggcgc cgttgatgaa aaaacagggt agcggttaca tccttaacgt ctcatcatac 2160

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aaggctggtc agcgggcaat ggccgaagtc tttgcgcgct tccttggccc ggagatacag 2280

atcaatgcca ttgcgccggg tccggtcgaa ggtgatcgct tgcgcggtac cggtgaacgt 2340

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cgttacgggg cacgtcaggt agtgatgatt gttgagacag aaaccggggc agagacaatg 3060

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<212> PRT
<213> Chloroflexus aurantiacus

<400> 191

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Val Ser Glu Gly Gln Val Thr Gly Thr Ser Glu Met Pro Pro Thr Gly
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Glu Glu Pro Ser Gly His Ala Glu Ser Lys Pro Pro Ala Ser Asp Pro
35 40 45

Met Ser Thr Pro Gly Thr Gly Gln Glu Gln Leu Pro Leu Ser Gly Ile
50 55 60

Arg Val Ile Asp Val Gly Asn Phe Leu Ala Gly Pro Tyr Ala Ala Ser
65 70 75 80

Ile Leu Gly Glu Phe Gly Ala Glu Val Leu Lys Ile Glu His Pro Leu
85 90 95

Gly Gly Asp Pro Met Arg Arg Phe Gly Thr Ala Thr Ala Arg His Asp
100 105 110

Ala Thr Leu Ala Trp Leu Ser Glu Ala Arg Asn Arg Lys Ser Val Thr
115 120 125

Ile Asp Leu Arg Gln Gln Glu Gly Val Ala Leu Phe Leu Lys Leu Val
130 135 140

Ala Lys Ser Asp Ile Leu Ile Glu Asn Phe Arg Pro Gly Thr Met Glu
145 150 155 160

Glu Trp Gly Leu Ser Trp Pro Val Leu Gln Ala Thr Asn Pro Gly Leu
165 170 175

Ile Met Leu Arg Val Ser Gly Tyr Gly Gln Thr Gly Pro Tyr Arg Arg
180 185 190

Arg Ser Gly Phe Ala His Ile Ala His Ala Phe Ser Gly Leu Ser Tyr
195 200 205

Leu Ala Gly Phe Pro Gly Glu Thr Pro Val Leu Pro Gly Thr Ala Pro
210 215 220

Leu Gly Asp Tyr Ile Ala Ser Leu Phe Gly Ala Ile Gly Ile Leu Ile
225 230 235 240

Ala Leu Arg His Lys Glu Gln Thr Gly Arg Gly Gln Leu Ile Asp Val
245 250 255

Gly Ile Tyr Glu Ala Val Phe Arg Ile Leu Asp Glu Ile Ala Pro Ala
260 265 270

Tyr Gly Leu Phe Gly Lys Ile Arg Glu Arg Glu Gly Ala Gly Ser Phe
275 280 285

Ile Ala Val Pro His Gly His Phe Arg Ser Lys Asp Gly Lys Trp Val
290 295 300

Ala Ile Ala Cys Thr Thr Asp Lys Met Phe Glu Arg Leu Ala Glu Ala
305 310 315 320

Met Glu Arg Pro Glu Leu Ala Ser Pro Glu Leu Tyr Gly Asp Gln Arg
325 330 335

Lys Arg Leu Ala Ala Arg Asp Ile Val Asn Gln Ile Thr Ile Glu Trp
340 345 350

Val Gly Ser Leu Thr Arg Asp Glu Val Met Arg Arg Cys Leu Glu Lys
355 360 365

Glu Val Pro Val Gly Pro Leu Asn Ser Ile Ala Asp Met Phe Asn Asp
370 375 380

Glu His Phe Leu Ala Arg Gly Asn Phe Ala Cys Ile Glu Ala Glu Gly
385 390 395 400

Ile Gly Glu Val Val Val Pro Asn Val Ile Pro Arg Leu Ser Glu Thr
405 410 415

Pro Gly Arg Val Thr Asn Leu Gly Pro Pro Leu Gly Asn Ala Thr Tyr
420 425 430

Glu Val Leu Arg Glu Leu Leu Asp Ile Ser Ala Glu Glu Ile Lys Arg
435 440 445

Leu Arg Ser Arg Lys Ile Ile
450 455

<210> 192
<211> 1368
<212> PRT
<213> Chloroflexus aurantiacus

<400> 192

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Cys Gly Gly Thr Cys Ala Gly Cys Cys Ala Ala Cys Gly Gly Ala Gly
35 40 45

Gly Thr Gly Thr Cys Ala Gly Ala Ala Gly Gly Ala Cys Ala Gly Gly
50 55 60

Thr Cys Ala Cys Cys Gly Gly Gly Ala Cys Ala Ala Gly Cys Gly Ala
65 70 75 80

Gly Ala Thr Gly Cys Cys Cys Cys Cys Cys Ala Cys Ala Gly Gly Ala
85 90 95

Gly Ala Ala Gly Ala Ala Cys Cys Ala Thr Cys Ala Gly Gly Ala Cys
100 105 110

Ala Cys Gly Cys Ala Gly Ala Ala Thr Cys Ala Ala Ala Gly Cys Cys
115 120 125

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Gly Cys Cys Gly Gly Cys Cys Ala Gly Thr Gly Ala Thr Cys Cys Gly
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Ala Thr Gly Ala Gly Cys Ala Cys Ala Cys Cys Gly Gly Gly Cys Ala
145                 150                 155                 160

Cys Cys Gly Gly Thr Cys Ala Gly Gly Ala Gly Cys Ala Gly Thr Thr
                165                 170                 175

Gly Cys Cys Gly Thr Thr Gly Ala Gly Thr Gly Gly Cys Ala Thr Thr
            180                 185                 190

Cys Gly Gly Gly Thr Cys Ala Thr Thr Gly Ala Thr Gly Thr Ala Gly
        195                 200                 205

Gly Thr Ala Ala Thr Thr Thr Thr Cys Thr Gly Gly Cys Cys Gly Gly
    210                 215                 220

Cys Cys Cys Gly Thr Ala Thr Gly Cys Thr Gly Cys Thr Thr Cys Cys
225                 230                 235                 240

Ala Thr Cys Cys Thr Gly Gly Gly Thr Gly Ala Ala Thr Thr Cys Gly
                245                 250                 255

Gly Thr Gly Cys Cys Gly Ala Gly Gly Thr Gly Cys Thr Cys Ala Ala
            260                 265                 270

Gly Ala Thr Cys Gly Ala Ala Cys Ala Cys Cys Cys Gly Cys Thr Gly
        275                 280                 285

Gly Gly Thr Gly Gly Cys Gly Ala Thr Cys Cys Gly Ala Thr Gly Cys
    290                 295                 300

Gly Thr Cys Gly Thr Thr Thr Cys Gly Gly Cys Ala Cys Thr Gly Cys
305                 310                 315                 320

Ala Ala Cys Thr Gly Cys Gly Cys Gly Cys Cys Ala Cys Gly Ala Thr
                325                 330                 335

Gly Cys Ala Ala Cys Ala Cys Thr Gly Gly Cys Cys Thr Gly Gly Cys
            340                 345                 350

Thr Gly Ala Gly Cys Gly Ala Gly Gly Cys Cys Cys Gly Thr Ala Ala
        355                 360                 365

Cys Cys Gly Thr Ala Ala Gly Thr Cys Gly Gly Thr Cys Ala Cys Gly
    370                 375                 380

Ala Thr Thr Gly Ala Thr Cys Thr Gly Cys Gly Thr Cys Ala Gly Cys
385                 390                 395                 400
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Ala Ala Gly Ala Gly Gly Gly Cys Gly Thr Thr Gly Cys Gly Cys Thr
405 410 415

Cys Thr Thr Thr Cys Thr Gly Ala Ala Gly Cys Thr Gly Gly Thr Cys
420 425 430

Gly Cys Cys Ala Ala Ala Thr Cys Cys Gly Ala Cys Ala Thr Thr Cys
435 440 445

Thr Gly Ala Thr Thr Gly Ala Ala Ala Ala Cys Thr Thr Thr Cys Gly
450 455 460

Cys Cys Cys Cys Gly Gly Thr Ala Cys Gly Ala Thr Gly Gly Ala Ala
465 470 475 480

Gly Ala Ala Thr Gly Gly Gly Gly Cys Thr Thr Gly Ala Gly Cys Thr
485 490 495

Gly Gly Cys Cys Thr Gly Thr Thr Thr Thr Gly Cys Ala Gly Gly Cys
500 505 510

Gly Ala Cys Gly Ala Ala Thr Cys Cys Cys Gly Gly Ala Cys Thr Gly
515 520 525

Ala Thr Thr Ala Thr Gly Cys Thr Gly Cys Gly Gly Gly Thr Gly Thr
530 535 540

Cys Gly Gly Gly Cys Thr Ala Thr Gly Gly Thr Cys Ala Gly Ala Cys
545 550 555 560

Cys Gly Gly Gly Cys Cys Gly Thr Ala Cys Cys Gly Thr Cys Gly Gly
565 570 575

Cys Gly Thr Thr Cys Gly Gly Gly Gly Thr Thr Thr Gly Cys Cys Cys
580 585 590

Ala Thr Ala Thr Thr Gly Cys Cys Cys Ala Cys Gly Cys Thr Thr Thr
595 600 605

Cys Ala Gly Cys Gly Gly Cys Cys Thr Cys Thr Cys Gly Thr Ala Thr
610 615 620

Cys Thr Gly Gly Cys Cys Gly Gly Gly Thr Thr Cys Cys Cys Cys Gly
625 630 635 640

Gly Cys Gly Ala Ala Ala Cys Gly Cys Cys Ala Gly Thr Cys Thr Thr
645 650 655

Gly Cys Cys Gly Gly Gly Ala Ala Cys Gly Gly Cys Ala Cys Cys Gly
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Cys Thr Cys Gly Gly Cys Gly Ala Cys Thr Ala Thr Ala Thr Cys Gly
675 680 685

Cys Cys Ala Gly Thr Cys Thr Gly Thr Thr Cys Gly Gly Gly Gly Cys
690 695 700

Gly Ala Thr Thr Gly Gly Gly Ala Thr Thr Thr Thr Gly Ala Thr Cys
705 710 715 720

Gly Cys Gly Cys Thr Gly Cys Gly Cys Cys Ala Cys Ala Ala Ala Gly
725 730 735

Ala Gly Cys Ala Gly Ala Cys Cys Gly Gly Ala Cys Gly Cys Gly Gly
740 745 750

Gly Cys Ala Gly Thr Thr Gly Ala Thr Cys Gly Ala Thr Gly Thr Cys
755 760 765

Gly Gly Gly Ala Thr Thr Thr Ala Cys Gly Ala Ala Gly Cys Gly Gly
770 775 780

Thr Cys Thr Thr Cys Cys Gly Gly Ala Thr Thr Cys Thr Gly Gly Ala
785 790 795 800

Thr Gly Ala Gly Ala Thr Thr Gly Cys Cys Cys Cys Gly Gly Cys Thr
805 810 815

Thr Ala Cys Gly Gly Thr Cys Thr Gly Thr Thr Cys Gly Gly Cys Ala
820 825 830

Ala Gly Ala Thr Thr Cys Gly Thr Gly Ala Ala Cys Gly Cys Gly Ala
835 840 845

Ala Gly Gly Gly Gly Cys Cys Gly Gly Gly Ala Gly Thr Thr Thr Thr
850 855 860

Ala Thr Thr Gly Cys Thr Gly Thr Thr Cys Cys Gly Cys Ala Thr Gly
865 870 875 880

Gly Cys Cys Ala Thr Thr Thr Cys Cys Gly Cys Thr Cys Gly Ala Ala
885 890 895

Gly Gly Ala Cys Gly Gly Cys Ala Ala Gly Thr Gly Gly Gly Thr Thr
900 905 910

Gly Cys Gly Ala Thr Thr Gly Cys Cys Thr Gly Thr Ala Cys Cys Ala
915 920 925

Cys Cys Gly Ala Cys Ala Ala Gly Ala Thr Gly Thr Thr Thr Gly Ala
930 935 940

Ala Cys Gly Gly Cys Thr Gly Gly Cys Cys Gly Ala Ala Gly Cys Ala
945 950 955 960

Ala Thr Gly Gly Ala Gly Cys Gly Cys Cys Cys Gly Gly Ala Ala Cys
965 970 975

Thr Gly Gly Cys Thr Thr Cys Gly Cys Cys Gly Gly Ala Ala Cys Thr
980 985 990

Gly Thr Ala Cys Gly Gly Cys Gly Ala Thr Cys Ala Ala Cys Gly Cys
995 1000 1005

Ala Ala Ala Cys Gly Gly Cys Thr Gly Gly Cys Ala Gly Cys Ala
1010 1015 1020

Cys Gly Cys Gly Ala Thr Ala Thr Thr Gly Thr Gly Ala Ala Cys
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Cys Ala Gly Ala Thr Cys Ala Cys Gly Ala Thr Thr Gly Ala Ala
1040 1045 1050

Thr Gly Gly Gly Thr Cys Gly Gly Thr Thr Cys Gly Thr Thr Gly
1055 1060 1065

Ala Cys Gly Cys Gly Cys Gly Ala Cys Gly Ala Gly Gly Thr Gly
1070 1075 1080

Ala Thr Gly Cys Gly Gly Cys Gly Thr Thr Gly Thr Cys Thr Gly
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Gly Ala Gly Ala Ala Gly Gly Ala Ala Gly Thr Thr Cys Cys Cys
1100 1105 1110

Gly Thr Thr Gly Gly Cys Cys Cys Ala Cys Thr Cys Ala Ala Cys
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Ala Gly Cys Ala Thr Cys Gly Cys Cys Gly Ala Thr Ala Thr Gly
1130 1135 1140

Thr Thr Cys Ala Ala Cys Gly Ala Cys Gly Ala Ala Cys Ala Thr
1145 1150 1155

Thr Thr Thr Cys Thr Gly Gly Cys Thr Cys Gly Cys Gly Gly Cys
1160 1165 1170

Ala Ala Cys Thr Thr Thr Gly Cys Cys Thr Gly Thr Ala Thr Cys
1175 1180 1185

Gly Ala Ala Gly Cys Cys Gly Ala Gly Gly Gly Thr Ala Thr Cys
1190 1195 1200

Gly Gly Cys Gly Ala Ala Gly Thr Gly Gly Thr Gly Gly Thr Thr
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Cys Cys Gly Ala Ala Cys Gly Thr Gly Ala Thr Cys Cys Cys Cys
1220 1225 1230

Ala Gly Ala Cys Thr Gly Thr Cys Ala Gly Ala Ala Ala Cys Ala
1235 1240 1245

Cys Cys Gly Gly Gly Ala Cys Gly Gly Gly Thr Gly Ala Cys Cys
1250 1255 1260

Ala Ala Cys Cys Thr Cys Gly Gly Cys Cys Cys Ala Cys Cys Gly
1265 1270 1275

Cys Thr Gly Gly Gly Gly Ala Ala Thr Gly Cys Cys Ala Cys Gly
1280 1285 1290

Thr Ala Thr Gly Ala Gly Gly Thr Gly Thr Thr Gly Cys Gly Cys
1295 1300 1305

Gly Ala Gly Cys Thr Gly Cys Thr Thr Gly Ala Thr Ala Thr Thr
1310 1315 1320

Thr Cys Thr Gly Cys Cys Gly Ala Ala Gly Ala Gly Ala Thr Cys
1325 1330 1335

Ala Ala Gly Cys Gly Thr Cys Thr Gly Cys Gly Cys Ala Gly Cys
1340 1345 1350

Cys Gly Cys Ala Ala Gly Ala Thr Thr Ala Thr Thr Thr Ala Gly
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<211> 405
<212> PRT
<213> Chloroflexus aurantiacus

<400> 193

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Asp Ala Ala Thr Val Ile Ala Ala Pro Phe Cys Ala Thr Leu Leu Gly
20 25 30

Glu Phe Gly Ala Asp Val Leu Lys Val Glu His Pro Ile Gly Gly Asp
35 40 45

Ala Leu Arg Arg Phe Gly Thr Pro Thr Ala Arg Gly Asp Thr Leu Thr
50 55 60

Trp Leu Ser Glu Ser Arg Asn Lys Arg Ser Val Thr Leu Asn Leu Gln
65 70 75 80

His Pro Glu Gly Ala Arg Val Phe Lys Glu Leu Ile Ala His Ser Asp
85 90 95

Val Leu Cys Glu Asn Phe Arg Pro Gly Thr Leu Glu Lys Trp Gly Leu
100 105 110

Gly Trp Asp Val Leu Ser Lys Ile Asn Pro Arg Leu Ile Met Leu Arg
115 120 125

Val Thr Gly Tyr Gly Gln Thr Gly Pro Tyr Arg Asp Arg Pro Gly Phe
130 135 140

Ala Arg Ile Ala His Ala Val Gly Gly Ile Ala Tyr Leu Ala Gly Met
145 150 155 160

Pro Lys Gly Thr Pro Val Thr Pro Gly Ser Thr Thr Leu Ala Asp Tyr
165 170 175

Met Thr Gly Leu Tyr Gly Cys Ile Gly Val Leu Leu Ala Leu Arg His
180 185 190

Arg Glu Gln Thr Gly Arg Gly Gln Tyr Ile Asp Ala Ala Leu Tyr Glu
195 200 205

Ser Val Phe Arg Cys Ser Asp Glu Leu Val Pro Ala Tyr Gly Met Tyr
210 215 220

Arg Lys Val Arg Glu Arg His Gly Ser His Tyr Asn Glu Phe Ala Cys
225 230 235 240

Pro His Gly His Phe Gln Thr Lys Asp Gly Lys Trp Val Ala Ile Ser
245 250 255

Cys Ala Thr Asp Lys Leu Phe Ala Arg Leu Ala Asn Ala Met Gly Arg
260 265 270

Pro Glu Leu Ala Ser Ser Ser Val Tyr Gly Asp Gln Lys Val Arg Leu
275 280 285

Ala His Ala Ser Asp Val Asn Glu Ile Val Arg Asp Trp Cys Ser Ser
290 295 300

Leu Thr Arg Ala Glu Val Leu Glu Arg Cys Tyr Ala Thr Ala Thr Pro
305 310 315 320

Ala Ala Pro Leu Asn Asp Ile Ala Asp Phe Phe Gly Asp Arg His Val
325 330 335

His Ala Arg Arg Asn Leu Val Ala Ile Asp Ala Glu Asp Leu Gly Glu
340 345 350

Thr Leu Ile Met Pro Asn Val Val Pro Lys Leu Ser Glu Thr Pro Gly
355 360 365

Ser Ile Arg Ser Leu Gly Pro Lys Leu Gly Glu His Thr Glu Glu Val
370 375 380

Leu Lys Glu Ile Leu Gly Met Cys Asp Glu Gln Ile Asn Asp Leu Arg
385 390 395 400

Ser Lys Arg Val Ile
405

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<211> 1218
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<400> 194
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gtagaacatc ctatcggcgg tgatgcgctg cgccgcttcg ggacgccgac cgcacgcggc 180

gatacgctga cctggctgag cgagtcgcgc aacaagcgtt cggttacgct caacctgcaa 240

catcccgaag gggcgcgtgt cttcaaagag ctgatcgccc actccgatgt gctgtgcgaa 300

aattttcgcc ccggtacgct ggaaaaatgg gggctgggtt gggatgtgtt gagcaagatc 360

aacccgcgcc tgattatgtt gcgggtcacc ggctatgggc agaccgggcc gtaccgtgat 420

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<213> Artificial Sequence

<220>
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<400> 195
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<210> 197
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<212> DNA
<213> sulfolobus tokodaii

<400> 201
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attgagtacg tacggatgct gagcaatcat ccgtacatca aacccgccta tctggctggg 120

aaagggtcag ttggcaaacc gtatggcgaa gtagtgcgct ggcagactgt gggccaagtt 180

cccaaagaaa ttgccgatat ggaaatcaag ccgactgatc cgaaactgat ggatgatgtt 240

gacatcatct ttagcccact gcctcaaggt gcggcaggac ccgttgagga acaatttgcg 300

aaagaaggat ttccggtcat ttccaattct ccggatcatc ggtttgatcc ggatgtccca 360

ctcctggtgc cagaactgaa tccgcacacc attagcctta ttgacgaaca gcgtaaacgc 420

cgtgaatgga aaggcttcat cgttacgacg ccgttatgca ccgcacaggg tgctgcgatc 480

ccattgggtg ccatcttcaa ggactacaaa atggatggcg cattcattac gaccattcag 540

tctcttagcg gtgcgggata tccgggtatt ccgtccctgg atgtggtgga taacattctg 600

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