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(54) CELL FACTORY HAVING IMPROVED IRON-SULFUR CLUSTER DELIVERY

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USINE DE CELLULES AYANT UNE MEILLEURE REMISE DE CLUSTERS FER-SOUFRE

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(56) References cited:
WO-A1-2009/091582
WO-A1-2017/103221
WO-A2-02/085293
WO-A2-2007/046943
CN-A- 106 086 052

- **YIRONG SUN ET AL: "Overproduction of [alpha]-Lipoic Acid by Gene Manipulated Escherichia coli", PLOS ONE, vol. 12, no. 1, 9 January 2017 (2017-01-09), page e0169369, XP055391544, DOI: 10.1371/journal.pone.0169369**
- **ANGELA S. FLEISCHHACKER ET AL: "Characterization of the [2Fe-2S] Cluster of Escherichia coli Transcription Factor IscR", BIOCHEMISTRY, vol. 51, no. 22, 5 June 2012 (2012-06-05), pages 4453-4462, XP055428859, US ISSN: 0006-2960, DOI: 10.1021/bi3003204 cited in the application**

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The complete document including Reference Tables and the Sequence Listing can be downloaded from the EPO website

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Description

Field of the invention

[0001] The invention relates to a genetically modified bacterial cell capable of improved iron-sulfur cluster delivery, characterized by a modified gene encoding a mutant Iron Sulfur Cluster Regulator (IscR) as well as one or more transgenes encoding polypeptides that enhance the biosynthesis of either biotin, lipoic acid or thiamine. The invention further relates to a method for producing either biotin, lipoic acid or thiamine using the genetically modified bacterium of the invention; as well as the use of the genetically modified bacterial cell for either biotin, lipoic acid or thiamine production.

Background of the Invention

[0002] Biotin (also known as vitamin B7 or vitamin H), and thiamine (also known as vitamin B1), are essential dietary vitamins for humans, because in common with other metazoans, they cannot produce biotin or thiamine. Lipoic acid (LA) is a sulfur-containing, vitamin-like antioxidant and is synthesized in small amounts in bacteria, plants and animals. All three are widely used as dietary supplements. The production of these vitamin or vitamin-like compounds currently relies on chemical synthesis, which is costly. Biosynthetic methods for their manufacture would provide an alternative, more cost effective means for meeting current and future needs.

[0003] Biotin is an essential cofactor for enzymes catalyzing certain carboxylation reactions, such as acetyl-CoA carboxylase (ACC). ACC, which is present in all life forms, produces malonyl-CoA, a key building block for fatty acid biosynthesis. In nature, biotin is synthesized by a linear pathway involving the fatty acid biosynthetic pathway. The initial substrate of biotin synthesis in *Escherichia coli* (*E. coli*) is malonyl-ACP, which is also the starting metabolite for fatty acid synthesis. Prior to entering the fatty acid cycle, malonyl-ACP is masked by the SAM (S-adenosylmethionine)-dependent methyltransferase, BioC, thereby generating a malonyl-ACP methyl ester. Subsequently, two rounds of fatty acid chain elongation yields the molecule pimeloyl-ester-ACP. Hydrolysis of the O-methyl group of the pimeloyl-ester-ACP by a dedicated esterase, BioH, allows this molecule to exit the fatty acid elongation cycle.

[0004] Subsequently, the intermediate, pimeloyl-ester-ACP, is converted to biotin via a biotin-specific pathway (figure 1A). In this pathway, BioF catalyzes the PLP-dependent decarboxylative aldol condensation of pimeloyl-ACP with alanine to yield KAPA (8-Amino-7-oxononanoate). BioA (and BioK) catalyzes the PLP-dependent transamination of KAPA to yield DAPA (7,8-diaminopelargonate), where the donor is SAM; with the by-product S-adenosyl-oxomethionine. BioD catalyzes the ATP-driven carboxylation and ring closure of DAPA to form the thiophane ring in desthiobiotin (DTB). The final step in the biotin synthesis pathway is one of the most complex reactions known, since it involves the introduction of a sulfur bridge between two hydrocarbons by BioB (biotin synthase), to yield biotin. BioB is an S-adenosyl-L-methionine (SAM or AdoMet) radical enzyme, which is found as a dimer; and comprises two iron-sulfur clusters: [2Fe-2S] $^{2+}$ and [4Fe-4S] $^{2+}$ in its active site. The sulfur atom, needed to create the thiophane ring in DTB, is believed to be recruited from the [2Fe-2S] $^{2+}$ cluster in BioB. As a consequence, the iron-sulfur cluster in the BioB dimer consumed in DTB synthesis is thought to be regenerated after each round of catalysis.

[0005] Lipoic acid (LA), in addition to being a potent scavenger of reactive oxygen species, and thus an important antioxidant, is also a co-factor for α-keto acid dehydrogenases. LA is synthesized *de novo* from an intermediate in fatty acid metabolism (figure 2). Three enzymes participating in LA synthesis in *E. coli* are LplA (lipoate-protein ligase), LipB (octanoyl protein ACP carrier protein: protein transferase), and LipA (lipoic acid synthase). LplA, encoded by the *lplA* gene, can catalyze the conjugation of exogenous octanoic acid to the unlipoylated-apo-lipoyl domain of the E2 subunit of a target enzyme in an ATP-dependent manner. LipB, encoded by the *lipB* gene, can catalyze the transfer of an octanoyl residue from ACP to the apo-lipoyl domain of the E2 subunit of a target enzyme. An *AceF* gene, encodes the lipoyl domain of the E2 subunit of pyruvate dehydrogenase. LipA, encoded by the *lipA* gene, is responsible for the formation of two C-S bonds. The LipA-driven reaction requires iron-sulfur clusters (4Fe-4S) and SAM (produced by the metK gene) in order to perform its function. Lipoic acid is mainly found in the cell as a protein-bound lipoamide moiety in a number of multi-enzyme complexes. Thiamine biosynthesis has been characterized in bacteria, some protozoans, plants, and fungi. The thiazole and pyrimidine moieties of thiamine are synthesized separately (figure 3). The pyrimidine moiety, 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate (HMP- P), is derived from 5-aminoimidazole ribotide (AIR), an intermediate in the de novo purine biosynthetic pathway. In Gram-negative bacteria, conversion of AIR to HMP-P is catalyzed in a radical S-adenosyl-L-methionine (SAM)-dependent reaction by the *thiC* gene product, HMP-P synthase, which binds 1 [4Fe-4S] cluster per subunit.

[0006] HMP-P is then phosphorylated to HMP-PP by *ThiD* kinase prior to coupling with the thiazole unit. The thiazole moiety, 5- (2-hydroxyethyl)-4-methylthiazole phosphate (HET-P), is derived from L-tyrosine and 1-deoxy-D-xylulose phosphate (DXP) and cysteine; where the sulfur atom likely derives from L-cysteine. Tyrosine lyase, encoded by the *thiH* gene, binds 1 [4Fe-4S] cluster per subunit, and catalyzes the radical-mediated cleavage of tyrosine to 2-iminoacetate and 4-cresol. Synthesis of the thiazole moiety requires expression of at least five genes *thiF*, *thiS, thiG, thiH* and *thiI.*

[0007] The pyrimidine and thiazole moieties are then combined to form TMP by the action of thiamine-phosphate synthase (EC 2.5.1.3) encoded by *thiE.* Thus TMP is the first product of all known thiamine biosynthetic pathways. In *E. coli* and other *Enterobacteriaceae*, TMP may be phosphorylated to the cofactor TPP by a thiamine-phosphate kinase (EC 2.7.4.16) encoded by *thiL* in the presence of ATP. Bacterial strains comprising a transgene expressing a thiamine mono-phosphate phosphatase (E.C 3.1.3.-) can convert TMP to thiamine and thereby enhance thiamine production.

[0008] The use of bacterial-based cell factories is a potential route for the biosynthetic production of biotin, lipoic acid and thiamine. The advantages of recombinant *E. coli* as a cell factory for production of bio-products are widely recognized due to the fact that: (i) it has unparalleled fast growth kinetics; with a doubling time of about 20 minutes when cultivated in glucose-salts media and under optimal environmental conditions, (ii) it easily achieves a high cell density; where the theoretical density limit of an *E. coli* liquid culture is estimated to be about 200 g dry cell weight/l or roughly 1×10^{13} viable bacteria/mL. Additionally, there are many molecular tools and protocols at hand for genetic modification of *E. coli*; as well as being an organism that is amenable to the expression of heterologous proteins; both of which may be essential for obtaining high-level production of desired bio-products.

[0009] In *E. coli,* the biotin operon structure is split into *bioA* and *bioBFCD* under the control of overlapping promoters on opposite strands (*bioO* locus), while *bioH* is located elsewhere on the *E. coli* chromosome. Expression of the biotin operon is down-regulated by a biotin-bound repressor (BirA); which binds to an operator in the biotin operon. BirA also functions as a biotin ligase, transferring biotin to cellular carboxylases. The switch in BirA function from biotin ligase to transcriptional repressor is regulated by the respective intracellular biotin and apo-carboxylase pools. Over-expression of the biotin operon (*bioA* and *bioBFCD*) in *E. coli* was reported to be inhibitory for growth (Ifuku, O. *et al*., 1995). Since the cause of this inhibition was unknown, this creates a stumbling block to enhancing biotin synthesis.

[0010] In general, there exists a need to identify the bottlenecks in these complex biosynthetic pathways such as to facilitate the production of biotin, lipoic acid and thiamine in bacterial-based cell factories (e.g. *E. coli*), that are tailor-made to overcome the diversity of factors that may limit their ability to both grow and produce elevated levels of their respective pathway enzymes.

[0011] In WO 02/085293 for example, the transcriptional regulator IscR that negatively regulates Fe-S cluster assembly, was deleted to increase lipoic acid production by an E. coli based cell factory.

Summary of the invention

[0012] According to a first embodiment, the invention provides a genetically modified bacterium, wherein the bacterium has an enhanced production of any one of biotin, lipoic acid or thiamine; wherein said bacterium comprises:

- a genetically modified endogenous *iscR* gene encoding a mutant IscR polypeptide, wherein the amino acid sequence of said mutant IscR polypeptide has at least 80% sequence identity to SEQ ID No: 2, 4, 6, 8, 10, 12 and 14, and wherein the amino acid sequence has at least one amino acid substitution selected from the group consisting of:
    - L15X, C92X, C98X, C104X, and H107X; wherein X is any amino acid other than the corresponding amino acid residue in SEQ ID No 2, 4, 6, 8, 10, 12 and 14, and
- at least one transgene encoding a polypeptide selected from among:
    - a polypeptide having biotin synthase activity (EC 2.8.1.6) for enhanced biotin production,
    - a polypeptide having lipoic acid synthase activity (EC 2.8.1.8) for enhanced lipoic acid production,
    - a polypeptide having HMP-P synthase activity (EC 4.1.99.17) for enhanced thiamine production, and
    - a polypeptide having tyrosine lyase activity (EC 4.1.99.19) for enhanced thiamine production.

[0013] Preferably, the at least one amino acid substitution in said mutant IscR polypeptide is selected from the group consisting of:

- L15X, wherein X is any one of F, Y, M and W;
- C92X, wherein X is any one of Y, A, V, I, G, L, M, F and W;
- C98X, wherein X is any one of A, V, I, G, L, F and W;
- C104X, wherein X is any one of A V, I, G, L, F and W; and
- H107X; wherein X, is any one of A, Y, M, F, W, V, I, G, and L.

[0014] The genetically modified bacterium that has an enhanced production of biotin according to the invention that comprises one transgene encoding a polypeptide having biotin synthase activity (EC 2.8.1.6) may further comprise additional transgenes encoding one or more polypeptides selected from the group consisting of:

- a polypeptide having SAM (S-adenosylmethionine)-dependent methyltransferase activity (BioC; EC 2.1.1.197);
- a polypeptide having 7-keto-8-aminopelargonic acid (KAPA) synthase activity (BioF; EC 2.3.1.47);
- a polypeptide having 7,8-Diaminopelargonic Acid (DAPA) Synthase activity (BioA; EC:2.6.1.62) or having L-lysine:8-amino-7-oxononanoate aminotransferase activity (BioK: EC:2.6.1.105);
- a polypeptide having Dethiobiotin (DTB) Synthetase activity (BioD; EC 6.3.3.3), and
- a polypeptide having Pimeloyl-[acyl-carrier protein] methyl ester esterase activity (BioH; EC 3.1.1.85) or a polypeptide having 6-carboxyhexanoate-CoA ligase activity (BioW; EC 6.2.1.14).

[0015] Preferably, the genetically modified bacterium that has an enhanced production of biotin according to the invention that comprises one transgene encoding a polypeptide having biotin synthase activity (EC 2.8.1.6) further comprises additional transgenes encoding polypeptides having SAM (S-adenosylmethionine)-dependent methyltransferase activity (BioC; EC 2.1.1.197); 7-keto-8-aminopelargonic acid (KAPA) synthase activity (BioF; EC 2.3.1.47); and 7,8-Diaminopelargonic Acid (DAPA) Synthase activity (BioA; EC:2.6.1.62).

[0016] The genetically modified bacterium that has an enhanced production of lipoic acid according to the invention that comprises one transgene encoding a .polypeptide having lipoic acid synthase activity (EC 2.8.1.8) may further comprise additional transgenes encoding one or more polypeptides selected from the group consisting of:

- a polypeptide having octanoyltransferase activity (EC 2.3.1.181), and
- a polypeptide comprising the dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase activity (EC 2.3.1.12), and
- a polypeptide having lipoate-protein ligase A (LplA; EC:6.3.1.20).

[0017] The genetically modified bacterium that has an enhanced production of thiamine according to the invention that comprises one transgene encoding a ThiC polypeptide having HMP-P synthase activity (EC 4.1.99.17), and/or one transgene encoding a ThiH polypeptide having tyrosine lyase activity (EC 4.1.99.19), may further comprise additional transgenes encoding one or more polypeptides selected from the group consisting of:

- a ThiF polypeptide having ThiS adenylyltransferase (EC 2.7.7.73) activity;
- a ThiE polypeptide having thiamine phosphate synthase (EC 2.5.1.3) activity;
- a ThiG polypeptide having thiazole synthase (E.C.2.8.1.10) activity;
- a ThiD polypeptide having phosphohydroxymethylpyrimidine kinase (EC 2.7.4.7) activity;
- a ThiS polypeptide having sulfur-carrier protein activity;
- a polypeptide having thiamine mono-phosphate phosphatase (E.C. 3.1.3.-) activity; and
- a ThiO polypeptide having glycine oxidase (EC 1.4.3.19) activity; and optionally, an additional transgene encoding:
- a ThiM polypeptide having hydroxyethylthiazole kinase activity (2.7.1.50).

[0018] Preferably, the genetically modified bacterial cell comprises transgenes encoding the polypeptides: ThiC (encoded by a *thiC* gene); ThiD (encoded by a *thiD* gene), ThiE (encoded by a *thiE* gene), ThiF (encoded by a *thiF* gene), sulfur-carrier protein (encoded by a *thiS* gene), ThiG (encoded by a *thiG* gene), TMP phophatase (encoded by a TMP phosphatase gene); and either ThiH (encoded by a *thiH* gene) or ThiO (encoded by a *thiO* gene). According to the embodiment, the cells may further comprise a transgene encoding the enzyme ThiM ((encoded by a *thiM* gene).

[0019] Preferably each of the at least one transgene and the one or more additional transgenes in the genetically modified bacterium of the invention is operably linked to a constitutive promoter (where the promoter may be operably linked to an operon comprising the transgenes).

[0020] The genetically modified bacterium of the invention is preferably a species of a genus selected from the group consisting of *Escherichia, Bacillus*, *Brevibacterium, Burkholderia, Campylobacter, Corynebacterium, Serratia, Lactobacillus, Lactococcus, Acinetobacter, Acetobacter* and *Pseudomonas*; more preferably a species of *Escherichia* or *Corynebacterium;* for example *Escherichia coli* or *Corynebacterium glutamicum.*

[0021] According to a second embodiment, the invention provides a method for producing biotin, comprising the steps of:

- introducing a genetically modified bacterium comprising a transgene encoding a polypeptide having biotin synthase activity (EC 2.8.1.6) according to the invention into a growth medium to produce a culture;
- cultivating the culture; and
- recovering biotin produced by said culture, and optionally purifying the recovered biotin.

[0022] According to a third embodiment, the invention provides a method for producing lipoic acid comprising the steps of:

◦ introducing a genetically modified bacterium comprising one transgene encoding a polypeptide having lipoic acid synthase activity (EC 2.8.1.8) according to the invention into a growth medium to produce a culture;
◦ cultivating the culture; and
◦ recovering lipoic acid produced by said culture, and optionally purifying the recovered lipoic acid.

[0023] According to a fourth embodiment, the invention provides a method for producing thiamine comprising the steps of:

◦ introducing a genetically modified bacterium a transgene encoding a polypeptide having HMP-P synthase activity (EC 4.1.99.17), and/or a transgene encoding a polypeptide having tyrosine lyase activity (EC 4.1.99.19) according to the invention into a growth medium to produce a culture;
◦ cultivating the culture; and
◦ recovering thiamine produced by said culture, and optionally purifying the recovered thiamine.

[0024] Preferably the growth medium used in the method for producing any one of biotin, lipoic acid and thiamine, comprises a carbon source selected from glucose, maltose, galactose, fructose, sucrose, arabinose, xylose, raffinose, mannose, lactose, or any combination thereof.

[0025] According to a fourth embodiment, the invention provides for the use of a genetically modified gene encoding a mutant iscR polypeptide to enhance biotin production in a bacterial cell expressing a transgene encoding a biotin synthase, wherein the amino acid sequence of the mutant iscR polypeptide has at least 80% sequence identity to SEQ ID No.: 2, 4, 6, 8, 10, 12 and 14; and wherein the amino acid sequence has at least one amino acid substitution selected from the group consisting of:L15X, Cys92X, Cys98X, Cys104X, and His107X; wherein X is any amino acid other than the corresponding amino acid residue in SEQ ID No.: 2, 4, 6, 8, 10, 12 and 14.

[0026] According to a fourth embodiment, the invention provides for the use of a genetically modified gene encoding a mutant iscR polypeptide to enhance production of any one of biotin, lipoic acid or thiamine in a bacterium, wherein said bacterium comprises and expresses at least one transgene encoding a polypeptide selected from among:

- a polypeptide having biotin synthase activity (EC 2.8.1.6),
- a polypeptide having lipoic acid synthase activity (EC 2.8.1.8),
- a polypeptide having HMP-P synthase activity (EC 4.1.99.17), and a polypeptide having tyrosine lyase activity (EC 4.1.99.19),

wherein said genetically modified gene is an endogenous *iscR* gene encoding a mutant IscR polypeptide, wherein the amino acid sequence of said mutant IscR polypeptide has at least 80% sequence identity to SEQ ID No: 2, 4, 6, 8, 10, 12 and 14, and
wherein the amino acid sequence has at least one amino acid substitution selected from the group consisting of:L15X, Cys92X, Cys98X, Cys104X, and His107X; wherein X is any amino acid other than the corresponding amino acid residue in SEQ ID No 2, 4, 6, 8, 10, 12 and 14.

[0027] According to a fifth embodiment, the invention provides for the use of a genetically modified bacterium according to the invention for enhanced production of biotin, lipoic acid or thiamine.

[0028] According to a sixth embodiment, the invention provides a genetically modified bacterium according to the invention for enhanced production of any one of biotin, lipoic acid or thiamine, wherein the bacterium further comprises one or more genes encoding polypeptides capable of mediating enhanced electron transfer from the electron donor NADPH to a $[4Fe\text{-}4S]^{2+}$ cluster of a SAM-radical iron-sulfur cluster enzyme; for example polypeptides of a flavodoxin/ferredoxin reductase and flavodoxin reduction system or a Pyruvate-flavodoxin/ferredoxin oxidoreductase system.

Brief description of the drawings

[0029]

Figure 1 Cartoon showing A) intermediates of the biotin pathway in bacteria and the respective enzymatic steps leading to synthesis of biotin. SAM: S-adenosyl-L -methionine, SAH: S-Adenosyl-L-homocysteine, CoA: coenzyme A, ACP: Acyl Carrier Protein, KAPA: 7-keto-8-aminopelargonic acid, AMTOD: S-adenosyl-2-oxo-4-thiomethylbutyrate, DAPA: 7,8-Diaminopelargonic Acid, DTB: desthiobiotin, 5'DOA: 5'-deoxyadenosine. B) *isc*-operon structure and role in Fe-S-cluster formation as well as the regulatory mechanism of IscR. The *isc* operon comprises an *iscR* gene encoding the IscR that regulates expression of the genes: *iscS* (cysteine desulphurase), *iscU* (scaffold), *iscA* (A-type protein), *HscB* (Dnaj-like co-chaperone), *HscA* (DnaK-like chaperone), and *fdx* (ferredoxin). IscR also reg-

ulates > 40 genes including the genes *hyaA, ydiU, erpA,* and *sufA*.

Figure 2 Cartoon showing intermediates of the lipoic acid pathway in bacteria and the respective enzymatic steps leading to lipoylated lipoyl domains (lipoic acid synthesis). The key enzymes in the pathway include LipA (lipoic acid synthase) and LipB (octanoyl protein ACP carrier protein: protein transferase), as well as the substrate SAM: S-adenosyl-L -methionine. LplA is a lipoate-protein ligase A; EC:6.3.1.20.

Figure 3 Cartoon showing intermediates of the thiamine pathway in bacteria and the respective enzymatic steps leading to synthesis of thiamine (THI); thiamine monophosphate (TMP) and thiamine diphosphate (TPP). Abbreviation of intermediates: 5-aminoimidazole ribonucleotide (AIR), 4-amino-2-methyl-5-(phosphooxymethyl)pyrimidine (HMP-P), 4-amino-2-methyl-5-(diphosphomethyl) pyrimidine (HMP-PP), 1-deoxy-D-xylulose 5-phosphate (DXP), dehydroglycine (DHG), 4-methyl-5-(2-phosphooxyethyl)thiazole (THZ-P), adenosine triphosphate (ATP), adenosine monophosphate (AMP), S-adenosyl-L-methionine (SAM), reduced nicotinamide adenine dinucleotide phosphate (NADPH), nicotinamide adenine dinucleotide phosphate (NADP+), reduced ferredoxin (Fdx red), oxidized ferredoxin (Fdx ox).

Figure 4 Graphical presentation of the cell density (measured at OD_{600}), measured over time, of a (E. *coli* BW25113) Δ*bioB* strain comprising an IPTG inducible *bioB* expression plasmid (right panel); and the reference (E. *coli* BW25113) strain comprising an IPTG inducible frameshifted *bioB* (premature stop codon) expression plasmid (left panel). OD_{620} was measured using a Multiskan and converted to OD_{600}, for 4 biological strain replicates grown in 200 μL mMOPS with 0.1 g/L DTB, 50 μg/mL kanamycin and 0 (dots) 0.01 (triangles) or 0.1 (squares) mM IPTG. Respective exponential growth rate values are shown in the adjacent boxes.

Figure 5 Graphical presentation of a scatter plot showing the final cell density (measured at OD_{600}) of cultures of *E. coli* BS1011 comprising plasmid pBS451 grown on 150 μL mMOPS medium with 40 μg/ml zeocin supplemented with zero or increasing concentrations of biotin, ranging up to 0.244 μg biotin/mL after incubation for 20 hours at 37°C with 275 rpm shake (as described under method for quantifying biotin). The stippled vertical grey lines identify an optimal concentration range of between 0.024 to 0.24 μg biotin/L for the biotin bioassay.

Figure 6 Bar diagram showing biotin production of 3 different *iscR* mutant strains expressing mutant IscR having amino acid mutations (BS1377, L15F), (BS1375, C92Y) and (BS1353, H107Y) and an *E. coli* BW25113 Δ*bioB* strain (BS1011, Ref) (see Table 1 for strains) in 4 biological replicates each comprising an IPTG-inducible *bioB* expression plasmid (pBS412). Strains were grown in 400 μL mMOPS with 0.1 g/l DTB and 50 μg/mL kanamycin for 24 H at 37°C with 275 rpm shake. Bars illustrate the mean biotin production value (height) and IPTG induction level (gray shade), black dots show biotin production from individual replicate cultures and the horizontal stippled line indicates the maximum biotin production from a reference wild type strain. Note that none of the strains produced detectable levels of biotin when cultured in the absence of IPTG.

Figure 7 Graphical presentation of the cell density and biotin production of the iscR mutant strain expressing the mutant IscR (BS1353, H107Y), and an *E. coli* BW25113 ΔbioB strain (BS1011, Ref), wherein each stain comprises an IPTG-inducible bioB gene expression plasmid (pBS412). The data represents the average measured OD_{600} of three biological replicates each of the *iscR* H107Y mutant strain (solid dark line) and the reference strain, *E. coli* BW25113 Δ*bioB* strain (stippled light grey) and biotin production by the respective *iscR* H107Y mutant strain (solid dark dots) and the reference strain, E. coli BW25113 ΔbioB strain (light gray dots) monitored over a period of 25 H. The strains were grown in 50 mL mMOPS with 0.1 g/l DTB, 0.01 (A) or 0.5 mM IPTG (B) and 50 μg/mL kanamycin in a 250 mL baffled shake flask at 37°C with 275 rpm shaking. Growth rates are shown in the black box.

Figure 8 Cartoon showing the IscR coding sequence annotated to show the location of the nucleotide and amino acid sequence mutations in the *iscR* genes of the identified mutant strains.

Figure 9 Cartoon showing the crystal structure (PDB entry 4HF1) of IscR dimer (grey) bound to *hya* DNA binding site (black) with L15F and H107Y *iscR* mutants indicated as sticks (WT amino acid in grey and mutant amino acid in black); and an expanded image highlighting the mutated residues.

Figure 10 Bar diagram showing biotin production of an *E. coli* strain comprising an IPTG-inducible *bioB* expression plasmid and either a plasmid comprising an *isc*-operon (*iscSUA-hscBA-fdx*, corresponding to a native *E. coli isc* operon structure lacking *iscR* gene) or the *E. coli suf*-operon (sufABCDSE) operably linked to a strong ribosomal binding site (RBS) and a T5 LacO repressed promoter from a medium copy number plasmid (p15A ori) or a control

plasmid. The control plasmid comprised an IPTG-inducible gene encoding GFP instead of the *suf*- or *isc*-operon. Biological triplicates of each strain were cultured in mMOPS with 100 μg/mL ampicillin and 50 μg/mL spectinomycin under low (0.01 mM IPTG) and high (0.1 mM IPTG) induction and providing 0.1 g/L (DTB) as substrate. The strains were grown in deep well plates for 24 hours at 37 degrees C with 275 rpm, after which biotin production was evaluated using a growth based bioassay. Bars illustrate the mean biotin production value (height) and IPTG induction level (gray shade), black dots shows biotin production from individual replicates and the crosses show end-point (end) cell density of each strain, measured as OD_{600}. Note that none of the strains produced detectable levels of biotin when induced with 0.01 mM IPTG.

Figure 11 Graphical presentation of the correlation between BioB protein expression levels and biotin production in 4 different samples performed in triplicate. The strains are BS1013 (E. *coli* BW25113, background strain) with pBS430 (*bioB* frameshift IPTG inducible plasmid), BS1011 (BS1013 with Δ*bioB*) with pBS412 (*bioB* IPTG inducible plasmid), BS1353 (BS1011 with *iscR H107Y* mutation) with pBS412. Strains were grown in mMOPS with 0.1 g/l DTB and IPTG as indicated in the graph.

Figure 12 Bar diagram showing biotin production of *E. coli* Δ*bioB* strains comprising an IPTG-inducible *bioB* expression plasmid and either of the following genomic variants of *iscR*: wild type (*iscR* WT), knock-out mutant encoding E22* glutamic acid mutated to a stop codon on position 22 (*iscR* KO), mutant (*iscR* C92Y) encoding a cysteine to tyrosine substitution at position 92. Bars illustrate the mean biotin production value (height) at given levels of IPTG induction (shade of gray), dots show biotin production from individual replicates. Biological triplicates of each strain were cultured in mMOPS with 100 μg/mL ampicillin under no (0 mM IPTG), low (0.01 mM IPTG) and high (0.1 mM IPTG) induction and providing 0.1 g/L DTB as substrate. Each strain was grown in a deep well plate for 24 hours at 37 degrees C with 275 rpm, after which biotin production was evaluated using a growth-based bioassay.

Figure 13 Bar diagram showing biotin production by an *E. coli* Δ*bioA* Δ*bioBFCD* strain comprising a biotin-operon plasmid and either of the following genomic variants of *iscR*: wild type (*iscR* WT), mutant *iscR* H107Y (encoding a histidine to tyrosine substitution at position 107) or mutant (*iscR* C92Y) encoding a cysteine to tyrosine substitution at position 92. Biological quadruplicates of each strain were cultured in mMOPS with 10 μg/mL tetracycline with and without providing 0.1 g/L desthiobiotin (DTB) as substrate. The strains were grown in deep well plates for 24 hours at 37 degrees C with 275 rpm, after which biotin production was evaluated using a growth based bioassay. Bars illustrate the mean biotin production value (height) and whether DTB was fed or not (shade of gray), black dots shows biotin production from individual replicates.

Figure 14 Bar diagram showing lipoic acid production (grey bars) and End (final) OD_{600} after 24 hours of production (crosses) of two different *iscR* mutant strains expressing mutant IscR having amino acid mutations (BS1375, C92Y) and (BS1353, H107Y) and a control strain (BS1011, *IscR* WT) (see Table 1 for strains) in 3 biological replicates each comprising a plasmid expressing IPTG inducible *lipA* (pBS993, see Table 4). Strains were grown in 400 μL mMOPS with 100 μg/mL ampicillin, 0.1 mM biotin, 0.6 g/l octanoic acid and 0.01 mM IPTG for 24 hours at 37°C with 275 rpm shake. Bars illustrate the mean lipoic acid production value (height), black dots show lipoic acid production from individual replicate cultures. The average lipoic acid production can be seen to increase 1.79-fold even though the end OD_{600} stays the same.

Figure 15 Graphical presentation of the cell density (measured at OD_{620}), measured over time, of the reference strain (*E. coli* BW25113) Δ*lipA* (WT, triangles); and the Δ*lipA* strain with a mutant *iscR* (C92Y) (C92Y, squares) both comprising an IPTG inducible *lipA* expression plasmid (pBS1037). OD_{620} was measured using a Multiskan, for 6 biological strain replicates grown in 200 μL mMOPS with 0.6 g/L octanoic acid, 100 μg/mL ampicillin and 0 to 0.04 mM IPTG (increasing darkness of grey shade). Respective growth rates (GR) are shown to the right.

Figure 16 Bar diagram showing thiamine production of two different *iscR* mutant strains expressing mutant IscR having amino acid mutations (BS2019, C92Y) and (BS2020, H107Y) and an *E. coli* BW25113 Δ*thiP*, *thiL** strain (BS750, Ref) (see Table 5 for strains) in 4 biological replicates each comprising a plasmid expressing the entire thiamine pathway genes, *thiCEFSGHMD* (pBS140). Strains were grown in 400 μL mMOPS with 50 μg/mL kanamycin for 24 hours at 37°C with 275 rpm shake. Bars illustrate the mean thiamine production value (height) in the supernatant as measured by thiochrome assay (including thiamine, TMP and TPP) corrected for end OD_{600}, black dots show thiamine production from individual replicate cultures. The OD normalized titer, can be seen to be improved 1.43-fold in mutant strains (BS2019 and BS2020) compared to the reference strain (BS750).

Figure 17 Bar diagram showing biotin production by *E. coli* Δ*bioABFCD iscR* H107Y (encoding a histidine to tyrosine

substitution at position 107) strains comprising IPTG-inducible BioB overexpression plasmid pBS679 alone (BS1937) or in addition pBS1112 (BS2185) with constitutive overexpression of FldA-Fpr or pBS1054 (BS2707) with constitutive overexpression of GFP. Each strain was cultured in mMOPS with 100μg/ml ampicillin, 0.1 g/L desthiobiotin (DTB) as substrate and either 0, 0.01, 0.025, 0.05, 0.075 or 0.1mM IPTG. The medium for BS2185 and BS2707 was identical except for the inclusion of 50μg/ml kanamycin. The strains were grown in deep well plates for 24 hours at 37 degrees C with 275 rpm, after which biotin production was evaluated using a growth based bioassay. Bars illustrate biotin production value (height) by the respective strains: BS1937 (black bars); BS2185 (grey bars); and BS2707 (checkered grey bars).

Figure 18 Bar diagram showing biotin production by *E. coli ΔbioABFCD iscR* H107Y (encoding a histidine to tyrosine substitution at position 107) strains comprising IPTG-inducible BioB overexpression plasmid pBS679. BS2185 comprises in addition pBS1112 with constitutive overexpression of FldA-Fpr. BS1937 was cultured in mMOPS with 100μg/ml ampicillin, 0.1 g/L desthiobiotin (DTB) as substrate and 0.025mM IPTG induction. The medium for BS2185 was identical, but included in addition 50μg/ml kanamycin. The strains were grown in deep well plates for 24 hours at 37 degrees C with 275 rpm, after which biotin production was evaluated using a growth based bioassay. Dark grey bars illustrate the mean biotin production value (height) (BS1937 n=6 and BS2185 n=8) and light grey bars illustrate end OD_{600}. Black dots show biotin production and end OD_{600} from individual replicates.

Definitions:

[0030] **Amino acid sequence identity:** The term "sequence identity" as used herein, indicates a quantitative measure of the degree of homology between two amino acid sequences of substantially equal length. The two sequences to be compared must be aligned to give a best possible fit, by means of the insertion of gaps or alternatively, truncation at the ends of the protein sequences. The sequence identity can be calculated as ((Nref-Ndif)100)/(Nref), wherein Ndif is the total number of non-identical residues in the two sequences when aligned and wherein Nref is the number of residues in one of the sequences. Sequence identity calculations are preferably automated using the BLAST program e.g. the BLASTP program (Pearson W.R and D.J. Lipman (1988)) (www.ncbi.nlm.nih.gov/cgi-bin/BLAST). Multiple sequence alignment is performed with the sequence alignment method ClustalW with default parameters as described by Thompson J., et al 1994, available at http://www2.ebi.ac.uk/clustalw/.

[0031] Preferably, the numbers of substitutions, insertions, additions or deletions of one or more amino acid residues in the polypeptide as compared to its comparator polypeptide is limited, i.e. no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 substitutions, no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 insertions, no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 additions, and no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 deletions. Preferably the substitutions are conservative amino acid substitutions: limited to exchanges within members of group 1: Glycine, Alanine, Valine, Leucine, Isoleucine; group 2: Serine, Cysteine, Selenocysteine, Threonine, Methionine; group 3: proline; group 4: Phenylalanine, Tyrosine, Tryptophan; Group 5: Aspartate, Glutamate, Asparagine, Glutamine.

[0032] **Amino acid abbreviations:** Leucine (L), Cysteine (C), and Histidine (H).

[0033] **Endogenous gene:** is a gene in a bacterial cell genome that is homologous in origin to a host bacterium (i.e. a native gene of the host bacterium). The endogenous gene may be genetically modified using tools known in the art whereby the genetically modified endogenous gene encodes a mutant polypeptide whose amino acid sequence differs at one or more position from the polypeptide encoded by the parent endogenous gene from which it was derived.

[0034] **Genome:** is the genetic material present in a cell or organism; said genome comprising all of the information needed to build and maintain that cell or organism; and includes the genetic material in both chromosome(s) and plasmid(s) present within the cell or organism.

[0035] **GFP:** Green Fluorescent Protein.

[0036] **gi number:** (genInfo identifier) is a unique integer which identifies a particular sequence, independent of the database source, which is assigned by NCBI to all sequences processed into Entrez, including nucleotide sequences from DDBJ/EMBL/GenBank, protein sequences from SWISS-PROT, PIR and many others.

[0037] **Isc pathway:** iron sulphur cluster pathway; encoded by the *isc* operon including the *iscR* gene.

[0038] **Multiskan:** filter-based microplate photometer; for measuring absorbance from 96 or 384-well plate formats in the wavelength range of 340 to 850 nm, including 600 - 620nm. Plates are incubated in the photometer at the selected temperature, of up to 50°C. The photometer is supplied by Thermo Scientific.

[0039] **Native gene:** endogenous gene in a bacterial cell genome, homologous to host bacterium.

[0040] **Non-native promoter:** in the context of a genetically modified bacterium of the present invention, is a promoter that is operably-linked to a gene or transgene in said cell, where said promoter would not be found operably-linked to said gene or transgene in a bacterial cell found in nature.

OD: Optical Density

[0041] **Transgene:** is an exogenous gene that has been introduced into the genome of a bacterium by means of genetic engineering. In the context of the present invention, said genome includes both chromosomal and episomal genetic elements.

Detailed description of the invention

[0042] A common feature of the biosynthetic pathways for the synthesis of biotin, lipoic acid and thiamine, is the requirement for one or more SAM or AdoMet radical enzymes to catalyze complex radical-mediated molecular rearrangements. Biotin synthase, lipoic acid synthase, HMP-P synthase, and tyrosine lyase are the respective enzymes known to catalyze these essential steps in these pathways. The failure of earlier attempts to elevate biotin biosynthesis in *E. coli* by overexpression of the biotin operon, or even by using a mutant biotin operon insusceptible to feedback regulation by the BirA repressor, was due to a strong inhibition of growth (Ifuku, O. *et al.,* 1995). In the absence of any evidence-based explanation for the observed growth inhibition; alternative approaches were needed to identify the cellular factors that may account for the toxicity of biotin synthase over-expression.

[0043] The solution to this problem, provided by the present invention, is shown to be equally applicable for enhancing the expression of biotin synthase, lipoic acid synthase, HMP-P synthase and tyrosine lyase in cells of a bacterial cell factory (for example *E. coli*). The approach pursued to solve this problem was to generate libraries of *E. coli* cells having evolved genomic diversity due to the accumulation of background mutations generated by imperfect error-correcting polymerases. Cells of such libraries were transformed with a plasmid comprising an IPTG-inducible *bioB* gene expression cassette. Candidate mutants were those cells in a library that were capable of growth in the presence of IPTG at a concentration sufficient to induce BioB expression toxicity in the parent *E.coli* strain from which the mutant cells were derived.

[0044] The genetic basis for the growth of the selected BioB-expressing mutant strains was established by whole genome sequencing. Surprisingly three of the strains were found to have mutations in the native Iron Sulfur Cluster Regulator gene (*iscR*); which encodes a pleiotropic transcription factor (IscR) [SEQ ID No.: 2]. Fe-S clusters are co-factors of many proteins and essential enzymes, endowing them with diverse biochemical abilities that are not solely required for the synthesis of S-containing compounds such as biotin, but also as sensors for redox- or iron-related stress conditions.

[0045] IscR exists in two states, either as an Fe-S cluster holo-protein, or as the apo-protein without the Fe-S cluster. Assembly of the Fe-S cluster of IscR is catalyzed by the Isc pathway encoded by the *isc* operon. The *isc* operon encodes firstly the regulator (IscR), followed by a cysteine desulphurase (IscS), a scaffold (IscU), an A-type protein (IscA), a Dnaj-like co-chaperone (HscB), a DnaK-like chaperone (HscA) and a ferredoxin (Fdx). In addition to being essential for the assembly of the IscR holoenzyme, the Isc pathway is the primary pathway for Fe-S cluster biogenesis in *E. coli* (Figure 1B).

[0046] The ratio between the two forms of IscR is determined by the cellular level of [2Fe-2S] clusters, which in turn is influenced by several factors including iron- and oxygen levels (Py, B. & Barras, 2010). Under iron-rich conditions, IscR exists mainly as the holoenzyme, which then acts as a transcriptional repressor of the *isc* operon. However, under iron-low conditions (low level of [2Fe-2S] clusters), IscR returns to its apo-protein state, allowing transcription of the *isc* operon. In its apo-protein state, IscR serves as an activator of the *sufABCDSE* operon, which catalyzes Fe-S cluster biogenesis under oxidative stress.

[0047] In addition to regulating expression of the two Fe-S-cluster assembly systems in *E. coli,* IscR regulates >40 genes involved in diverse mechanisms of action such as oxidative stress mechanisms (e.g. *sodA*), specific and global regulators (e.g. *yqjI* and *soxS*), amino acid biosynthesis (e.g *argE*) and a range of genes with unknown functions. The role of IscR is further complicated by the fact that the IscR regulatory landscape changes between aerobic and anaerobic conditions (Martin, and Imlay, 2012; Giel et al., 2006).

[0048] In view of the homeostatic role of IscR; and its role in global gene regulation; the consequences of any modification of its regulatory properties are unpredictable and probably profound for cellular metabolism. Furthermore, cellular conditions where Fe-S cluster biogenesis is increased, due to elevated expression of both the sulfur formation (suf) and isc pathways creates the risk that the accumulated Fe-S clusters generate peroxide radicals by fenton reactions.

[0049] In this light, it was highly unexpected that IscR should be found so important for the activity and toxicity of cellular BioB, as demonstrated by the three isolated individual mutants. Furthermore, the impact of the mutant IscR protein on biotin biosynthesis was unexpected, since over-expression of the *isc* operon or *suf* operons giving an increased capacity to synthesize and assemble Fe-S clusters was not found to enhance biotin production in cells over-expressing *bioB* (see example 1, Figure 10). Further, removal of cellular iscR regulation by knock-out of the *iscR* gene also failed to enhance biotin production in the cell (see example 1, figure 12).

[0050] The three different mutations in the IscR protein that eliminated the toxicity of BioB expression in the mutant cells, were single amino acid substitutions of the amino acids L15 [SEQ ID No.: 16], C92 [SEQ ID No.: 18] and H107

[SEQ ID No.: 20] (figure 8). Two of the three mutations correspond precisely to those residues in IscR, each of which is known to be essential for the formation of the IscR holo-protein. IscR, as seen in *E. coli,* has an unusual Fe-S cluster ligation mechanism, whereby the residues essential for Fe-S cluster ligation are C92, C98, and C104, as well as H107. This atypical ligation may confer a lower stability of holoenzyme state of IscR relative to other Fe-S proteins that in turn accounts for the switch to the apo-protein state during low Fe-S conditions (Fleischhacker et al., 2012).

[0051] While not wishing to be bound by theory, this suggests that homeostatic control of Fe-S cluster biogenesis and global gene regulation required for cell growth are uniquely preserved in cells expressing a mutant *iscR* gene of the invention, while facilitating the assembly of iron-sulfur cluster containing enzymes (biotin synthase, lipoic acid synthase, HMP-P synthase and tyrosine lyase) even during their over-expression.

[0052] In summary, the inventors have identified a mutant *iscR* gene encoding a mutant IscR protein, characterized by the lack of one or more of amino acid residues required for ligation of Fe-S clusters, such that the expressed mutant IscR protein exists solely in the apo-protein form. Synthesis of iron-sulfur cluster containing enzymes is shown to constitute a significant bottleneck in efforts to enhance production of either biotin, lipoic acid or thiamine in bacteria. The solution to this problem, as provided by the present invention, is facilitated by over-expression of these enzymes in a cell factory comprising a gene encoding a mutant IscR protein that exists in the apo-protein state. The various embodiments of the invention are described in more detail below.

I A genetically modified bacterial cell for production of biotin

[0053] The present invention provides a genetically modified bacterial cell capable of producing enhanced levels of biotin. The bacterial cell is genetically modified to express a mutant IscR in substitution for a wild type IscR, as well as comprising a transgene encoding a biotin synthase (biotin synthase having EC 2.8.1.6). Optionally, the genetically modified bacterial cell may further comprise one or more additional transgenes encoding polypeptides that catalyze additional steps in the biotin pathway (figure 1A). An increase in the levels of those polypeptides that catalyze steps in the biotin pathway enhances the synthesis of both intermediates in the biotin pathway, and the end product of the pathway (biotin) in the bacterial cell.

[0054] The mutant IscR polypeptide, expressed by the genetically modified bacterial cell, is derived from a wild-type member of a family of IscR polypeptides characterized by a polypeptide backbone (apo-protein). The amino acid sequence of a wild-type member of a family of IscR polypeptides has at least 70, 75, 80, 85, 90, 95, 96, 98, 100% amino acid sequence identity to a sequence selected from any one of: SEQ ID No.: 2, 4, 6, 8, 10, 12 and 14. The amino acid sequence of the mutant IscR polypeptide according to the invention differs from the amino acid sequence of the corresponding wild-type IscR polypeptide from which it was derived by at least one amino acid substitution; wherein said substitution is selected from L15X, C92X, C98X, C104X, and H107X; wherein X, the substituting amino acid, is any amino acid other than the amino acid found at the corresponding position in the wild type IscR from which the mutant was derived.

[0055] In alternative embodiments, the amino acid substitution in the mutant IscR is selected from either L15X, wherein X is any amino acid other than L, more preferably X is selected from phenylalanine (F), tyrosine (Y), methionine (M) and tryptophan (W); C92X, wherein X is any amino acid other than C, more preferably X is selected from tyrosine (Y), alanine (A), methionine (M), phenylalanine (F) and tryptophan (W); C98X, wherein X is any amino acid other than C, more preferably X is selected from alanine (A), valine (V), isoleucine (I), leucine (L), phenylalanine (F) and tryptophan (W); Cys104X, wherein X is any amino acid other than C, more preferably X is selected from alanine (A), valine (V), isoleucine (I), leucine (L), phenylalanine (F), and tryptophan (W); and His107X, wherein X is any amino acid other than H, more preferably X is selected from alanine (A), tyrosine (Y), valine (V), isoleucine (I), and leucine (L). For example, the amino acid substitution in the mutant IscR may be selected from among L15F, C92Y, C92A, C98A, Cys104A, H107Y, and H107A.

[0056] The mutant IscR expressed by the genetically modified bacterial cell of the invention (instead of a wild type IscR), is encoded by a genetically modified gene, located in the genome of the bacterial cell, either on the chromosome or on a self-replicating plasmid. The genetically modified *iscR* gene in the chromosome can be located in the genome at the same position of the wild-type *iscR* gene in the native genome. The genome of the genetically modified bacterial cell of the invention lacks a native wild type *iscR* gene, since the native wild type *iscR* gene is either deleted or directly substituted by the genetically modified *iscR* gene. The promoter driving expression of the genetically modified *iscR* gene may be the native promoter of the wild type *iscR* gene from which the genetically modified *iscR* gene was derived or replaced by. Alternatively, the promoter may be a heterologous constitutive or inducible promoter. When the promoter is a heterologous constitutive promoter, then a suitable promoter includes: *apFab* family [SEQ ID Nos.:230-232] while a suitable inducible promoter includes: *pBad* (arabinose inducible [SEQ ID No.:233] and *LacI* [SEQ ID No.:234]. Suitable terminators include members of the apFAB terminator family including [SEQ ID No.: 235-237].

[0057] A polypeptide having biotin synthase activity (EC 2.8.1.6) according to the invention is a polypeptide having biotin synthase activity catalyzing the conversion of desthiobiotin (DTB) into biotin. The members of this family of biotin synthases are encoded by genes found in bacteria belonging to a wide range of genera. The amino acid sequence of

the polypeptide having biotin synthase activity has at least 70, 75, 80, 85, 90, 95, 96, 98, 100% amino acid sequence identity to a sequence selected from any one of: SEQ ID No.: 22 (origin: *Escherichia coli*); SEQ ID No.: 27 (origin: *Candidatus Chloracidobacterium thermophilum* B); SEQ ID No.: 29 (origin: *Streptomyces lydicus*); SEQ ID No.: 31 (origin: *Paracoccus denitrificans*); SEQ ID No.: 33 (origin: *Paracoccus denitrificans PD1222*); SEQ ID No.: 35 (origin: *Agrobacterium vitis*); SEQ ID No.: 37 (origin: *Ruegeria pomeroyi*); SEQ ID No.: 39 (origin: *Agrobacterium fabrum*); SEQ ID No.: 41 (origin: *Wolbachia endosymbiont of Cimex lectularius*); SEQ ID No.: 43 (origin: *Sphingomonas paucimobilis*); SEQ ID No.: 45 (origin: *Acidithiobacillus ferrivorans*); SEQ ID No.: 47 (origin: *Gallionella capsiferriformans*); SEQ ID No.: 49 (origin: *Ralstonia eutropha*); SEQ ID No.: 51 (origin: *Bordetella parapertussis*); SEQ ID No.: 53 (origin: *Pusillimonas sp.*); SEQ ID No.: 55 (origin: *Cenarchaeum symbiosum sp.*); SEQ ID No.: 57 (origin: *Alicyclobacillus acidocaldarius sp.*); SEQ ID No.: 59 (origin: *Geobacillus thermoglucosidasius*); SEQ ID No.: 61 (origin: *Bacillus subtilis*); SEQ ID No.: 63 (origin: *Lysinibacillus sphaericus*); SEQ ID No.: 65 (origin: *Methylococcus capsulatus*); SEQ ID No.: 67 (origin: *Leclercia adecarboxylata*); SEQ ID No.: 69 (origin: *Chromohalobacter salexigens*); SEQ ID No.: 71, 73, 75, 77, 79, 81, 83, 85, 87 (origin: *Pseudomonas spp*).

[0058] The polypeptides that are encoded by the additional transgenes in the genetically modified bacterial cell, and whose activity serves to enhance the synthesis of both intermediates and products of the biotin pathway, are as follows:

a) a polypeptide having SAM (S-adenosylmethionine)-dependent methyltransferase activity (**BioC;** EC 2.1.1.197); such as a polypeptide with an amino acid sequence having 80, 85, 90, 95 or 100% sequence identity to SEQ ID No.:89;

b) a polypeptide having 7-keto-8-aminopelargonic acid (KAPA) synthase activity (**BioF;** EC 2.3.1.47), such as a polypeptide with an amino acid sequence having 80, 85, 90, 95 or 100% sequence identity to SEQ ID No.: 91;

c) a polypeptide having 7,8-Diaminopelargonic Acid (DAPA) Synthase activity (**BioA;** EC:2.6.1.62) such as a polypeptide with an amino acid sequence having 80, 85, 90, 95 or 100% sequence identity to SEQ ID No.: 93; or L-lysine:8-amino-7-oxononanoate aminotransferase (**BioK;** EC:2.6.1.105) such as a polypeptide with an amino acid sequence having 80, 85, 90, 95 or 100% sequence identity to SEQ ID No.: 97;

d) a polypeptide having Desthiobiotin (DTB) Synthetase activity (**BioD;** E.C 6.3.3.3), such as a polypeptide with an amino acid sequence having 80, 85, 90, 95 or 100% sequence identity to SEQ ID No.:95; and optionally

f) a polypeptide having Pimeloyl-[acyl-carrier protein] methyl ester esterase (**BioH;** EC:3.1.1.85) such as a polypeptide with an amino acid sequence having 80, 85, 90, 95 or 100% sequence identity to SEQ ID No.:99; or

g) a polypeptide having 6-carboxyhexanoate-CoA ligase activity (**BioW;** EC 6.2.1.14); such as a polypeptide with an amino acid sequence having 80, 85, 90, 95 or 100% sequence identity to SEQ ID No.:101;

[0059] The transgene encoding BioB together with one or more additional transgenes encoding polypeptides that catalyze additional steps in the biotin pathway, are located in the genome of the genetically modified bacterial cell, either integrated into the bacterial cell chromosome or on a self-replicating plasmid. The transgenes encoding BioB and one or more enzymes in the biotin pathway enzymes (BioABFCD and H or W) may be present in the genome within one or more operon.

[0060] The promoter driving expression of the transgene encoding BioB together with one or more additional transgenes is preferably a non-native promoter, which may be a heterologous constitutive-promoter or an inducible-promoter. When the promoter is a heterologous constitutive promoter, then a suitable promoter includes apFab family [SEQ ID Nos.:230-232] while a suitable inducible promoter includes: pBad (arabinose inducible [SEQ ID No.:233] and LacI [SEQ ID No.:234]. Suitable terminators include members of the apFAB terminator family including [SEQ ID No.: 235-237]. The selected promoter and terminator may be operably linked to the coding sequence for BioB; and to the coding sequences of the one or more coding sequences for the BioC, BioD, BioA, BioF, and either BioW or BioH polypeptides or may be operably linked to the one or more operon encoding the selected Bio polypeptides.

II A method for producing and detecting biotin using a genetically modified bacterium according to the invention

[0061] Biotin can be produced and exported using genetically modified bacterial cells of the invention (e.g. genetically modified *E. coli* cells) by introducing the cells into a culture medium suitable for supporting growth as well as comprising a carbon source suitable for the biosynthesis of biotin; and finally recovering the biotin produced by the culture, as illustrated in the Examples.

[0062] The genetically modified bacterial cells of the invention comprising a transgene encoding a biotin synthase (BioB) will produce enhanced levels of biotin when the supplied carbon source includes desthiobiotin (DTB). When the

genetically modified bacterial cells of the invention additionally comprise transgenes encoding each of BioA, BioF, BioC, BioD, and BioH or BioW they will produce biotin when the supplied carbon source is selected from among glucose, maltose, galactose, fructose, sucrose, arabinose, xylose, raffinose, mannose, and lactose (example 1, figure 13).

[0063] A method for quantifying extracellular biotin produced by a genetically modified bacterial cell of the invention is described in example 1.5. The method is a bioassay, based on measuring the growth of a biotin-starved overnight culture of BS1011 comprising plasmid pBS451 in a biotin-deficient growth medium that is supplemented with the extracellular growth medium derived from culturing cells of the invention. A biotin bioassay calibration curve is prepared by measuring the growth of the biotin-starved overnight culture, when supplemented with a known concentration range of biotin standards, as shown in figure 5.

III A genetically modified bacterial cell for production of lipoic acid

[0064] The present invention provides a genetically modified bacterial cell capable of producing enhanced levels of lipoic acid. The bacterial cell is genetically modified to express a mutant IscR, according to the invention (see Section I), in substitution for a wild type IscR, as well as comprising a transgene encoding a lipoic acid synthase (EC 2.8.1.8). LipA catalyzes the conversion of covalently attached octanoyl-domains to lipoyl domains by facilitating the formation of two sulfur bonds. Optionally, the genetically modified bacterial cell may further comprise one or more additional transgenes encoding polypeptides that catalyze additional steps in the lipoic acid synthesis pathway (figure 2), more specifically the encoded polypeptide LipB; EC:2.3.1.181 of for example the *lipB* gene, and the encoded polypeptide E2; EC:2.3.1.12 of for example the *aceF* gene. An increase in the levels of those polypeptides that catalyze steps in the lipoic acid pathway enhances the synthesis of both intermediates in the pathway, and the end product of the pathway in the bacterial cell. An additional transgene encoding LplA, a lipoate-protein ligase A; EC:6.3.1.20, serves to facilitate synthesis of lipoic acid in cells fed with octanoic acid; by catalysing the transfer of an octanoyl moiety onto an activated lipoyl domain.

[0065] The lipoic acid synthases are encoded by genes found in a wide range of bacteria and fungi belonging to a wide range of genera. The amino acid sequence of the polypeptide having lipoic synthase activity has at least 70, 75, 80, 85, 90, 95, 96, 98, 100% amino acid sequence identity to a sequence selected from any one of: SEQ ID No.: 103 (origin: *Escherichia coli*); SEQ ID No.: 105 (origin: *Bacillus subtilis*); SEQ ID No.: 107 (origin: *Saccharomyces cerevisiae*); SEQ ID No.: 109 (origin: *Pseudomonas putida*); SEQ ID No.: 111 (origin: *Bacteroides fragilis*); and SEQ ID No.: 113 (origin: *Streptomyces coelicolor*).

[0066] The polypeptides that are encoded by the additional transgenes in the genetically modified bacterial cell, and whose activity serves to enhance the synthesis of both intermediates and products of the lipoic acid pathway, are as follows:

a) a polypeptide having octanoyltransferase activity (for transfer of an octanoyl residue from ACP to the apo-lipoyl domain of the E2 subunit of a target enzyme activity; LipB; EC: 2.3.1.181, such as a polypeptide with an amino acid sequence having 80, 85, 90, 95 or 100% sequence identity to SEQ ID No.:115 (origin: *Escherichia coli*) or SEQ ID No.:117 (origin: *Shigella flexneri*);

b) a polypeptide comprising the dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase (E2; EC:2.3.1.12), such as a polypeptide with an amino acid sequence having 80, 85, 90, 95 or 100% sequence identity to SEQ ID No.:119 (origin: *Escherichia coli*), or SEQ ID No.:121 (origin: *Klebsiella oxytoca*) or SEQ ID No.: 239 (hybrid sequence).

c) a polypeptide having lipoate-protein ligase A (LplA; EC:6.3.1.20) activity, such as a polypeptide with an amino acid sequence having 80, 85, 90, 95 or 100% sequence identity to SEQ ID No.:123 (origin: *Escherichia coli*) or SEQ ID No.:125 (origin: *Klebsiella oxytoca*).

[0067] The transgene encoding lipoic acid synthase together with one or more additional transgenes encoding polypeptides that catalyze additional steps in the lipoic acid pathway, are located in the genome of the genetically modified bacterial cell, either integrated into the bacterial cell chromosome or on a self-replicating plasmid. The transgene encoding LipA and one or more of the transgenes (*lpB, lplA,* and *AceF*) encoding enzymes in the lipoic acid pathway enzymes may be present in the genome within one or more operon.

[0068] The promoter driving expression of the transgene encoding LipA and one or more additional transgenes is preferably a non-native promoter, which may be a heterologous constitutive-promoter or an inducible-promoter. When the promoter is a heterologous constitutive promoter, then a suitable promoter includes the *apFab* family [SEQ ID Nos.:230-232], while a suitable inducible promoter includes: pBad (arabinose inducible [SEQ ID No.:233] and LacI [SEQ ID No.:234]. Suitable terminators include members of the *apFAB* terminator family including [SEQ ID No.: 235-237].The selected promoter and terminator may be operably linked to the respective gene, either to provide individual gene

regulation or for regulation of an operon.

IV A method for producing and detecting lipoic acid using a genetically modified bacterium to the invention

[0069] Lipoic acid can be produced using genetically modified bacterial cells of the invention (e.g. genetically modified *E. coli* cells) by introducing the cells into a suitable culture medium; and finally recovering the lipoic acid produced by the cells, as illustrated in the example 2 and figure 14).

[0070] The genetically modified bacterial cells of the invention comprising a transgene encoding a lipoic acid synthase (LipA) will produce lipoic acid when the supplied carbon source includes Octanoic Acid (OA). The cells will produce lipoic acid when supplied with a suitable carbon source for example a source selected from among glucose, maltose, galactose, fructose, sucrose, arabinose, xylose, raffinose, mannose, and lactose.

[0071] A method for quantifying cellular lipoic acid produced by a genetically modified bacterial cell of the invention is described in example 2. The method is a bioassay, based on measuring the growth of a lipoic acid-dependent auxotrophic *E. coli* strain on a minimal medium supplemented with the lipoic acid extracted from cells of the invention.

V A genetically modified bacterial cell for production of thiamine

[0072] The present invention provides a genetically modified bacterial cell capable of producing enhanced levels of thiamine. The bacterial cell is genetically modified to express a mutant IscR, according to the invention (see section I), in substitution for a wild type IscR, as well as comprising a transgene encoding a phosphomethylpyrimidine synthase, also called HMP-P synthase (EC 4.1.99.17), encoded by *thiC*; or a tyrosine lyase (also called 2-iminoacetate synthase (EC 4.1.99.19) encoded by *thiH*.

[0073] The genetically modified bacterial cell may further comprise one or more additional transgenes encoding polypeptides that catalyze additional steps in the thiamine synthesis pathway (figure 3). An increase in the levels of those polypeptides that catalyze steps in the thiamine pathway enhances the synthesis of both intermediates in the pathway, and the end product of the pathway in the bacterial cell. For example the bacterial cell may further comprise one or more transgenes encoding: ThiE thiamine phosphate synthase (EC 2.5.1.3); a [ThiS] adenylyltransferase (EC 2.7.7.73) (encoded e.g. by the *thiF* gene); a ThiG thiazole synthase (E.C.2.8.1.10); ThiS sulfur-carrier protein; a ThiD phosphohydroxymethylpyrimidine kinase (EC 2.7.4.7) and a thiamine mono-phosphate phosphatase (E.C. 3.1.3.-); a ThiO glycine oxidase (EC 1.4.3.19); and a ThiM hydroxyethylthiazole kinase (2.7.1.50).

[0074] HMP-P synthases are found in a wide range of bacteria and fungi belonging to a wide range of genera. The amino acid sequence of the polypeptide having HMP-P synthase activity has at least 70, 75, 80, 85, 90, 95, 96, 98, 100% amino acid sequence identity to a sequence selected from any one of: SEQ ID No.: 201 (origin: *Escherichia coli*); SEQ ID No.: 203 (origin: *Synechococcus_elongatus*); SEQ ID No.: 205 (origin: *Corynebacterium glutamicum*); SEQ ID No.: 207 (origin *Candidatus Baumannia cicadellinicola*). Tyrosine lyases are also called 2-iminoacetate synthase (EC 4.1.99.19). The amino acid sequence of the polypeptide having HMP-P synthase activity has at least 70, 75, 80, 85, 90, 95, 96, 98, 100% amino acid sequence identity to the sequence of: SEQ ID No.:217.

[0075] The polypeptides that are encoded by the one or more additional transgenes in the genetically modified bacterial cell, and whose activity serves to enhance the synthesis of both intermediates and products of the thiamine pathway, are as follows:

a) a polypeptide having [ThiS] adenylyltransferase (EC 2.7.7.73) activity, such as a polypeptide with an amino acid sequence having 80, 85, 90, 95 or 100% sequence identity to SEQ ID No.:211;

b) a polypeptide having thiamine phosphate synthase (EC 2.5.1.3) activity, such as a polypeptide with an amino acid sequence having 80, 85, 90, 95 or 100% sequence identity to SEQ ID No.:209;

c) a polypeptide having thiazole synthase (E.C.2.8.1.10) activity, such as a polypeptide with an amino acid sequence having 80, 85, 90, 95 or 100% sequence identity to SEQ ID No.:215;

d) a polypeptide having phosphohydroxymethylpyrimidine kinase (EC 2.7.4.7) activity, such as a polypeptide with an amino acid sequence having 80, 85, 90, 95 or 100% sequence identity to SEQ ID No.:225;

e) a polypeptide having glycine oxidase (EC 1.4.3.19) activity; such as a polypeptide with an amino acid sequence having 80, 85, 90, 95 or 100% sequence identity to a sequence selected from among SEQ ID No.:219, 221, and 223;

f) a polypeptide having ThiS sulfur-carrier activity such as a polypeptide with an amino acid sequence having 80, 85, 90, 95 or 100% sequence identity to SEQ ID No.:213;

g) a polypeptide having thiamine mono-phosphate phosphatase (E.C. 3.1.3.-) activity; such as a polypeptide with an amino acid sequence having 80, 85, 90, 95 or 100% sequence identity to a sequence selected from any one of SEQ ID No.:127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199; and

h) a polypeptide having ThiM hydroxyethylthiazole kinase activity (2.7.1.50), such as a polypeptide with an amino acid sequence having 80, 85, 90, 95 or 100% sequence identity to SEQ ID No.:227.

[0076] Preferably, the genetically modified bacterial cell comprises transgenes encoding the enzymes: ThiC (encoded by a *thiC* gene); ThiD (encoded by a *thiD* gene), ThiE (encoded by a *thiE* gene), ThiF (encoded by a *thiF* gene), sulfur-carrier protein (encoded by a *thiS* gene), ThiG (encoded by a *thiG* gene), TMP phosphatase (encoded by a TMP phosphatase gene); and either ThiH (encoded by a *thiH* gene) or ThiO (encoded by a *thiO* gene). According to the embodiment, the cells may further comprise a transgene encoding the enzyme ThiM ((encoded by a *thiM* gene).

[0077] Thiamine synthesis levels in the genetically modified bacterium of the invention may be further enhanced by mutation of the endogenous *thiL* gene that encodes thiamine-phosphate kinase. The mutant *thiL* gene has nucleotide sequence of SEQ ID No.: 228 and compared to the parent wild-type gene has a mutation at nucleotides 133-135 (GGT to GAC) that encodes a polypeptide having a G133D substitution [SEQ ID No.: 229].

[0078] The transgene encoding encoding HMP-P synthase (EC 4.1.99.17), encoded by *thiC;* or tyrosine lyase (EC 4.1.99.19) encoded by *thiH*, together with one or more additional transgenes encoding polypeptides that catalyze additional steps in the thiamine pathway, are located in the genome of the genetically modified bacterial cell, either integrated into the bacterial cell chromosome or on a self-replicating plasmid. The *thiC* or *thiH* transgene and one or more of the transgenes encoding enzymes in the thiamine pathway enzymes may be present in the genome within one or more operon.

[0079] The promoter driving expression of the *thiC* or *thiH* transgene and one or more additional transgenes is preferably a non-native promoter, which may be a heterologous constitutive- promoter or an inducible-promoter. When the promoter is a heterologous constitutive promoter, then a suitable promoter includes *apFab* family [SEQ ID Nos.:230-232] while a suitable inducible promoter includes: *pBad* (arabinose inducible [SEQ ID No.:233] and *LacI* [SEQ ID No.:234]. Suitable terminators include members of the *apFAB* terminator family including [SEQ ID No.: 235-237]. The selected promoter and terminator may be operably linked to the respective gene, either to provide individual gene regulation or for regulation of an operon.

VI A method for producing and detecting thiamine using a genetically modified bacterium according to the invention

[0080] Thiamine, thiamine monophosphate (TMP) and thiamine diphosphate (TPP) can be produced using genetically modified bacterial cells of the invention (e.g. genetically modified *E. coli* cells) by introducing the cells into a suitable culture medium; and finally recovering the thiamine, and additionally TPP and TMP produced by the cells, as illustrated in the Example 3 and figure 16.

[0081] The genetically modified bacterial cells of the invention comprising a transgene encoding a HMP-P synthase will produce thiamine, TPP and TMP when the supplied carbon source is selected from among glucose, maltose, galactose, fructose, sucrose, arabinose, xylose, raffinose, mannose, and lactose.

[0082] A method for quantifying thiamine produced by a genetically modified bacterial cell of the invention is described in example 3; and may include the use of High Pressure Liquid Chromatography, relative to a thiamine standard.

VII Methods for engineering a genetically modified bacterial cell for production of biotin, lipoic acid or thiamine

[0083] Integration and self-replicating vectors suitable for cloning and introducing one or more transgene encoding one or more a polypeptide having an enzymatic activity associated with the synthesis of biotin, lipoic acid or thiamine in a bacterial cell of the invention are commercially available and known to those skilled in the art (see, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, 1989). Bacterial cells are genetically engineered by the introduction into the cells of heterologous DNA. Heterologous expression of genes encoding one or more polypeptide having an enzymatic activity associated with biotin, lipoic acid or thiamine synthesis in a bacterial cell of the invention is demonstrated in Example 1, 2 and 3 respectively.

[0084] A nucleic acid molecule, that encodes one or more polypeptide having an enzymatic activity associated with biotin, lipoic acid or thiamine synthesis according to the invention, can be introduced into a host cell by means of a self-replicating vector or optionally integrated into the host cell genome using methods and techniques that are standard in the art. For example, nucleic acid molecules can be introduced by standard protocols such as transformation including chemical transformation and electroporation, transduction, particle bombardment, etc. Expressing the nucleic acid mol-

ecule encoding the enzymes of the claimed invention also may be accomplished by integrating the nucleic acid molecule into the genome.

[0085] Genetic modification of the native endogenous *iscR* gene in a bacterial cell of the invention can be performed by deletion (knockout) of the endogenous *iscR* gene and insertion/substitution with a transgene encoding a mutant IscR polypeptide as defined in section I, by applying standard recombineering methods to suitable parent bacterial cell (Datsenko KA, et al.; 2000).

[0086] The genetically modified bacterial cell according to the invention, for the production of biotin, lipoic acid or thiamine may be a bacterium, a non-exhaustive list of suitable bacteria is given as follows: a species belonging to the genus selected from the group consisting of: *Escherichia, Brevibacterium, Burkholderia, Campylobacter, Corynebacterium, Pseudomonas, Serratia, Lactobacillus, Lactococcus, Acetobacter, Acinetobacter, Pseudomonas,* etc.

[0087] Preferred bacterial species of the invention are *Escherichia coli, Pseudomonas putida, Serratia marcescens and Corynebacterium glutamicum.*

VIII Biotin production capacity of genetically modified bacterial cells of the invention is enhanced by increased electron transfer.

[0088] SAM-radical iron-sulfur cluster enzymes containing an oxidized $[4Fe\text{-}4S]^{2+}$ cluster, e.g. BioB, ThiC and LipA, need electron transfer for reduction to a $[4Fe\text{-}4S]^{+}$ cluster. Only the reduced $[4Fe\text{-}4S]^{+}$ cluster is able generate the SAM-radical needed for catalysis. The electron transfer from the electron donor NADPH to the $[4Fe\text{-}4S]^{2+}$ cluster can be mediated by a flavodoxin/ferredoxin reductase (Fpr) and flavodoxin (FldA) reduction system or by a Pyruvate-flavodoxin/ferredoxin oxidoreductase system.

[0089] In a further embodiment, the genetically modified bacterial cell according to the present invention, that is capable of producing either biotin, lipoic acid or thiamine, further comprises one or more genes selected from the group: a gene encoding a flavodoxin/ferredoxin-NADP reductase (EC: 1.18.1.2 and EC 1.19.1.1); a gene encoding a pyruvate-flavodoxin/ferredoxin oxidoreductase (EC 1.2.7); a gene encoding a flavodoxin; a gene encoding a ferredoxin; a gene encoding a flavodoxin and a ferredoxin-NADP reductase. Promoter(s), operably-linked to each of said one or more genes are capable of enhancing expression of said one or more genes in said bacterium; wherein each said one or more genes may be a native gene or a transgene. Preferably, the operably-linked promoter, enhances expression of said one or more genes in said bacterium to a level greater than in the parent bacterium from which the genetically-modified bacterium of the invention was derived. Preferably, the genetically modified bacterial cell according to the present invention comprises a gene encoding a flavodoxin/ferredoxin-NADP reductase (EC:1.18.1.2 and EC 1.19.1.1) and a gene encoding a flavodoxin; or a single gene comprising coding sequences for both a flavodoxin and a ferredoxin-NADP reductase. Additionally said genetically modified bacterial cell may further comprise a gene encoding a ferredoxin.

[0090] Overexpression of genes expressing components of the electron transfer pathway in genetically modified bacterial cells of the present invention, enhances the cellular activity of their SAM-radical iron-sulfur cluster enzymes (as illustrated in Example 4 for biotin-producing cells of the invention).

[0091] Preferably, when the polypeptide encoded by a native gene or transgene in the genetically modified bacterial cell of the invention has flavodoxin/ferredoxin reductase activity (EC:1.18.1.2 and EC 1.19.1.1), it has an amino acid sequence having 80, 85, 90, 95 or 100% sequence identity to a sequence selected from any one of: SEQ ID No.: 241 (origin: *fpr gene* from *E. coli*); SEQ ID No.:243 (origin: *yumC* gene from *Bacillus subtilis* 168); SEQ ID No.:245 (origin: *fpr-I* gene from *Pseudomonas putida* KT2440); SEQ ID No.:247 (origin: SVEN_0113 gene from *Streptomyces venezuelae* ATCC 10712 -); SEQ ID No.:249 (origin: Cgl2384 gene from *Corynebacterium glutamicum* ATTCC 13032), and SEQ ID No.:251 (origin: SJN15614.1 gene from *Sphingobacterium* sp. JB170.

[0092] Preferably, when the polypeptide encoded by a native gene or transgene in the genetically modified bacterial cell of the invention has pyruvate-flavodoxin/ferredoxin oxidoreductase activity (EC 1.2.7), it has an amino acid sequence having 80, 85, 90, 95 or 100% sequence identity to a sequence selected from any one of: SEQ ID No.: 253 (origin: *YdbK* gene from *E. coli* K12 MG1655); SEQ ID No.: 255 (origin: *por* gene from *Geobacter sulfurreducens* AM-1); SEQ ID No.: 257 (origin: Sfla_2592 gene from *Streptomyces pratensis* ATCC 33331; SEQ ID No.: 259 (origin: RM25_0186 gene from *Propionibacterium freudenreichii* DSM 20271); SEQ ID No.: 261 (origin: *nifJ* gene from *Synechocystis sp.* PCC 6803)

[0093] Preferably, when the polypeptide encoded by a native gene or transgene in the genetically modified bacterial cell of the invention is a flavodoxin, it has an amino acid sequence having 80, 85, 90, 95 or 100% sequence identity to a sequence selected from any one of: SEQ ID No.: 263 (origin: *fldA* gene from *Escherichia coli* K12 MG1655); SEQ ID No.: 265 (origin: *fldB* gene from *Escherichia coli* K12 MG1655); SEQ ID No.: 267 (origin: *ykuN* gene from *Bacillus subtilis* 168); SEQ ID No.: 269 (origin: *isiB* gene from *Synechocystis sp.* PCC 6803; SEQ ID No.: 271 (origin: *wrbA* gene from *Streptomyces venezuelae* ATCC 10712); SEQ ID No.: 273 (origin: PRK06242 gene from *Methanococcus aeolicus* Nankai-3).

[0094] Preferably, when the polypeptide encoded by a native gene or transgene in the genetically modified bacterial cell of the invention is a ferredoxin, it has an amino acid sequence having 80, 85, 90, 95 or 100% sequence identity to

a sequence selected from any one of: SEQ ID No.: 275 (origin: *fdx* gene from *E. coli*); SEQ ID No.: 277 (origin: *fer* gene from *Bacillus subtilis* 168); SEQ ID No.: 279 (origin: *fdxB* gene from *Corynebacterium glutamicum* ATTCC 13032); SEQ ID No.: 281 (origin: *fdx* gene from *Synechocystis sp.* PCC 6803); SEQ ID No.: 283 (origin: SVEN_7039 gene from *Streptomyces venezuelae* ATCC 10712); SEQ ID No.: 285 (origin: *fdx* gene from *Methanococcus aeolicus* Nankai-3).

[0095] A promoter(s) that is capable of enhancing gene expression when operably-linked of a native gene or to a transgene encoding polypeptides of the electron transport pathway in said bacterium is preferably a non-native promoter. Said promoter may be a member of the constitutive apFAB309 promoter family [SEQ ID Nos.:230-232]. Preferably said non-native promoter, when operably-linked to said native gene or transgene enhances expression of said encoded polypeptide(s) in said genetically modified bacterium to a level greater than the parent bacterium from which it was derived. Suitable terminators that may be operably-linked to said native gene or transgene includes the apFAB378 terminator family [SEQ ID No.: 235-237].

Examples

Example 1: Identification and characterization of genetically modified *E. coli* strains capable of enhanced biotin production

1 Methods

[0096] 1.1: The following strains of *Escherichia coli* used in the examples are listed below.

Table 1: Strains

Name	Description
BS1013	*E. coli* K-12 BW25113 parent strain having genotype: rrnB3 ΔlacZ4787 hsdR514 Δ(araBAD)567 Δ (rhaBAD)568 rph-1
BS1011	Δ*bioB* [1](JW0758-1) derived from *E. coli* K-12 BW25113
BS1353	BS1011 derivative comprising a H107Y mutation in *iscR*
BS1113	BS1011 derivative comprising pBS412 plasmid giving IPTG - inducible BioB expression
BS1375	BS1011 derivative comprising a C92Y mutation in *iscR*
BS1377	BS1011 derivative comprising a L15F mutation in *iscR*
[1] Nucleotide sequence of Δ*bioB* gene prior to deletion was SEQ ID No. 21	

[0097] 1.2: The following plasmids used in the examples are listed below.

Table 2: Plasmids

Name	Description
pBS412	BioB [SEQ ID No: 22] overexpression plasmid (kanR, SC101) from a T5 lacO repressed promoter
pBS430	pBS412 with frame shift mutation early in *bioB* [1](kanR, SC101) from a T5 lacO repressed promoter [SEQ ID No.: 25]
pBS451	Constitutively expressed GFP [SEQ ID No.: 287] (zeoR, p15A)
pBS281	*E. coli isc* operon (iscSUA-hscBA-fdx) from an IPTG inducible T5 promoter cloned in a medium copy number plasmid (p15A ori)
pBS282	*E. coli suf* operon (*sufABCDSE*) from an IPTG inducible T5 promoter
	cloned in a medium copy number plasmid (p15A ori)
pBS231	A medium copy number plasmid (p15A ori) expressing a gene encoding a sfGFP protein from an IPTG inducible T5 promoter
pBS936	Native biotin-operon from *E. coli* with "type 9" mutation in *bio* operator site (Ifuku et al., 1993)
[1]: Nucleotide sequence of *bioB* frameshift gene has SEQ ID No. 23	

1.3 Media and additives:

[0098] The growth media (mMOPS) used in each example had the following composition: 1.32 mM K2HPO4; 2 g/l D-glucose; 0.0476 mg/l calcium pantothenate; 0.0138 mg/l p-aminobenzoic acid; 0.0138 mg/l p-hydroxybenzoic acid; 0.0154 mg/l 2,3-dihydroxybenzoic acid, and 1x modified MOPS buffer.

[0099] 10 x modified MOPS comprises 0.4 M MOPS (3-(N-morpholino)propane sulfonic acid); 0.04 M Tricine; 0.1 mM $FeSO_4 \cdot 7H_2O$; 95 mM NH_4Cl; 2.76 mM K_2SO_4; 5 μM $CaCl_2 \cdot 2H_2O$; 5.25 mM $MgCl_2$; 0.5 M NaCl; and 5000x dilution of micronutrient stock solution.

Micronutrient stock solution:

Component	Formula	FW	Grams per 50 mL
ammonium molybdate	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	1235.9	0.009
boric acid	H_3BO_3	61.83	0.062
cobalt chloride	$CoCl_2$	237.9	0.018
cupric sulfate	$CuSO_4$	249.7	0.006
manganese chloride	$MnCl_2$	197.9	0.040
zinc sulfate	$ZnSO_4$	287.5	0.007

[0100] The following antibiotic stocks were employed: ampicillin (amp, 100 mg/mL), kanamycin (kan, 50 mg/mL), zeocin (zeo, 40 mg/mL); that were added to growth media as indicated to obtain a 1000x dilution.

1.4 Establishing of *E. coli* strain libraries:

[0101] *E. coli* libraries having evolved genomic diversity were derived from cells of *E. coli* strain BS1011 comprising plasmid pBS412 by subjecting the cells to stationary overnight culture in mMOPS medium supplemented with kan (MOPS-kan), preparing a 100 x dilution of resulting culture in mMOPS-kan and repeating the consecutive steps of overnight culture and dilution 5 times.

[0102] This procedure creates genetic diversity by allowing the accumulation of background mutation generated by imperfect error-correcting polymerases. After each round of culture and dilution a sample of the cell culture was plated on mMOPS plates with IPTG (see below), to detect the evolution of cells adapted to tolerate enhanced BioB expression. Cells of each library were then transformed with the BioB over-expression plasmid, pBS412.

1.5 Selection of mutant strains

[0103] A selection assay was developed by plating respectively 10^4, 10^5, 10^6 and 10^7 cells, derived from an o/n culture in mMOPS-kan of BS1011 comprising pBS412, on a series of 1.5 % agar plates comprising mMOPS (∅=9cm) comprising IPTG concentrations of either 0, 0.0001, 0.001, 0.01, 0.1 and 1 mM. The plates were then incubated at 37°C for up to 36 hours and cell growth was evaluated at intervals. Under these conditions, induction of BioB expression from pBS412 with 0.1 mM IPTG was found to prevent growth of up to 10^5 cells, while induction with 1 mM IPTG prevented growth of at least 10^7 cells when plated on a single petri dish. A selection pressure comprising induction with 1mM IPTG for a cell population of 10^5 cells was found optimal for identifying strains with higher robustness towards BioB expression; and accordingly was implemented as follows:

1) Approximately 10^5 cells from each library, as described in section 1.4, were plated on each mMOPS-kan -1 mM IPTG agar plate, and incubated at 37°C for maximum 24 hours.

2) Single colonies were grown in mMOPS-kan liquid medium to produce pre-cultures, that were then evaluated for their biotin production by means of a biotin bioassay (as described in section 1.6 below) that was performed in mMOPS-kan supplemented with either 0.00, 0.01 or 0.1 mM IPTG. Cells of each pre-culture were preserved as glycerol stocks in 20% glycerol.

3) Colonies producing more than 1.5 mg biotin/l (detected as extracellular biotin) were re-streaked on mMOPS-kan agar plates, incubated at 37°C for up to 24 hours, and then re-bioassayed for biotin production in biological replicates (as detailed in section 1.6 below).

4) Cells of the selected biotin over-producing strains were evaluated as follows:

a) Whole genome sequencing to identify genetic mutations in the genome of cells of selected strains as compared to the genome of the parent strain BS1011 was performed on DNA isolated from cells of the selected strain as follows: Selected cells were grown in 5-10 mL mMOPS-kan and the cells were subsequently harvested; genomic DNA was isolated from the harvested cells using Invitrogen Purelink genomic DNA extraction kit: (https://www.thermofisher.com/order/catalog/product/K182001); the extracted DNA was subjected to whole genome sequencing.

b) Curing cells of the selected strains of their pBS412 plasmid; then re-transforming the cells of the cured strain with the pBS412 plasmid; and finally redoing bioassay of cultures of cells of the transformed strains for biotin production. The steps of curing cells of their pBS412 plasmid were performed by growing the cells in rich, Luria Broth (LB) media with 1 mM IPTG without antibiotics overnight at 37 °C; streaking out cells of the resulting culture on LB agar plates and incubating overnight at 37 °C. Single colonies from the agar plates were diluted in 50 μl LB media and 5 μl were used to spot on LB and LB-amp agar plates, which were incubated overnight at 37 °C. Those single colonies that grew on LB plates, but not on LB-amp plates, were re-streaked on LB plates to obtain single colonies, which were used as cured strain for re-transformation. Biotin production by cells of the transformed strains where measured in biological replicates (as detailed in section 1.6 below). Briefly, biotin production re-evaluated for biological replicates in mMOPS-kan supplemented with 0.00, 0.01 or 0.1 mM IPTG. In parallel, the growth rates of the cells of each transformed strain were measured in 200μL mMOPS-kan medium in a microtiter plate sealed with transparent breathable seal at 37°C with "fast shaking" for aerobic growth, for a period of 24 hours in a Multiskan FC. Cell growth was monitored by measuring OD620 every 30 minutes.

1.6 Bioassay for quantifying biotin production

[0104] Pre-cultures were each prepared from a selected single cell colony in 400 μL mMOPS-kan in a 96 deep-well plate, incubated at 37°C with shake at 275 rpm for 16-18 hours. Production cultures were produced by inoculating 400 μL mMOPS-kan, supplemented with 0.1 g/L desthiobiotin (DTB), and optionally comprising IPTG at a final concentration of up to 1 mM, in a 96 deep-well plate, with 4 μL of the pre-culture sufficient to provide an initial OD600 of ~0.03. Cultures were then grown at 37°C with 275 rpm shake for 24 hours. Cells in the 96 deep-well plate were pelleted by centrifuging at 4000 G for 8 minutes after measuring OD_{600} of the cultures. The supernatant from each culture supernatant was diluted to a concentration range of 0.05 nM to 0.50 nM biotin in ultrapure (Milli-Q) water. In parallel, >5 biotin standards in the concentration range of 0.1 nM (0. 024 μg biotin/L) to 1 nM (0.24 μg biotin/L), were prepared in Milli-Q water. 15 μL of each diluted supernatant and each of the biotin standards was then added to a well of a microtiter plate; wherein each well comprised 135 μL of a biotin-starved overnight culture of BS1011 comprising plasmid pBS451; and where the overnight culture was diluted to an initial OD_{620} of 0.01 in mMOPS supplemented with zeocin. The plate was sealed with a breathable seal and incubated at 37°C with 275 rpm shaking for 20 hours before OD_{600} was measured. A biotin bioassay calibration curve obtained with this bioassay, using a range of biotin standards, is shown in figure 5.

1.7 Identification of genomic mutations

[0105] For all Next-Generation Sequencing (NGS) data, CLC genomic workbench version 9.5.3 (supplied by Qiagen) was used to identify mutations in the genome of cells of selected strains as compared to the parent strain genome (single or a few substitutions, deletions or insertions by Variant Detection and bigger insertions/deletions by InDels and Structural Variants). A cut-off of 85% were used to define "significant mutations" meaning that a mutation should be present in more than 85% of the population of DNA molecules (genomes) isolated from cells of a given bacterial strain, in order to distinguish genome mutations from erroneous nucleotides introduced by the sequencing procedure.

[0106] The genome accession number CP009273 from NCBI was used as the reference sequence, while taking account of the Keio *ΔbioB* scar mutation whose sequence was confirmed by sequencing.

1.8 Characterizing proteomics landscape of *iscR* mutant

[0107] Protein content of BS1013 + pBS430, BS1011 + pBS412 and BS1353 + pBS412 at 0.025 mM IPTG induction levels as well as BS1353 + pBS412 at 1 mM IPTG induction were determined by a recently developed approach combining LC-MS and efficient protein extraction (Schmidt et al, 2015). 3 peptides were chosen as minimum number of identified peptides for analysis along with a peptide threshold of 2.0 % FDR. Significant changes in protein expression are reported with a 0.5% confidence interval based on Analysis of Variance (ANOVA) with Benjamini-Hochberg correction for multiple

testing using a Scaffold Viewer 4.7.5.

[0108] Strains were grown in mMOPS with IPTG induction for approximately 10 generations, until OD_{600} of 0.5 were reached (exponential phase). 10^8 cells were harvested by centrifugation at 4°C at maximum speed; washed once in ice-cold PBS buffer; re-pelleted by centrifugation at 4°C at maximum speed and snap-frozen in liquid nitrogen, after removing PBS buffer.

2. Results

2.1 Overexpression of BioB is toxic

[0109] *E. coli* retaining its native biotin synthase gene (*bioB*) but expressing an IPTG-inducible frameshifted *E. coli bioB* gene (encoding non-functional biotin synthase due to a premature stop codon) from a low-copy plasmid (Sc101 origin of replication) is able to grow aerobically in mMOPS-kan medium with or without IPTG. This is illustrated in Figure 4 (left panel), showing the exponential growth curve of the *E. coli* BW25113, BioB frameshift mutant. In contrast, over-expression of a functional biotin synthase gene (*bioB*) in the *E. coli* knock-out strain Δ*bioB*, is toxic for growth, causing a very significant extension in the lag phase. This is illustrated in Figure 4 (right panel), that shows the growth of *E. coli* knock-out strain Δ*bioB* expressing an *E. coli bioB* gene from an IPTG-inducible T5 promoter on a low-copy plasmid (Sc101 origin of replication). As seen in figure 4, induction of increasing *bioB* expression in response to increasing IPTG levels (darkness of grey) significantly affects the lag-phase while the growth rate is affected slightly (black boxes).

2.2 Isolation of iscR mutant strains having enhanced biotin production titers

[0110] *E. coli* libraries having evolved genomic diversity (see sections 1.4 and 1.5) were screened for strains with improved tolerance for *bioB* gene expression and increased biotin production. Whole genome sequencing of the selected strains led to the identification of three unique mutants each comprising an Iron-sulfur cluster regulator (*iscR*) gene encoding an iscR polypeptide having one of the amino acid substitutions: L15F, C92Y and H107Y, and where the amino acid sequence of the encoded regulators is SEQ ID No.: 16, 18 and 20 respectively. Biotin production levels were measured using a bioassay, as described in section 1.6 (and figure 5). The biotin production titers for each of the *iscR* mutant strains as well as the *E. coli* BW25113 Δ*bioB* reference strain are shown in figure 6 for 4 biological replicates (black dots) grown in mMOPS supplemented with 0.1 g/l DTB in the absence or presence of two different IPTG concentration levels (increasing IPTG with darker grey). Biotin production in the reference strain was inhibited at IPTG levels of above 0.01 mM, which corresponds to IPTG levels that are toxic for growth of the reference strain (see figure 4), while the *iscR* mutant strains both grew and produced biotin at IPTG levels of 0.01 - 0.1mM IPTG. All three *iscR* mutant strains produce approximately 1.5 fold more biotin than the reference strain (stippled line) at an IPTG concentration of 0.01 mM. The *iscR* mutant strains produce up to 2-fold more (~3.2 mg biotin/l) at an IPTG concentration of 0.1 mM, than the highest production titer from the reference strain (~1.5 mg biotin/l).

2.3 Biotin production and growth in an IscR H107Y mutant strain

[0111] The growth profile and biotin production titer of the *iscR* (H107Y) mutant strain was characterized in 50 mL mMOPS supplemented with 0.1 g/l DTB in a 250 mL shake-flask experiment at two different IPTG induction levels (0.01 mM in figure 7A) and 0.5 mM (figure 7B). At low IPTG levels (figure 7A), the IscR mutant strain (dark grey) and the reference strain (light grey) were similar with respect to growth and biotin production titers, with a final titer of ~1.1 mg biotin/l. However, at high IPTG induction levels (0.5 mM in figure 7B) growth of the reference strain (light grey) was severely inhibited, while the IscR mutant strain retained the same growth profile as at low IPTG induction levels. Furthermore, the biotin production titers of the IscR mutant strain increased around 2-fold, up to ~2.2 mg biotin/l after 25 hours of growth.

2.4 Mechanism of action of IscR mutations

[0112] An enhanced biotin tolerance phenotype was clearly demonstrated for all three of the identified IscR mutant strains, as seen in figure 6. The ability of the C92 mutation (C92Y) to enhance biotin tolerance is suggested to be due to a role for C92 in the [Fe-S] cluster binding properties of IscR. Loss of [Fe-S] cluster binding properties due to the C92Y mutation is proposed to inactivate the Isc-operon repression behavior of IscR. At the same time, it is proposed that the promoter function of IscR remains intact in the C92Y mutant IscR, such that it retains its function in activating other pathways essential for multiple cellular processes. A similar essential role in providing the [Fe-S] cluster binding properties of IscR is attributed to H107; where the H107Y mutation is similarly able to enhance biotin tolerance in *E. coli.* The L15F in IscR is also proposed to disrupt iron-sulfur cluster binding and thereby partially overcome iron-sulfur

cluster depletion. Figure 9 shows the position of the L15 and H107 in IscR when bound to DNA (binding site of hya, PDB entry 4HF1), and it can be seen that L15 is positioned at the inside of each of the IscR subunits. Phenylalanine is a significantly larger amino acid than leucine, and may interfere with the three-dimensional folding of the protein.

2.5 Overexpression of the *isc*-operon or *suf* operon in *E. coli* strains alone is not sufficient to enhance biotin production

[0113] In order to determine the direct effect of overexpressing the *isc*-operon (*iscSUA-hscBA-fdx,* corresponding to the native *E. coli* operon structure minus the *iscR* gene) or the *suf*-operon (sufABCDSE corresponding to the native *E. coli* operon structure) on biotin production in *E. coli,* each operon was cloned into a medium copy number plasmid (p15A ori) placed under the control of a strong RBS and an IPTG inducible T5 promoter. A plasmid, comprising a gene encoding a super folder Green Fluorescent Protein (sfGFP) in substitution for the isc- or suf-operon, was employed as a control. The respective plasmids were transformed into cells of an *E. coli* strain comprising an IPTG-inducible *bioB* expression plasmid. Biological triplicate colonies comprising one of: 1) IPTG-inducible *isc*-operon, 2) IPTG-inducible suf-operon or 3) IPTG-inducible GFP (control) in addition to the IPTG-inducible *bioB* expression plasmid were assayed for biotin production (as described in section 1.5) following cultivation in 400 μL mMOPS with 100 μg/mL ampicillin and 50 μg/mL spectinomycin under low (0.01 mM IPTG) and high (0.1 mM IPTG) induction.

[0114] From the graph (figure 10) it can be seen that although biotin production was IPTG-inducible in all strains; the IPTG concentration needed to reach detectable biotin production levels was increased from 0.01 mM IPTG to 0.1 mM IPTG, when compared to the reference and mutant *iscR* strains shown in figure 6. Furthermore, biotin production titers were significantly decreased by the overexpression of *isc*- or suf-operons, when compared to both the *sfGFP* strain in figure 10. Additionally, overexpression of *isc*-operon depressed biotin production even more than overexpression of suf-operon. Taken together with the observed increase in biotin production seen in mutant strains having a single point mutation in *iscR* (figure 6), it is unlikely that the resulting de-repression of *isc*-operon in these strains is the only/main reason for improved biotin production in those strains.

2.6 BioB protein contents correlates with biotin production

[0115] To investigate the molecular effects of BioB overexpression in wild type and mutant background strains, proteomics measurements were carried out for a wild type background strain: BS1013 holding pBS430; a wild type *iscR* strain with a *bioB* production plasmid: BS1011 holding pBS412; and a mutant *iscR* strain with a *bioB* production plasmid: BS1353 holding pBS412. All strains were grown in mMOPS with 0.1 g/L DTB and 0.025 mM IPTG. The latter strain was additionally grown at 1 mM IPTG induction. Cells were harvested for proteomics analysis in exponential phase, while the remaining cell culture were kept incubating for 24 hours in total, before biotin production were measured using the bioassay described elsewhere.

[0116] From the graph (figure 11) the measured BioB protein level strongly correlates with the biotin production (R^2 value of 0.96). The linear correlation shows that facilitating enhanced BioB expression is a key to improve biotin production in IscR mutant cell factories. The ANOVA analysis of the proteomics data revealed a significant increase in expression (95% confidence interval, p-value of 0.00166) in additional 29 proteins. Among these are members of the *isc*-operon (IscA and IscS) and suf-operon (SufB and SufS).

2.7 Biotin production is not enhanced in *iscR* knockout mutants

[0117] A translational knockout of the *iscR* gene was introduced into a BW25113 *ΔbioB* strain by MAGE, by converting the codon encoding glutamic acid on position 22 in iscR (E, GAA) into a stopcodon (*, TGA). Successful conversion of the codon was verified by PCR amplification of the region followed by Sanger sequencing. Strains with genes encoding wild type *iscR, iscR* knockout (E22*), and mutant *iscR* (C92Y) were transformed with IPTG-inducible *bioB* plasmid pBS412, and tested for biotin production in biological replicates (n=3) grown in mMOPS supplemented with 0.1 g/l DTB and 50 μg/l kanamycin at three different IPTG induction levels (0, 0.01, and 0.1 mM) as described above.

[0118] No significant differences in biotin production were observed between the *iscR* knockout (*iscR* KO) and the wild type *iscR* (*iscR* WT) strains when inducing *bioB* expression by IPTG induction. This provides evidence that knocking out *iscR* does not improve biotin production. Significant improvement in biotin production was again observed for the mutant *iscR* encoding IscR C92Y substitution as compared to both *iscR* WT and *iscR* KO strains.

2.8 De-novo biotin production is enhanced in *iscR* mutant strains of the invention

[0119] A BW25113 *E. coli* strain from which both the *bioA* gene and the entire biotin-operon (Δ*bioB-bioD*) were deleted and comprising either *iscR* WT, *iscR* H107Y mutant or *iscR* C92Y mutant genes, were transformed with a tetracycline resistant plasmid, constitutively overexpressing the native *E. coli bioA* and biotin-operon, with a single point mutation in

the *bioO* operator site (Type 9 mutation, Ifuku et al., 1993). Biotin production was evaluated for the three different strains in biological replicates (n=4) in mMOPS (2 g glucose/l) with 10 μg/ml tetracycline with and without the addition of 0.1 g/l DTB as described above (figure 13).

[0120] A significant increase in biotin titers were observed for all three strains when the substrate, DTB, was added to the growth medium, indicating that the *bioB* enzyme reaction itself, converting DTB to biotin, is no longer a bottleneck for biotin production in these strains (figure 13). Furthermore, a significant increase in de-novo production of biotin from glucose was observed for both *iscR* mutant strains as compared to the *iscR* WT strain. On view of these results it may deduced that the mutant *iscR* strains of the invention all support enhanced biotin production from both the direct precursor, DTB, and from glucose.

Example 2: Engineering and characterization of genetically modified *E. coli* strains capable of enhanced lipoic acid production

[0121] The following strains of *Escherichia coli* used in the example are listed below.

Table 3: Strains

Name	Description
BS1912	Δ*lipA* derived from *E. coli* K-12 BW25113
BS2114	BS1912 derivate comprising a H107Y mutation in *iscR*

[0122] The following plasmids used in the example are listed below.

Table 4: Plasmids

Name	Description
pBS993	LipA [SEQ ID No.: 103] overexpression plasmid (kanR, SC101) from a T5 lacO repressed promoter [SEQ ID No.: 234] with additional constitutive expression of AceF [SEQ ID No.:119] (medium RBS)
pBS1037	pBS993 derivate with p15A origin of replication instead of SC101 and low RBS strength for AceF [SEQ ID No.:119] expression
pBS451	Constitutively expressed GFP [SEQ ID No.: 287] (zeoR, p15A)

[0123] An IPTG-inducible transgene encoding LipA [SEQ ID No.: 103], cloned on plasmid pBS1037, was introduced into an *E. coli* host strain comprising the native *iscR* gene or into an *E. coli* host strain in which the native *iscR* gene was substituted by a mutant *iscR* gene encoding an IscR protein having an C92Y or H107Y substitution, as described in Example 1; the two strains further comprising a knock-out of the *bioB* or *lipA*. The strains were cultured in mMOPS medium (as described in section 1.3) supplemented with 0.1 mM biotin (for Δ*bioB* strains), IPTG for induction of *lipA* expression and 0.6 g/l octanoic acid as substrate, for 24 hours at 37°C, before measuring of free lipoic acid in the supernatant. For Δ*lipA* strains (BS1912 and BS2114, Table 3) growth was followed during the 24 hours of growth at 37°C.

[0124] Lipoic acid, produced by cultured cells of the described strains, was measured from the supernatant by means of a bioassay similar to the one described in section 1.6 using BS1912 comprising pBS451. The growth-based bioassay, for quantification of lipoic acid, was performed using an auxotrophic *E. coli* single Δ*lipA* mutant strain that is incapable of synthesizing lipoic acid (Herbert and Guest, 1975) (BS1912 comprising pBS451). Free lipoic acid concentrations in the supernatant were determined by measuring the growth of the lipoic acid auxotroph strain in a minimal media with 50 nN na-succinate as carbon source, supplemented with lipoic acid recovered from production strains, as the sole source of lipoic acid. A lipoic acid bioassay calibration curve was performed in parallel, where the auxotrophic stain was grown on minimal media, supplemented with a known concentration range of lipoic acid standards.

[0125] The tests demonstrate that over-expression of a *lipA* gene in an *E. coli* strain comprising a gene encoding a mutant form of the IscR protein (IscR protein having an C92Y or H107Y substitution) leads to both a more stable production and an 80% increase in the lipoic acid titer as compared to over-expression of the *lipA* gene in a parent *E. coli* strain comprising a gene encoding the native form of the IscR protein (Figure 14). Thus the standard deviation in the production titers of the *iscR* WT strain (BS1011 with pBS993) is 2.73, whereas it is 1.42 for *iscR* C92Y (BS1375 with pBS993) and as low as 0.11 for *iscR* H107Y (BS1353 with pBS993) (see Table 1 for strain references). Based on the average production titers of the individual strains, lipoic acid production is improved 1.79-fold in mutant strains compared to WT strain (see Figure 14).

[0126] Overexpression of LipA showed a clear tendency to reduce the growth rate in response to increased induction

of LipA by IPTG (darker shaded grey, figure 15) for both WT *iscR* strain (triangles, BS1912 comprising pBS1037) and *iscR* mutant strain (squares, BS2114 comprising pBS1037). However, the growth rate for the WT *iscR* strain was more severely reduced as compared to the mutant *iscR* strain for all IPTG induction levels tested between 0.01 mM and 0.03 mM (see Figure 15).

Example 3 Engineering and characterization of genetically modified E. *coli* strains capable of enhanced thiamine production

[0127] The following strains of *Escherichia coli* used in the example are listed below.

Table 5: Strains

Name	Description
BS750	BS1013 derivative, BW25113 Δ*thiP* and comprising a point mutation in the native *thiL* gene: codon 133 from GGT to GAC causing G133D substitution in the encoded TMP kinase.
BS2019	BS750 derivative comprising a C92Y mutation in *iscR*
BS2020	BS750 derivative comprising a H107Y mutation in *iscR.*

[0128] The following plasmids used in the example are listed below.

Table 6: Plasmids

Name	Description
pBS140	Vector comprising the *E. coli* thiamine pathway genes *thiCEFSGHMD;* constructed from a combination of the *thiC* operon (functionally linked to an apFAB46 promoter (SEQ ID No.:147) and an apFAB377 terminator (SEQ ID No.:153)) and the *thiM* operon (functionally linked to an apFAB71 promoter (SEQ ID No.: 149)) and an apFAB378 terminator (SEQ ID No.: 152)).
pBS100	Empty vector used for construction of pBS140
pBS93	Vector comprising synthetic gene encoding *Arabidopsis thaliana* AT5G32470.1 phosphatase codon optimized for expression in *E. coli* functionally-linked to a pFAB70 promoter (SEQ ID No.:148) and apFAB381 terminator (SEQ ID No.:154)
pBS209	Vector based on pBS140 with an additional thiC from *E. coli*

[0129] The thiamine pathway genes *thiCEFSGHMD* cloned on plasmid pBS140, were introduced into an *E. coli* host strain (BS750) comprising the native *iscR* gene (as reference strain); as well as into derivatives of this reference strain in which the native *iscR* gene is substituted by a mutant *iscR* gene encoding an IscR protein having an C92Y or H107Y substitution respectively (BS2019 and BS2020), as described in Example 1. The strains were cultured in mMOPS medium (as described in section 1.3) for 24 hours at 37°C in individual wells of a deep culture plate.

[0130] Extracellular and intracellular thiamine, TMP and TPP produced by the cultured cells of the described strains, was recovered and extracted as follows: 0.4mL of each culture were harvested at 4°C by centrifugation in the cultivation plate at 4000 x g for 5 minutes. All remaining steps are performed on ice. 40 μL of supernatant was gently removed for analysis of extracellular TPP, TMP and thiamine. After decanting the remaining supernatant; the culture plate was inverted to remove residual medium and then vortexed. 100μL ice-cold HPLC grade methanol was added to each well of the culture plate; and the cells were vortexed again. After incubation on ice for a minimum of 20 minutes cell debris was pelleted by centrifugation at 4000 x g for 5 minutes. The supernatant was used as intracellular extract for further analysis.

[0131] In order to detect TPP, TMP and thiamine using a fluorescence detector, the thiamine compounds produced by each culture were derivatized into thiochromes, which are strongly fluorescent. All steps were performed at room temperature. 40μl volumes of the extracellular and intracellular extracts was added to 80μl of 4M potassium acetate and mixed by pipetting. 40μl of freshly prepared 3.8mM potassium ferricyanide in 7M NaOH was added and mixed. The reaction was quenched by addition of 40μl freshly prepared 0.06% H_20_2 in saturated KH_2PO_4. The extracts were neutralized by addition of 47μL 6M HCl and then analyzed by HPLC or direct fluorescence measurement using a Multiskan. All derivatized compounds were quantified using fluorescence standard curves of freshly prepared of TPP, TMP and thiamine standards that are derivatized to thiochromes in parallel with the analyzed extracts.

[0132] The tests demonstrate that over-expression of the thiamine pathway genes, which comprise the *thiC* gene and

the *thiH* gene in combination with a TMP phosphatase gene (At5g32470), in host *E. coli* strains (BS2019 and BS2020) comprising a gene encoding a mutant form of the IscR protein (IscR protein having an C92Y or H107Y substitution) leads to enhanced biosynthesis of thiamine, TMP and TPP, in particular thiamine, as compared to over-expression in host *E. coli* strain (BS750) comprising a gene encoding the native form of the IscR protein.

[0133] More specifically, the tests showed an increase of 1.43 fold in OD-normalized extracellular production of thiamines (thiamine, TMP and TPP) between a strain with WT *iscR* (BS750) and a strain encoding an *iscR* mutant (BS2020, H107Y or BS2019, C92Y) when using pBS140 (figure 16).

Example 4 Overexpression of a flavodoxin/ferredoxin reductase (Fpr) and flavodoxin (FldA) reduction system to increase productivity of genetically modified E. coli strains capable of producing biotin

1 Methods

[0134] 1.1: The following strains of *Escherichia coli* used in the examples are listed below.

Table 7: Strains

Name	Description
BS1013	*E. coli* K-12 BW25113 parent strain having genotype: rrnB3 ΔlacZ4787 hsdR514 Δ(araBAD)567 Δ (rhaBAD)568 rph-1
BS1011	ΔbioB (JW0758-1) derived from E. coli K-12 BW25113
BS1353	BS1011 derivative comprising a H107Y mutation in iscR
BS1615	BS1011 derivative with additional deletion of ΔbioAFCD
BS1937	BS1615 derivative comprising pBS679 plasmid giving IPTG - inducible BioB expression
BS2185	BS1615 derivative comprising pBS679 plasmid giving IPTG - inducible BioB expression and pBS1112 giving constitutive FldA-Fpr expression
BS2707	BS1615 derivative comprising pBS679 plasmid giving IPTG - inducible BioB expression and pBS1054 giving constitutive GFP expression

[0135] The following plasmids used in the example are listed below.

Table 8: Plasmids

Name	Description
pBS679	BioB [SEQ ID No.: 22] overexpression plasmid (ampR, pSC101) from a T5 lacO repressed promoter [SEQ ID No: 25] and
pBS1054	GFP [SEQ ID No.: 276] overexpression plasmid (kanR, pBR322) from a constitutive apFAB309 promoter [SEQ ID No.: 291] with an apFAB378 terminator [SEQ ID No.: 292].
pBS1112	FldA-Fpr overexpression plasmid (kanR, pBR322) from a constitutive apFAB306 promoter (apFAB306-FldA-Fpr gene- apFAB378 terminator [SEQ ID No: 288])

[0136] An IPTG-inducible transgene encoding BioB was cloned on plasmid pBS679; a constitutively-regulated transgene encoding GFP was cloned on plasmid pBS1054; and a constitutively-regulated transgene comprising a synthetic operon encoding FldA-Fpr was cloned on plasmid pBS1112. pBS679 was introduced into an *E. coli* host strain (BS1615) in which the native *iscR* gene was substituted by a mutant *iscR* gene encoding an IscR protein having an H107Y substitution, as described in Example 1, and further comprising a knock-out of the *bioAFCD* genes resulting in the strain BS1937. The strain BS1937 was then further transformed with either plasmid pBS1054 or pBS1112 resulting in the strains BS2707 (control strain) and BS2185, respectively.

[0137] The strains were cultured in mMOPS medium (as described in example 1.3) with appropriate antibiotic(s), 0.1g/L DTB as substrate for BioB-mediated catalysis, and supplemented with either 0, 0.01, 0.025, 0.05, 0.075 or 0.1 mM IPTG for inducing expression of the *BioB* gene. The cells were incubated for 24 hours at 37 degrees C in individual wells of a deep well culture plate. End ODs were estimated, supernatants were harvested by centrifugation and biotin quantified from the supernatants by a biotin bioassay carried out as described in example 1.6.

[0138] As shown in figure 12 and figure 17 for strain BS1937, when *BioB* gene expression in *E. coli* cells comprising a genetically modified endogenous *iscR* gene, are induced with increasing concentrations of IPTG, the cells show a corresponding progressive increase in biotin production. Biotin production in these genetically modified cells is further enhanced by the co-expression of a transgene encoding FldA-Fpr (strain BS2185) when compared with its parent strain BS1937, and with a control strain expressing a transgene encoding GFP instead of FldA-Fpr.

[0139] Biotin production of of strain BS2185 comprising a gene encoding a mutant form of the IscR protein (IscR protein having an H107Y substitution) and a plasmid for overexpression of BioB (pBS679) and FldA-Fpr genes (pBS1112) is 2.12-fold enhanced compared to the control strain BS1937 (no overexpression of FldA-Fpr genes) (figure 18).

References

[0140]

Datsenko KA, Wanner BL. (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A.;97(12):6640-5.

Fleischhacker, A. S. et al. (2012) Characterization of the [2Fe-2S] cluster of Escherichia coli transcription factor IscR. Biochemistry 51: 4453-4462.

Giel, J. L., Rodionov, D., Liu, M., Blattner, F. R. & Kiley, P. J. (2006) IscR-dependent gene expression links iron-sulphur cluster assembly to the control of O2-regulated genes in Escherichia coli. Mol. Microbiol. 60, 1058-1075.

Herbert AA, Guest JR. (1975) Lipoic acid content of Escherichia coli and other microorganisms. Arch Microbiol.;106(3):259-66. Epub 1975/12/31. pmid:814874

Ifuku, O. et al. Sequencing analysis of mutation points in the biotin operon of biotin-overproducing Escherichia coli mutants. Biosci Biotechnol Biochem 57, 760-765 (1993).

Ifuku, O. et al., (1995) "Molecular analysis of growth inhibition caused by overexpression of the biotin operon in Escherichia coli." Bioscience, biotechnology, and biochemistry 59(2):184-189.

Martin, J. E. & Imlay, J. A. (2012) Replication during periods of iron starvation. 80, 319-334.

Py, B. & Barras, F. (2010) Building Fe-S proteins: bacterial strategies. Nat. Rev. Microbiol. 8, 436-446.

Schmitt, A, Kochanowski K., Vedelaar S., Ahrné E., Volkmer B., Callipo L., Knoops K., Bauer M., Aebersold R., & Heinemann M., (2015) The quantitative and condition-dependent Escherichia coli proteome. Nature Biotechnology 2015; doi:10.1038

Claims

1. A genetically modified bacterium; wherein the bacterium has an enhanced production of biotin or lipoic acid or thiamine; wherein said bacterium comprises:

a) genetically modified endogenous iscR gene encoding a mutant IscR polypeptide, wherein the amino acid sequence of said mutant IscR polypeptide has at least 80% amino acid sequence identity to a sequence selected from the group consisting of SEQ ID No: 2, 4, 6, 8, 10, 12 and 14, and wherein said amino acid sequence has at least one amino acid substitution selected from the group consisting of:

i) L15X, C92X, C98X, C104X, and H107X; wherein X is any amino acid other than the corresponding amino acid residue in SEQ ID No.: 2, 4, 6, 8, 10, 12 and 14;

and

b) at least one transgene encoding a polypeptide selected from the group consisting of:

ii) a polypeptide having biotin synthase activity (EC2.8.1.6);
iii) a polypeptide having lipoic acid synthase activity (EC2.8.1.8);
iv) a polypeptide having HMP-P synthase activity (EC4.1.99.17); and
v) a polypeptide having tyrosine lyase activity (EC4.1.99.19).

2. A genetically modified bacterium for enhanced production of biotin or lipoic acid or thiamine according to claim 1, wherein said at least one amino acid substitution in said mutant IscR polypeptide is selected from the group consisting of:

a) L15X, wherein X is any one of F, Y, M and W;

b) C92X, wherein X is any one of Y, A, M, F and W;
c) C98X, wherein X is any one of A, V, I, L, F and W;
d) C104X, wherein X is any one of AV, I, L, F and W; and
e) H107X; wherein X, is any one of A, Y, V, I, and L.

3. A genetically modified bacterium according to claims 1 or 2, wherein said at least one transgene encodes a polypeptide having biotin synthase activity (EC 2.8.1.6), further comprising additional transgenes encoding one or more polypeptides selected from the group consisting of:

a) a polypeptide having SAM (S-adenosylmethionine)-dependent methyltransferase activity (EC 2.1.1.197);
b) a polypeptide having 7-keto-8-aminopelargonic acid (KAPA) synthase activity (EC 2.3.1.47);
c) a polypeptide having 7,8-Diaminopelargonic Acid (DAPA) Synthase activity (EC:2.6.1.62 or EC2.6.1.105);
d) a polypeptide having Dethiobiotin (DTB) Synthetase activity (EC 6.3.3.3);
e) a polypeptide having Pimeloyl-[acyl-carrier protein] methyl ester esterase (EC 3.1.1.85); and
f) a polypeptide having 6-carboxyhexanoate-CoA ligase activity (EC 6.2.1.14);

wherein said bacterium is for enhanced production of biotin.

4. A genetically modified bacterium according to claim 1 or 2, wherein said at least one transgene encodes a polypeptide having lipoic acid synthase activity (EC 2.8.1.8), further comprising additional transgenes encoding one or more polypeptides selected from the group consisting of:

a) a polypeptide having octanoyltransferase activity (EC 2.3.1.181);
b) a polypeptide comprising the dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase (EC 2.3.1.12); and
c) a polypeptide having lipoate-protein ligase A activity (EC:6.3.1.20);

wherein said bacterium is for enhanced production of lipoic acid.

5. A genetically modified bacterium according to claim 1 or 2, wherein said at least one transgene encodes a polypeptide having HMP-P synthase activity (EC 4.1.99.17), and/or one transgene encoding a polypeptide having tyrosine lyase activity (EC 4.1.99.19), further comprising additional transgenes encoding one or more polypeptides selected from the group consisting of:

a) a ThiF polypeptide having ThiS adenylyltransferase activity (EC2.7.7.73);
b) a ThiE polypeptide having thiamine phosphate synthase activity (EC 2.5.1.3);
c) a ThiG polypeptide having thiazole synthase activity (E.C.2.8.1.10);
d) a ThiD polypeptide having phosphohydroxymethylpyrimidine kinase activity (EC 2.7.4.7);
e) a ThiO polypeptide having glycine oxidase activity (EC 1.4.3.19);
f) a ThiS polypeptide having sulfur-carrier protein activity;
g) a ThiM polypeptide having hydroxyethylthiazole kinase activity (EC2.7.1.50); and
h) a polypeptide having thiamine mono-phosphate phosphatase activity (E.C. 3.1.3.-);

wherein said bacterium is for enhanced production of thiamine.

6. A genetically modified bacterium according to claim 5, wherein said bacterium comprises the additional transgenes encoding:

a) a ThiC polypeptide having HMP-P synthase activity (EC4.1.99.17);
b) a ThiH polypeptide having tyrosine lyase activity (EC 4.1.99.19) or a ThiO polypeptide having glycine oxidase activity (EC 1.4.3.19);
c) a ThiF polypeptide having ThiS adenylyltransferase activity (EC2.7.7.73);
d) a ThiE polypeptide having thiamine phosphate synthase activity (EC 2.5.1.3);
e) a ThiG polypeptide having thiazole synthase activity (E.C.2.8.1.10);
f) a ThiD polypeptide having phosphohydroxymethylpyrimidine kinase activity (EC 2.7.4.7);
g) a ThiS polypeptide having sulfur-carrier protein activity; and
h) a polypeptide having thiamine mono-phosphate phosphatase (E.C. 3.1.3.-) activity.

7. A genetically modified bacterium according to any one of claims 1 to 6, wherein said at least one transgene and said one or more additional transgenes are operably linked to a constitutive promoter.

8. A genetically modified bacterium according to any one of claims 1 to 7, wherein said bacterium is a genus of bacterium selected from the group consisting of Escherichia, Bacillus, Brevibacterium, Burkholderia, Campylobacter, Corynebacterium, Pseudomonas, Serratia, Lactobacillus, Lactococcus, Acinetobacter, Pseudomonas, and Acetobacter.

9. A method for producing biotin, comprising the steps of:

a) introducing a genetically modified bacterium according to any one of claims 1 - 3, 7 and 8 wherein said at least one transgene encodes a polypeptide having biotin synthase activity (EC 2.8.1.6), into a growth medium to produce a culture;
b) cultivating the culture; and
c) recovering biotin produced by said culture, and optionally purifying the recovered biotin.

10. A method for producing lipoic acid comprising the steps of:

a) introducing a genetically modified bacterium according to any one of claims 1, 2, 4, 7 and 8 wherein said at least one transgene encodes a polypeptide having lipoic acid synthase activity (EC 2.8.1.6), into a growth medium to produce a culture;
b) cultivating the culture; and
c) recovering lipoic acid produced by said culture, and optionally purifying the recovered lipoic acid.

11. A method for producing thiamine comprising the steps of:

a) introducing a genetically modified bacterium according to any one of claims 1, 2, 5 - 8 wherein said at least one transgene encodes a polypeptide having HMP-P synthase activity (EC 4.1.99.17), and/or a transgene encoding a polypeptide having tyrosine lyase activity (EC 4.1.99.19) into a growth medium to produce a culture;
b) cultivating the culture; and
c) recovering thiamine produced by said culture, and optionally purifying the recovered thiamine.

12. A method for producing any one of biotin, lipoic acid and thiamine according to any one of claims 9 to 11, wherein the growth medium comprises a carbon source selected from among glucose, maltose, galactose, fructose, sucrose, arabinose, xylose, raffinose, mannose, and lactose, or any combination thereof.

13. Use of a genetically modified gene encoding a mutant iscR polypeptide to enhance production of any one of biotin, lipoic acid or thiamine in a genetically modified bacterium, wherein said bacterium comprises and expresses at least one transgene encoding a polypeptide selected from the group consisting of:

i) a polypeptide having biotin synthase activity (EC2.8.1.6);
ii) ii) a polypeptide having lipoic acid synthase activity (EC2.8.1.8);
iii) iii) a polypeptide having HMP-P synthase activity (EC4.1.99.17); and
iv) iv) a polypeptide having tyrosine lyase activity (EC4.1.99.19); and

wherein said genetically modified gene is an endogenous iscR gene encoding a mutant IscR polypeptide, wherein the amino acid sequence of said mutant IscR polypeptide has at least 80% amino acid sequence identity to SEQ ID No.: 2, 4, 6, 8, 10, 12 and 14, and wherein the amino acid sequence has at least one amino acid substitution selected from the group consisting of: L15X, C92X, C98X, C104X, and H107X; wherein Xis any amino acid other than the corresponding amino acid residue in SEQ ID No 2, 4, 6, 8, 10, 12 and 14.

14. Use of a genetically modified gene encoding a mutant iscR polypeptide to enhance production of any one of biotin, lipoic acid or thiamine in a genetically modified bacterium according to claim 13, wherein said at least one amino acid substitution in said mutant IscR polypeptide is selected from the group consisting of:

a) L15X, wherein X is any one of F, Y, M and W;
b) C92X, wherein X is any one of Y, A, M, F and W;
c) C98X, wherein X is any one of A, V, I, L, F and W; and
d) C104X, wherein X is any one of AV, I, L, F and W; and e. H107X; wherein X, is any one of A, Y, V, I, and L.

15. Use of a genetically modified bacterium according to any one of claims 1- 8 for enhanced production of any one of biotin, lipoic acid or thiamine.

16. A genetically modified bacterium according to any one of claims 1 - 8, wherein said bacterium further comprises one or more genes selected from the group:

a) a gene encoding a flavodoxin/ferredoxin-NADP reductase (EC:1.18.1.2 and EC 1.19.1.1);
b) a gene encoding a pyruvate-flavodoxin/ferredoxin oxidoreductase (EC 1.2. 7);
c) a gene encoding a flavodoxin;
d) a gene encoding a ferredoxin; and
e) a gene encoding a flavodoxin and a ferredoxin-NADP reductase;

wherein said one or more genes are operably-linked to a non-native promoter capable of enhancing expression of said one or more gene in said bacterium, and wherein said one or more gene may be a native gene or a transgene.

17. Use of a genetically modified bacterium according to claim 16 for enhanced production of any one of biotin, lipoic acid or thiamine.

18. A method for producing any one of biotin, lipoic acid and thiamine according to any one of claims 9 to 12, wherein said genetically modified bacterium further comprises one or more genes selected from the group:

a) a gene encoding a flavodoxin/ferredoxin-NADP reductase (EC:1.18.1.2 and EC 1.19.1.1);
b) a gene encoding a pyruvate-flavodoxin/ferredoxin oxidoreductase (EC 1.2.7);
c) a gene encoding a flavodoxin;
d) a gene encoding a ferredoxin; and
e) a gene encoding a flavodoxin and a ferredoxin-NADP reductase; wherein said one or more genes are operably-linked to a non• native promoter capable of enhancing expression of said one or more gene in said bacterium, and wherein said one or more gene may be a native gene or a transgene.

Patentansprüche

1. Gentechnisch modifiziertes Bakterium; wobei das Bakterium eine verstärkte Produktion von Biotin oder Liponsäure oder Thiamin aufweist; wobei das Bakterium Folgendes umfasst:

a) ein genetisch modifiziertes endogenes iscR-Gen, das für ein mutiertes IscR-Polypeptid codiert, wobei die Aminosäuresequenz des mutierten IscR-Polypeptids wenigstens 80 % Aminosäuresequenzidentität mit einer Sequenz aufweist, die aus der Gruppe ausgewählt ist, die aus SEQ ID NO: 2, 4, 6, 8, 10, 12 und 14 besteht, und wobei die Aminosäuresequenz wenigstens eine Aminosäuresubstitution aufweist, die aus der Gruppe ausgewählt ist, die aus Folgendem besteht:

i) L15X, C92X, C98X, C104X und H107X; wobei X eine beliebige andere Aminosäure als der entsprechende Aminosäurerückstand in SEQ ID NO: 2, 4, 6, 8, 10, 12 und 14 ist;

und
b) wenigstens ein Transgen, das für ein Polypeptid codiert, das aus der Gruppe ausgewählt ist, die aus Folgendem besteht:

ii) einem Polypeptid, das Biotin-Synthase-Aktivität (EC 2.8.1.6) aufweist;
iii) einem Polypeptid, das Liponsäure-Synthase-Aktivität (EC 2.8.1.8) aufweist;
iv) einem Polypeptid, das HMP-P-Synthase-Aktivität (EC 4.1.99.17) aufweist; und
v) einem Polypeptid, das Tyrosin-Lyase-Aktivität (EC 4.1.99.19) aufweist.

2. Genetisch modifiziertes Bakterium für die verstärkte Produktion von Biotin oder Liponsäure oder Thiamin nach Anspruch 1, wobei die wenigstens eine Aminosäuresubstitution in dem mutierten IscR-Polypeptid aus der Gruppe ausgewählt ist, die aus Folgendem besteht:

a) L15X, wobei X F, Y, M oder W ist;

b) C92X, wobei X Y, A, M, F oder W ist;
c) C98X, wobei X A, V, I, L, F oder W ist;
d) C104X, wobei X A, V, I, L, F oder W ist; und
e) H107X; wobei X A, Y, V, I oder L ist.

3. Genetisch modifiziertes Bakterium nach Anspruch 1 oder 2, wobei das wenigstens eine Transgen für ein Polypeptid codiert, das Biotin-Synthase-Aktivität (EC 2.8.1.6) aufweist, ferner umfassend zusätzliche Transgene, die für ein oder mehrere Polypeptide codieren, die aus der Gruppe ausgewählt sind, die aus Folgendem besteht:

a) einem Polypeptid, das SAM(S-Adenosylmethionin)-abhängige Methyl-Transferase-Aktivität (EC 2.1.1.197) aufweist;
b) einem Polypeptid, das 7-Keto-8-aminopelargonsäure(7-keto-8-aminopelargonic acid - KAPA)-Synthase-Aktivität (EC 2.3.1.47) aufweist;
c) einem Polypeptid, das 7,8-Diaminopelargonsäure-(7,8-Diaminopleargonic Acid - DAPA)-Synthase-Aktivität (EC: 2.6.1.62 oder EC 2.6.1.105) aufweist;
d) einem Polypeptid, das Dethiobiotin(DTB)-Synthetase-Aktivität (EC 6.3.3.3) aufweist;
e) einem Polypeptid, das Pimeloyl-[Acyl-Trägerprotein]-Methylester-Esterase (EC 3.1.1.85) aufweist; und
f) einem Polypeptid, das 6-Carboxyhexanoat-CoA-Ligase-Aktivität (EC 6.2.1.14) aufweist;

wobei das Bakterium für die verstärkte Produktion von Biotin ist.

4. Genetisch modifiziertes Bakterium nach Anspruch 1 oder 2, wobei das wenigstens eine Transgen für ein Polypeptid codiert, das Liponsäure-Synthase-Aktivität (EC 2.8.1.8) aufweist, ferner umfassend zusätzliche Transgene, die für ein oder mehrere Polypeptide codieren, die aus der Gruppe ausgewählt sind, die aus Folgendem besteht:

a) einem Polypeptid, das Octanoyl-Transferase-Aktivität (EC 2.3.1.181) aufweist;
b) einem Polypeptid, umfassend die Dihydrolipoyllysinrückstand-Acetyl-Transferasekomponente von Pyruvat-Dehydrogenase (EC 2.3.1.12); und
c) einem Polypeptid, das Lipoat-Protein-Ligase-A-Aktivität (EC: 6.3.1.20) aufweist; wobei das Bakterium für die verstärkte Produktion von Liponsäure ist.

5. Genetisch modifiziertes Bakterium nach Anspruch 1 oder 2, wobei das wenigstens eine Transgen für ein Polypeptid codiert, das HMP-P-Synthase-Aktivität (EC 4.1.99.17) aufweist, und/oder ein Transgen, das für ein Polypeptid codiert, das Tyrosin-Lyase-Aktivität (EC 4.1.99.19) aufweist, ferner umfassend zusätzliche Transgene, die für ein oder mehrere Polypeptide codieren, die aus der Gruppe ausgewählt sind, die aus Folgendem besteht:

a) einem ThiF-Polypeptid, das ThiS-Adenylyl-Transferase-Aktivität (EC 2.7.7.73) aufweist;
b) einem ThiE-Polypeptid, das Thiaminphosphat-Synthase-Aktivität (EC 2.5.1.3) aufweist;
c) einem ThiG-Polypeptid, das Thiazol-Synthase-Aktivität (EC 2.8.1.10) aufweist;
d) einem ThiD-Polypeptid, das Phosphohydroxymethylpyrimidin-Kinase-Aktivität (EC 2.7.4.7) aufweist;
e) einem ThiO-Polypeptid, das Glycin-Oxidase-Aktivität (EC 1.4.3.19) aufweist;
f) einem ThiS-Polypeptid, das Schwefelträgerprotein-Aktivität aufweist;
g) einem ThiM-Polypeptid, das Hydroxyethylthiazol-Kinase-Aktivität (EC 2.7.1.50) aufweist; und
h) einem Polypeptid, das Thiaminmonophosphat-Phosphatase-Aktivität (EC 3.1.3.-) aufweist;

wobei das Bakterium für die verstärkte Produktion von Thiamin ist.

6. Genetisch modifiziertes Bakterium nach Anspruch 5, wobei das Bakterium die zusätzlichen Transgene umfasst, die für Folgendes codieren:

a) ein ThiC-Polypeptid, das HMP-P-Synthase-Aktivität (EC 4.1.99.17) aufweist;
b) ein ThiH-Polypeptid, das Tyrosin-Lyase-Aktivität (EC 4.1.99.19) aufweist, oder ein ThiO-Polypeptid, das Glycin-Oxidase-Aktivität (EC 1.4.3.19) aufweist;
c) ein ThiF-Polypeptid, das ThiS-Adenylyl-Transferase-Aktivität (EC 2.7.7.73) aufweist;
d) ein ThiE-Polypeptid, das Thiaminphosphat-Synthase-Aktivität (EC 2.5.1.3) aufweist;
e) ein ThiG-Polypeptid, das Thiazol-Synthase-Aktivität (EC 2.8.1.10) aufweist;
f) ein ThiD-Polypeptid, das Phosphohydroxymethylpyrimidin-Kinase-Aktivität (EC 2.7.4.7) aufweist;
g) ein ThiS-Polypeptid, das Schwefelträgerprotein-Aktivität aufweist; und

h) ein Polypeptid, das Thiaminmonophosphatphosphatase(EC 3.1.3.-)-Aktivität aufweist.

7. Genetisch modifiziertes Bakterium nach einem der Ansprüche 1 bis 6, wobei das wenigstens eine Transgen und das eine oder die mehreren zusätzlichen Transgene an einen konstitutiven Promotor wirkgebunden sind.

8. Genetisch modifiziertes Bakterium nach einem der Ansprüche 1 bis 7, wobei das Bakterium eine Bakteriengattung ist, die aus der Gruppe ausgewählt ist, die aus Escherichia, Bacillus, Brevibacterium, Burkholderia, Campylobacter, Corynebacterium, Pseudomonas, Serratia, Lactobacillus, Lactococcus, Acinetobacter, Pseudomonas und Acetobacter besteht.

9. Verfahren zum Produzieren von Biotin, umfassend die folgenden Schritte:

a) Einführen eines genetisch modifizierten Bakteriums nach einem der Ansprüche 1-3, 7 und 8, wobei das wenigstens eine Transgen für ein Polypeptid codiert, das Biotin-Synthase-Aktivität (EC 2.8.1.6) aufweist, in ein Wachstumsmedium, um eine Kultur zu produzieren;
b) Kultivieren der Kultur; und
c) Zurückgewinnen von Biotin, das durch die Kultur produziert wird, und optional Reinigen des zurückgewonnenen Biotins.

10. Verfahren zum Produzieren von Liponsäure, umfassend die folgenden Schritte:

a) Einführen eines genetisch modifizierten Bakteriums nach einem der Ansprüche 1, 2, 4, 7 und 8, wobei das wenigstens eine Transgen für ein Polypeptid codiert, das Liponsäure-Synthase-Aktivität (EC 2.8.1.6) aufweist, in ein Wachstumsmedium, um eine Kultur zu produzieren;
b) Kultivieren der Kultur; und
c) Zurückgewinnen von Liponsäure, die durch die Kultur produziert wird, und optional Reinigen der zurückgewonnenen Liponsäure.

11. Verfahren zum Produzieren von Thiamin, umfassend die folgenden Schritte:

a) Einführen eines genetisch modifizierten Bakteriums nach einem der Ansprüche 1, 2, 5-8, wobei das wenigstens eine Transgen für ein Polypeptid codiert, das HMP-P-Synthase-Aktivität (EC 4.1.99.17) aufweist, und/oder eines Transgens, das für ein Polypeptid codiert, das Tyrosin-Lyase-Aktivität (EC 4.1.99.19) aufweist, in ein Wachstumsmedium, um eine Kultur zu produzieren;
b) Kultivieren der Kultur; und
c) Zurückgewinnen von Thiamin, das durch die Kultur produziert wird, und optional Reinigen des zurückgewonnenen Thiamins.

12. Verfahren zum Produzieren von Biotin, Liponsäure oder Thiamin nach einem der Ansprüche 9 bis 11, wobei das Wachstumsmedium eine Kohlenstoffquelle umfasst, die aus Glucose, Maltose, Galactose, Fructose, Saccharose, Arabinose, Xylose, Raffinose, Mannose und Lactose oder einer beliebigen Kombination davon ausgewählt ist.

13. Verwendung eines genetisch modifizierten Gens, das für ein mutiertes iscR-Polypeptid codiert, um die Produktion von Biotin, Liponsäure oder Thiamin in einem genetisch modifizierten Bakterium zu verstärken, wobei das Bakterium wenigstens ein Transgen umfasst und exprimiert, das für ein Polypeptid codiert, das aus der Gruppe ausgewählt ist, die aus Folgendem besteht:

i) einem Polypeptid, das Biotin-Synthase-Aktivität (EC 2.8.1.6) aufweist;
ii) ii) einem Polypeptid, das Liponsäure-Synthase-Aktivität (EC 2.8.1.8) aufweist;
iii) iii) einem Polypeptid, das HMP-P-Synthase-Aktivität (EC 4.1.99.17) aufweist; und
iv) iv) einem Polypeptid, das Tyrosin-Lyase-Aktivität (EC 4.1.99.19) aufweist; und

wobei das genetisch modifizierte Gen ein endogenes iscR-Gen ist, das für ein mutiertes IscR-Polypeptid codiert, wobei die Aminosäuresequenz des mutierten IscR-Polypeptids wenigstens 80 % Aminosäuresequenzidentität mit SEQ ID NO: 2, 4, 6, 8, 10, 12 und 14 aufweist, und wobei die Aminosäuresequenz wenigstens eine Aminosäuresubstitution aufweist, die aus der Gruppe ausgewählt ist, die aus Folgendem besteht: L15X, C92X, C98X, C104X und H107X; wobei X eine andere Aminosäure als der entsprechende Aminosäurerückstand in SEQ ID NO 2, 4, 6, 8, 10, 12 und 14 ist.

14. Verwendung eines genetisch modifizierten Gens, das für ein mutiertes iscR-Polypeptid codiert, um die Produktion von Biotin, Liponsäure oder Thiamin in einem genetisch modifizierten Bakterium nach Anspruch 13 zu verstärken, wobei die wenigstens eine Aminosäuresubstitution in dem mutierten IscR-Polypeptid aus der Gruppe ausgewählt ist, die aus Folgendem besteht:

    a) L15X, wobei X F, Y, M oder W ist;
    b) C92X, wobei X Y, A, M, F oder W ist;
    c) C98X, wobei X A, V, I, L, F oder W ist; und
    d) C104X, wobei X A, V, I, L, F oder W ist; und
    e) H107X; wobei X A, Y, V, I oder L ist.

15. Verwendung eines genetisch modifizierten Bakteriums nach einem der Ansprüche 1-8 für die verstärkte Produktion von Biotin, Liponsäure oder Thiamin.

16. Genetisch modifiziertes Bakterium nach einem der Ansprüche 1-8, wobei das Bakterium ferner ein oder mehrere Gene umfasst, die aus der folgenden Gruppe ausgewählt sind:

    a) ein Gen, das für eine Flavodoxin-/Ferredoxin-NADP-Reduktase (EC: 1.18.1.2 und EC 1.19.1.1) codiert;
    b) ein Gen, das für eine Pyruvat-Flavodoxin-/Ferredoxin-Oxidoreduktase (EC 1.2.7) codiert;
    c) ein Gen, das für ein Flavodoxin codiert;
    d) ein Gen, das für ein Ferredoxin codiert; und
    e) ein Gen, das für ein Flavodoxin und eine Ferredoxin-NADP-Reduktase codiert;

    wobei das eine oder die mehreren Gene an einen nicht-nativen Promotor wirkgebunden sind, der in der Lage ist, eine Expression des einen oder der mehreren Gene in dem Bakterium zu verstärken, und wobei das eine oder die mehreren Gen ein natives Gen oder ein Transgen sein können.

17. Verwendung eines genetisch modifizierten Bakteriums nach Anspruch 16 für die verstärkte Produktion von Biotin, Liponsäure oder Thiamin.

18. Verfahren zum Produzieren von Biotin, Liponsäure oder Thiamin nach einem der Ansprüche 9 bis 12, wobei das genetisch modifizierte Bakterium ferner ein oder mehrere Gene umfasst, die aus der folgenden Gruppe ausgewählt sind:

    a) ein Gen, das für eine Flavodoxin-/Ferredoxin-NADP-Reduktase (EC: 1.18.1.2 und EC 1.19.1.1) codiert;
    b) ein Gen, das für eine Pyruvat-Flavodoxin-/Ferredoxin-Oxidoreduktase (EC 1.2.7) codiert;
    c) ein Gen, das für ein Flavodoxin codiert;
    d) ein Gen, das für ein Ferredoxin codiert; und
    e) ein Gen, das für ein Flavodoxin und eine Ferredoxin-NADP-Reduktase codiert;

    wobei das eine oder die mehreren Gene an einen nicht-nativen Promotor wirkgebunden sind, der in der Lage ist, die Expression des einen oder der mehreren Gene in dem Bakterium zu verstärken, und wobei das eine oder die mehreren Gene ein natives Gen oder ein Transgen sein können.

Revendications

1. Bactérie génétiquement modifiée ; la bactérie ayant une production améliorée de biotine ou d'acide lipoïque ou de thiamine ; ladite bactérie comprenant :

    a) le gène iscR endogène génétiquement modifié codant pour un polypeptide IscR mutant, la séquence d'acides aminés dudit polypeptide IscR mutant ayant au moins 80 % d'identité de séquence d'acides aminés avec une séquence choisie dans le groupe constitué de SEQ ID N° : 2, 4, 6, 8, 10, 12 et 14, et ladite séquence d'acides aminés ayant au moins une substitution d'acides aminés choisie dans le groupe constitué de :

        i) L15X, C92X, C98X, C104X et H107X ; où X représente n'importe quel acide aminé autre que le résidu d'acide aminé correspondant dans SEQ ID N° : 2, 4, 6, 8, 10, 12 et 14 ;

et

b) au moins un transgène codant pour un polypeptide choisi dans le groupe constitué :

ii) d'un polypeptide ayant une activité de biotine synthase (EC 2.8.1.6) ;
iii) d'un polypeptide ayant une activité d'acide lipoïque synthase (EC 2.8.1.8) ;
iv) d'un polypeptide ayant une activité de HMP-P synthase (EC 4.1.99.17) ; et
v) d'un polypeptide ayant une activité de tyrosine lyase (EC 4.1.99.19).

2. Bactérie génétiquement modifiée pour une production améliorée de biotine ou d'acide lipoïque ou de thiamine selon la revendication 1, dans laquelle ladite au moins une substitution d'acides aminés dans ledit polypeptide IscR mutant est choisie dans le groupe constitué de :

a) L15X, où X représente l'un quelconque parmi F, Y, M et W ;
b) C92X, où X représente l'un quelconque parmi Y, A, M, F et W ;
c) C98X, où X représente l'un quelconque parmi A, V, I, L, F et W ;
d) C104X, où X représente l'un quelconque parmi A, V, I, L, F et W ; et
e) H107X ; où X représente l'un quelconque parmi A, Y, V, I et L.

3. Bactérie génétiquement modifiée selon les revendications 1 ou 2, dans laquelle ledit au moins un transgène code pour un polypeptide ayant une activité de biotine synthase (EC 2.8.1.6), comprenant en outre des transgènes supplémentaires codant pour un ou plusieurs polypeptides choisis dans le groupe constitué :

a) d'un polypeptide ayant une activité de méthyltransférase SAM (S-adénosylméthionine)-dépendante (EC 2.1.1.197) ;
b) d'un polypeptide ayant une activité d'acide 7-céto-8-aminopélargonique (KAPA) synthase (EC 2.3.1.47) ;
c) d'un polypeptide ayant une activité d'acide 7,8-diaminopélargonique (DAPA) synthase (EC : 2.6.1.62 ou EC 2.6.1.105) ;
d) d'un polypeptide ayant une activité de déthiobiotine (DTB) synthétase (EC 6.3.3.3) ;
e) d'un polypeptide ayant la pimeloyl-[protéine porteuse d'acyle] methyl ester estérase (EC 3.1.1.85) ; et
f) d'un polypeptide ayant une activité de 6-carboxyhexanoate-CoA ligase (EC 6.2.1.14) ; ladite bactérie étant destinée à une production améliorée de biotine.

4. Bactérie génétiquement modifiée selon la revendication 1 ou 2, dans laquelle ledit au moins un transgène code pour un polypeptide ayant une activité d'acide lipoïque synthase (EC 2.8.1.8), comprenant en outre des transgènes supplémentaires codant pour un ou plusieurs polypeptides choisis dans le groupe constitué :

a) d'un polypeptide ayant une activité d'octanoyltransférase (EC 2.3.1.181) ;
b) d'un polypeptide comprenant le composant dihydrolipoyllysine-résidus acétyltransférase de la pyruvate déshydrogénase (EC 2.3.1.12) ; et
c) d'un polypeptide ayant une activité de lipoate-protéine ligase A (EC : 6.3.1.20) ;

ladite bactérie étant destinée à une production améliorée d'acide lipoïque.

5. Bactérie génétiquement modifiée selon la revendication 1 ou 2, dans laquelle ledit au moins un transgène code pour un polypeptide ayant une activité de HMP-P synthase (EC 4.1.99.17), et/ou un transgène codant pour un polypeptide ayant une activité de tyrosine lyase (EC 4.1.99.19), comprenant en outre des transgènes supplémentaires codant pour un ou plusieurs polypeptides choisis dans le groupe constitué :

a) d'un polypeptide ThiF ayant une activité d'adénylyltransférase ThiS (EC 2.7.7.73) ;
b) d'un polypeptide ThiE ayant une activité de thiamine phosphate synthase (EC 2.5.1.3) ;
c) d'un polypeptide ThiG ayant une activité de thiazole synthase (EC 2.8.1.10) ;
d) d'un polypeptide ThiD ayant une activité de phosphohydroxyméthylpyrimidine kinase (EC 2.7.4.7) ;
e) d'un polypeptide ThiO ayant une activité de glycine oxydase (EC 1.4.3.19) ;
f) d'un polypeptide ThiS ayant une activité de protéine porteuse de soufre ;
g) d'un polypeptide ThiM ayant une activité d'hydroxyéthylthiazole kinase (EC 2.7.1.50) ; et
h) d'un polypeptide ayant une activité de thiamine monophosphate phosphatase (EC 3.1.3.-);

ladite bactérie étant destinée à une production améliorée de thiamine.

6. Bactérie génétiquement modifiée selon la revendication 5, dans laquelle ladite bactérie comprend les transgènes supplémentaires codant pour :

a) un polypeptide ThiC ayant une activité de HMP-P synthase (EC 4.1.99.17);
b) un polypeptide ThiH ayant une activité de tyrosine lyase (EC 4.1.99.19) ou un polypeptide ThiO ayant une activité de glycine oxydase (EC 1.4.3.19) ;
c) un polypeptide ThiF ayant une activité de ThiS adénylyltransférase (EC 2.7.7.73) ;
d) un polypeptide ThiE ayant une activité de thiamine phosphate synthase (EC 2.5.1.3) ;
e) un polypeptide ThiG ayant une activité de thiazole synthase (EC 2.8.1.10) ;
f) un polypeptide ThiD ayant une activité de phosphohydroxyméthylpyrimidine kinase (EC 2.7.4.7) ;
g) un polypeptide ThiS ayant une activité de protéine porteuse de soufre ; et
h) un polypeptide ayant une activité de thiamine monophosphate phosphatase (EC 3.1.3.-).

7. Bactérie génétiquement modifiée selon l'une quelconque des revendications 1 à 6, dans laquelle ledit au moins un transgène et ledit un ou plusieurs transgènes supplémentaires sont liés de manière fonctionnelle à un promoteur constitutif.

8. Bactérie génétiquement modifiée selon l'une quelconque des revendications 1 à 7, dans laquelle ladite bactérie est un genre de bactérie choisie dans le groupe constitué d'Escherichia, Bacillus, Brevibacterium, Burkholderia, Campylobacter, Corynebacterium, Pseudomonas, Serratia, Lactobacillus, Lactococcus, Acinetobacter, Pseudomonas et Acetobacter.

9. Procédé de production de biotine, comprenant les étapes consistant à :

a) introduire une bactérie génétiquement modifiée selon l'une quelconque des revendications 1 à 3, 7 et 8, ledit au moins un transgène codant pour un polypeptide ayant une activité de biotine synthase (EC 2.8.1.6), dans un milieu de croissance pour produire une culture ;
b) cultiver la culture ; et
c) récupérer la biotine produite par ladite culture, et éventuellement purifier la biotine récupérée.

10. Procédé de production d'acide lipoïque comprenant les étapes consistant à :

a) introduire une bactérie génétiquement modifiée selon l'une quelconque des revendications 1, 2, 4, 7 et 8, ledit au moins un transgène codant pour un polypeptide ayant une activité d'acide lipoïque synthase (EC 2.8.1.6), dans un milieu de croissance pour produire une culture ;
b) cultiver la culture ; et
c) récupérer l'acide lipoïque produit par ladite culture, et éventuellement purifier l'acide lipoïque récupéré.

11. Procédé de production de thiamine comprenant les étapes consistant à :

a) introduire une bactérie génétiquement modifiée selon l'une quelconque des revendications 1, 2, 5 à 8, ledit au moins un transgène codant pour un polypeptide ayant une activité de HMP-P synthase (EC 4.1.99.17), et/ou un transgène codant pour un polypeptide ayant une activité de tyrosine lyase (EC 4.1.99.19) dans un milieu de croissance pour produire une culture ;
b) cultiver la culture ; et
c) récupérer la thiamine produite par ladite culture, et éventuellement purifier la thiamine récupérée.

12. Procédé de production de l'une quelconque parmi la biotine, l'acide lipoïque et la thiamine selon l'une quelconque des revendications 9 à 11, dans lequel le milieu de croissance comprend une source de carbone choisie parmi le glucose, le maltose, le galactose, le fructose, le saccharose, l'arabinose, le xylose, le raffinose, le mannose et le lactose, ou toute combinaison de ceux-ci.

13. Utilisation d'un gène génétiquement modifié codant pour un polypeptide iscR mutant pour améliorer la production de l'une quelconque parmi la biotine, l'acide lipoïque ou la thiamine dans une bactérie génétiquement modifiée, ladite bactérie comprenant et exprimant au moins un transgène codant pour un polypeptide choisi dans le groupe constitué :

i) d'un polypeptide ayant une activité de biotine synthase (EC 2.8.1.6) ;

ii) d'un polypeptide ayant une activité d'acide lipoïque synthase (EC 2.8.1.8) ;
iii) d'un polypeptide ayant une activité de HMP-P synthase (EC 4.1.99.17) ; et
iv) d'un polypeptide ayant une activité de tyrosine lyase (EC 4.1.99.19) ; et

ledit gène génétiquement modifié étant un gène iscR endogène codant pour un polypeptide IscR mutant, la séquence d'acides aminés dudit polypeptide IscR mutant ayant au moins 80 % d'identité de séquence d'acides aminés avec SEQ ID N : 2, 4, 6, 8, 10, 12 et 14, et la séquence d'acides aminés ayant au moins une substitution d'acides aminés choisie dans le groupe constitué de : L15X, C92X, C98X, C104X et H107X ; X représentant tout acide aminé autre que le résidu d'acide aminé correspondant dans les SEQ ID N : 2, 4, 6, 8, 10, 12 et 14.

14. Utilisation d'un gène génétiquement modifié codant pour un polypeptide iscR mutant pour améliorer la production de l'une quelconque parmi la biotine, l'acide lipoïque ou la thiamine dans une bactérie génétiquement modifiée selon la revendication 13, ladite au moins une substitution d'acides aminés dans ledit polypeptide IscR mutant étant choisie dans le groupe constitué de :

a) L15X, où X représente l'un quelconque parmi F, Y, M et W ;
b) C92X, où X représente l'un quelconque parmi Y, A, M, F et W ;
c) C98X, où X représente l'un quelconque parmi A, V, I, L, F et W ; et
d) C104X, où X représente l'un quelconque parmi A, V, I, L, F et W ; et e. H107X ; où X représente l'un quelconque parmi A, Y, V, I et L.

15. Utilisation d'une bactérie génétiquement modifiée selon l'une quelconque des revendications 1 à 8 pour une production améliorée de l'une quelconque parmi la biotine, l'acide lipoïque ou la thiamine.

16. Bactérie génétiquement modifiée selon l'une quelconque des revendications 1 à 8, dans laquelle ladite bactérie comprend en outre un ou plusieurs gènes choisis dans le groupe :

a) d'un gène codant pour une flavodoxine/ferrédoxine-NADP réductase (EC : 1.18.1.2 et EC 1.19.1.1) ;
b) d'un gène codant pour une pyruvate-flavodoxine/ferrédoxine oxydoréductase (EC 1.2.7) ;
c) d'un gène codant pour une flavodoxine ;
d) d'un gène codant pour une ferrédoxine ; et
e) d'un gène codant pour une flavodoxine et une ferrédoxine-NADP réductase ;

lesdits un ou plusieurs gènes étant liés de manière fonctionnelle à un promoteur non endogène capable d'améliorer l'expression dudit un ou plusieurs gènes dans ladite bactérie, et ledit un ou plusieurs gènes pouvant être un gène endogène ou un transgène.

17. Utilisation d'une bactérie génétiquement modifiée selon la revendication 16 pour une production améliorée de l'une quelconque parmi la biotine, l'acide lipoïque ou la thiamine.

18. Procédé de production de l'une quelconque parmi la biotine, l'acide lipoïque et la thiamine selon l'une quelconque des revendications 9 à 12, dans lequel ladite bactérie génétiquement modifiée comprend en outre un ou plusieurs gènes choisis dans le groupe :

a) d'un gène codant pour une flavodoxine/ferrédoxine-NADP réductase (EC : 1.18.1.2 et EC 1.19.1.1) ;
b) d'un gène codant pour une pyruvate-flavodoxine/ferrédoxine oxydoréductase (EC 1.2.7) ;
c) d'un gène codant pour une flavodoxine ;
d) d'un gène codant pour une ferrédoxine ; et
e) d'un gène codant pour une flavodoxine et une ferrédoxine-NADP réductase ; lesdits un ou plusieurs gènes étant liés de manière fonctionnelle à un promoteur non endogène capable d'améliorer l'expression dudit un ou plusieurs gènes dans ladite bactérie, et ledit un ou plusieurs gènes pouvant être un gène endogène ou un transgène.

Figure 1

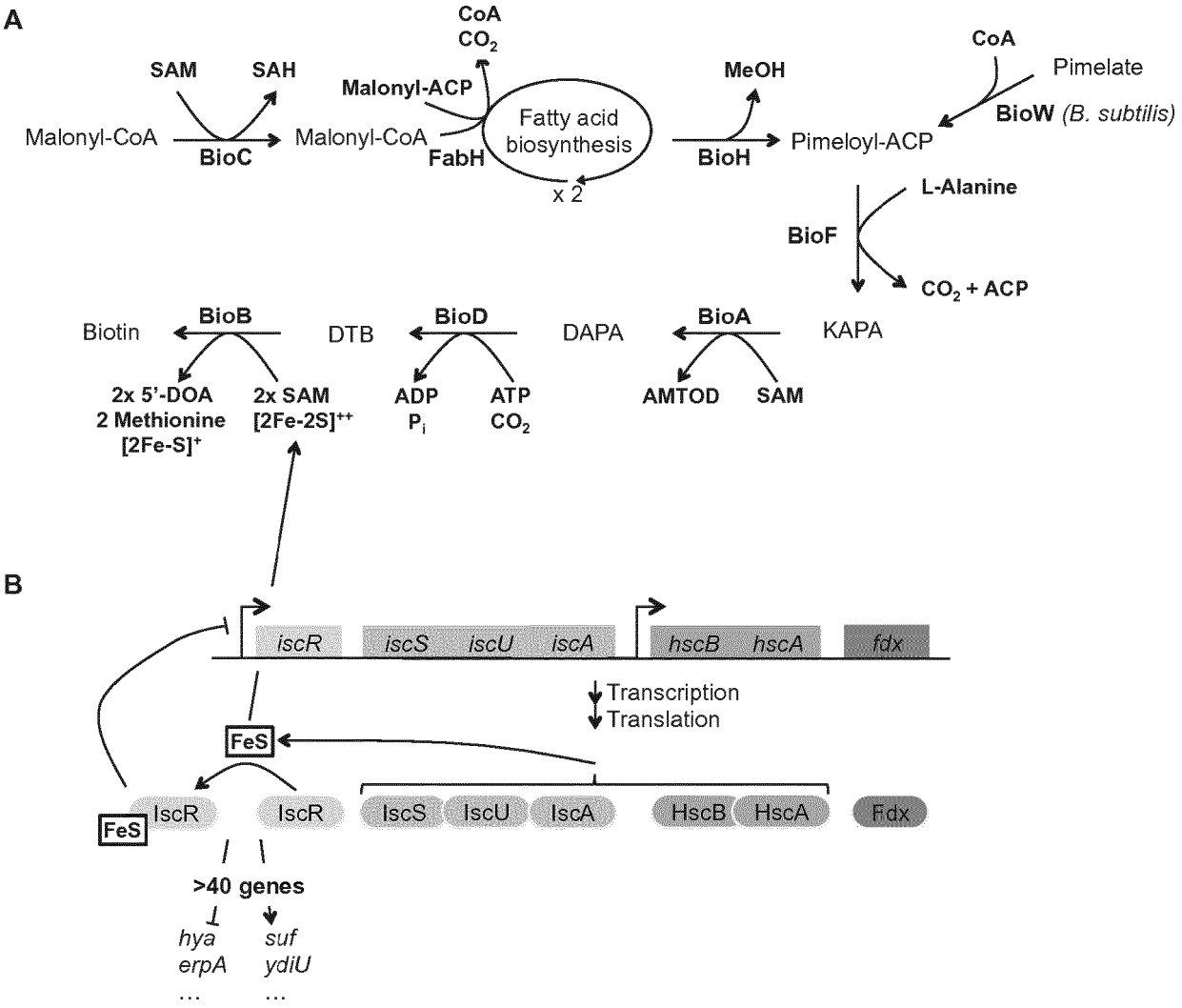

A
CoA
CO_2
SAM
SAH
Malonyl-ACP
MeOH
CoA
Pimelate
BioW (B. subtilis)
Malonyl-CoA
BioC
Malonyl-CoA
FabH
Fatty acid biosynthesis
x 2
BioH
Pimeloyl-ACP
L-Alanine
BioF
CO_2 + ACP
Biotin
BioB
DTB
BioD
DAPA
BioA
KAPA
2x 5'-DOA
2 Methionine
[2Fe-S]+
2x SAM
[2Fe-2S]++
ADP
P_i
ATP
CO_2
AMTOD
SAM
B
iscR
iscS
iscU
iscA
hscB
hscA
fdx
Transcription
Translation
FeS
FeS
IscR
IscR
IscS
IscU
IscA
HscB
HscA
Fdx
>40 genes
hya
erpA
...
suf
ydiU
...

Figure 2

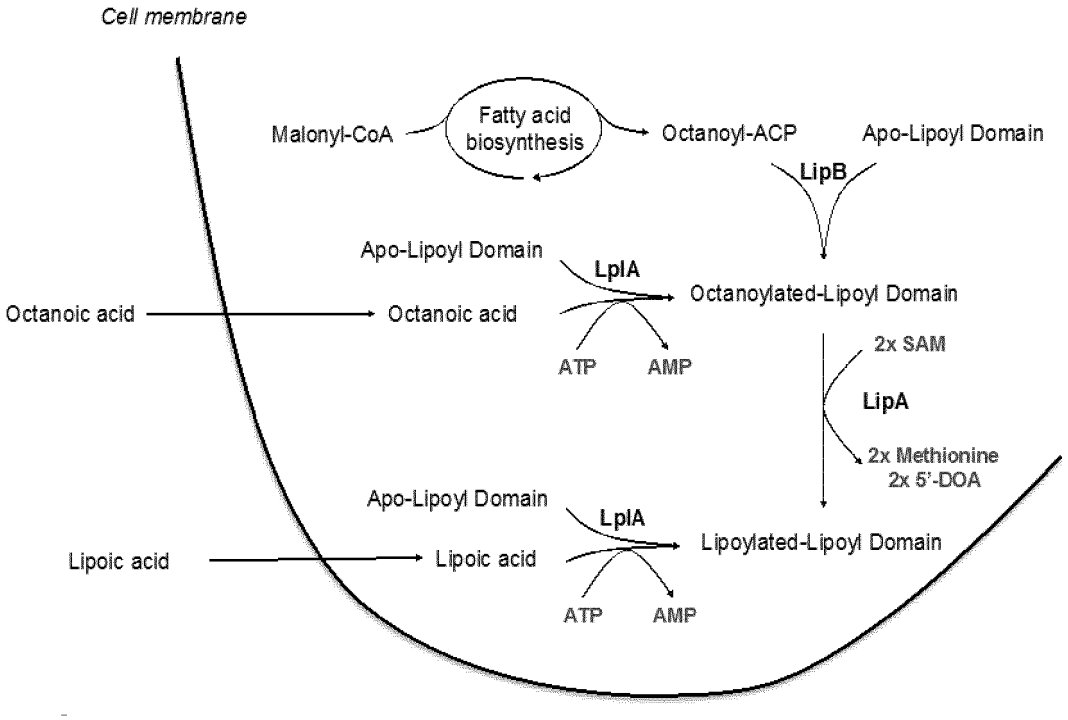

Cell membrane
Malonyl-CoA
Fatty acid biosynthesis
Octanoyl-ACP
Apo-Lipoyl Domain
LipB
Apo-Lipoyl Domain
LplA
Octanoic acid
Octanoic acid
Octanoylated-Lipoyl Domain
ATP
AMP
2x SAM
LipA
2x Methionine
2x 5'-DOA
Apo-Lipoyl Domain
LplA
Lipoic acid
Lipoic acid
Lipoylated-Lipoyl Domain
ATP
AMP

Figure 3

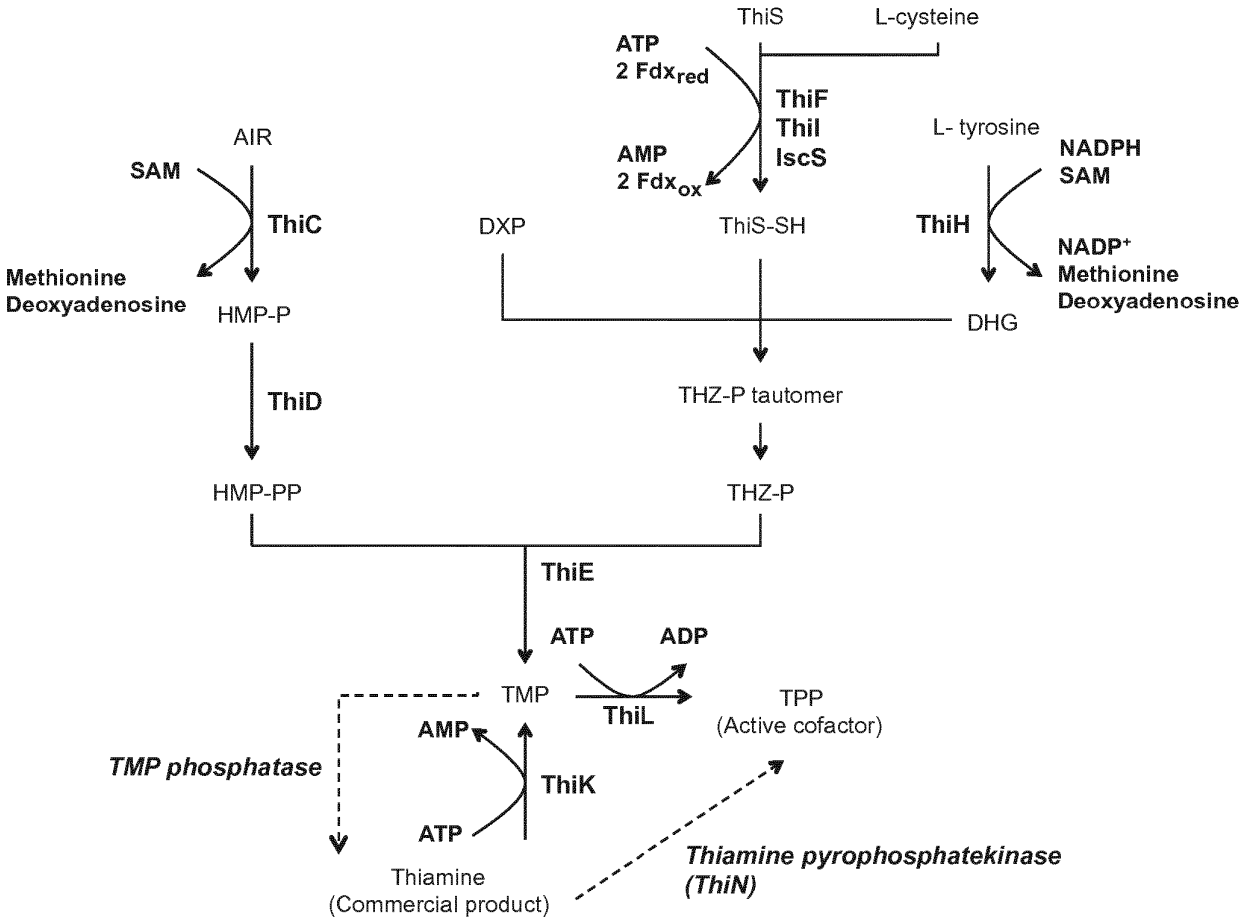

ThiS
L-cysteine
ATP
2 Fdx$_{red}$
ThiF
ThiI
IscS
AMP
2 Fdx$_{ox}$
L- tyrosine
NADPH
SAM
AIR
SAM
ThiC
Methionine
Deoxyadenosine
HMP-P
DXP
ThiS-SH
ThiH
NADP$^+$
Methionine
Deoxyadenosine
DHG
ThiD
THZ-P tautomer
HMP-PP
THZ-P
ThiE
ATP
ADP
TMP
ThiL
TPP
(Active cofactor)
AMP
TMP phosphatase
ThiK
ATP
Thiamine
(Commercial product)
Thiamine pyrophosphatekinase
(ThiN)

Figure 4

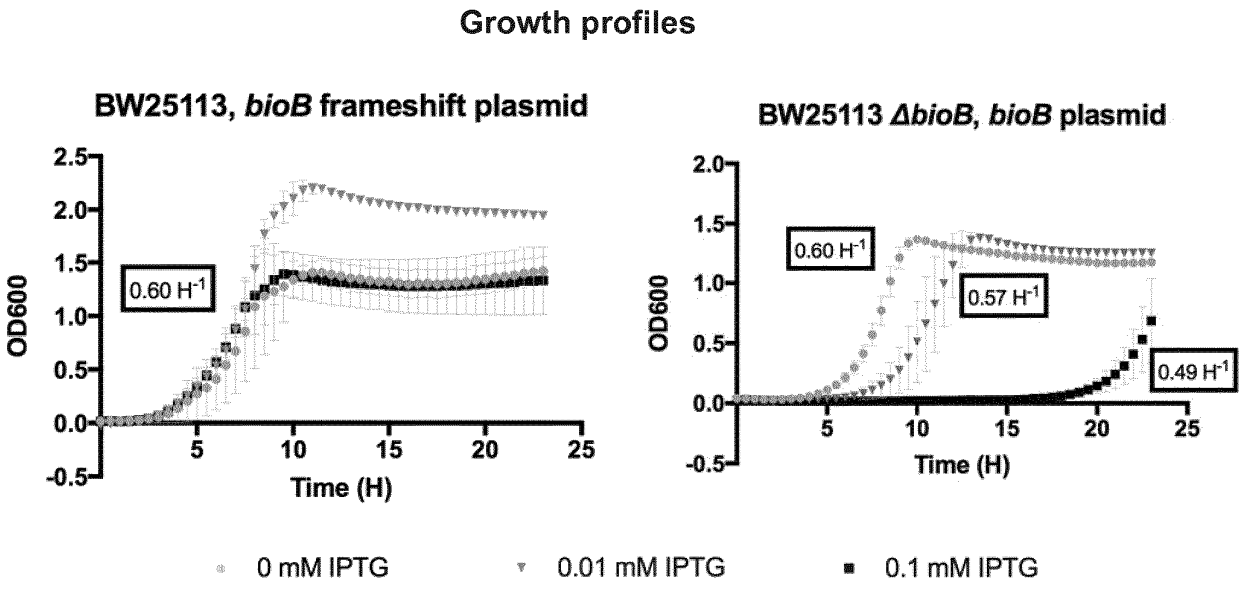


Figure 5

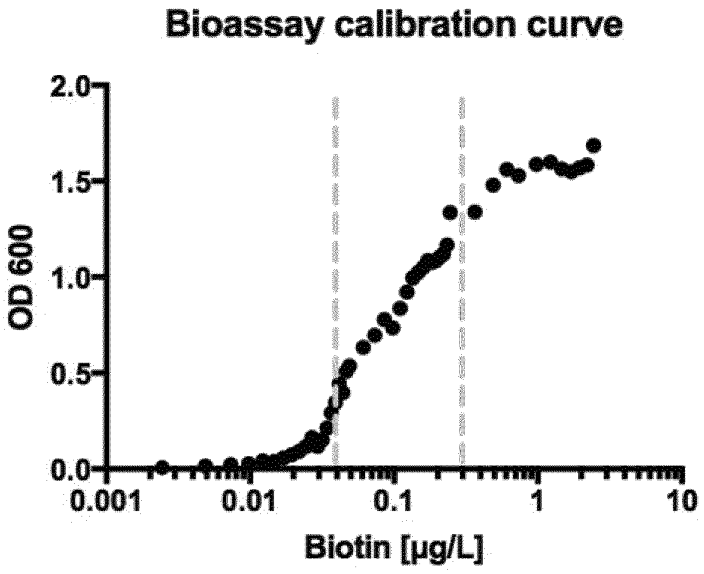


Figure 6

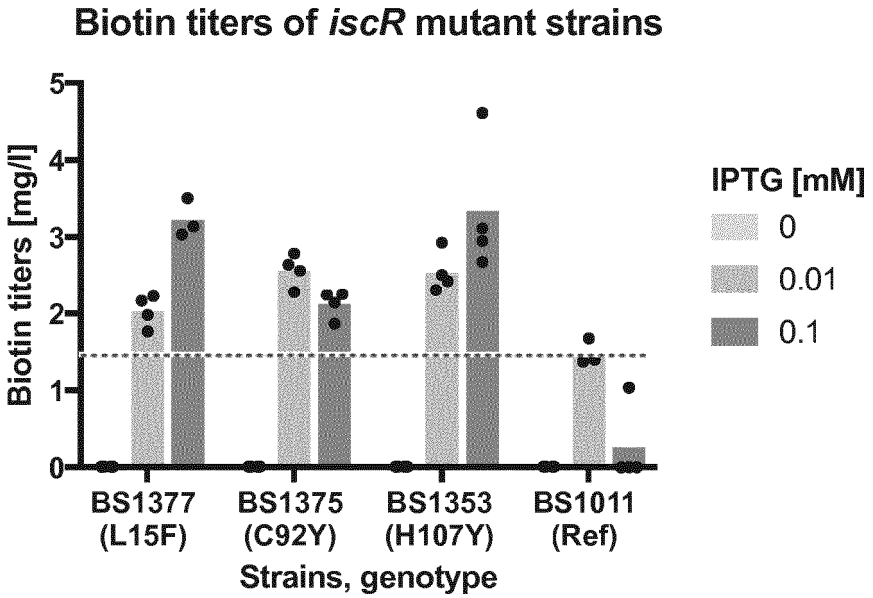

Biotin titers of *iscR* mutant strains
Biotin titers [mg/l]
5
4
3
2
1
0
BS1377 (L15F)
BS1375 (C92Y)
BS1353 (H107Y)
BS1011 (Ref)
Strains, genotype
IPTG [mM]
0
0.01
0.1

Figure 7

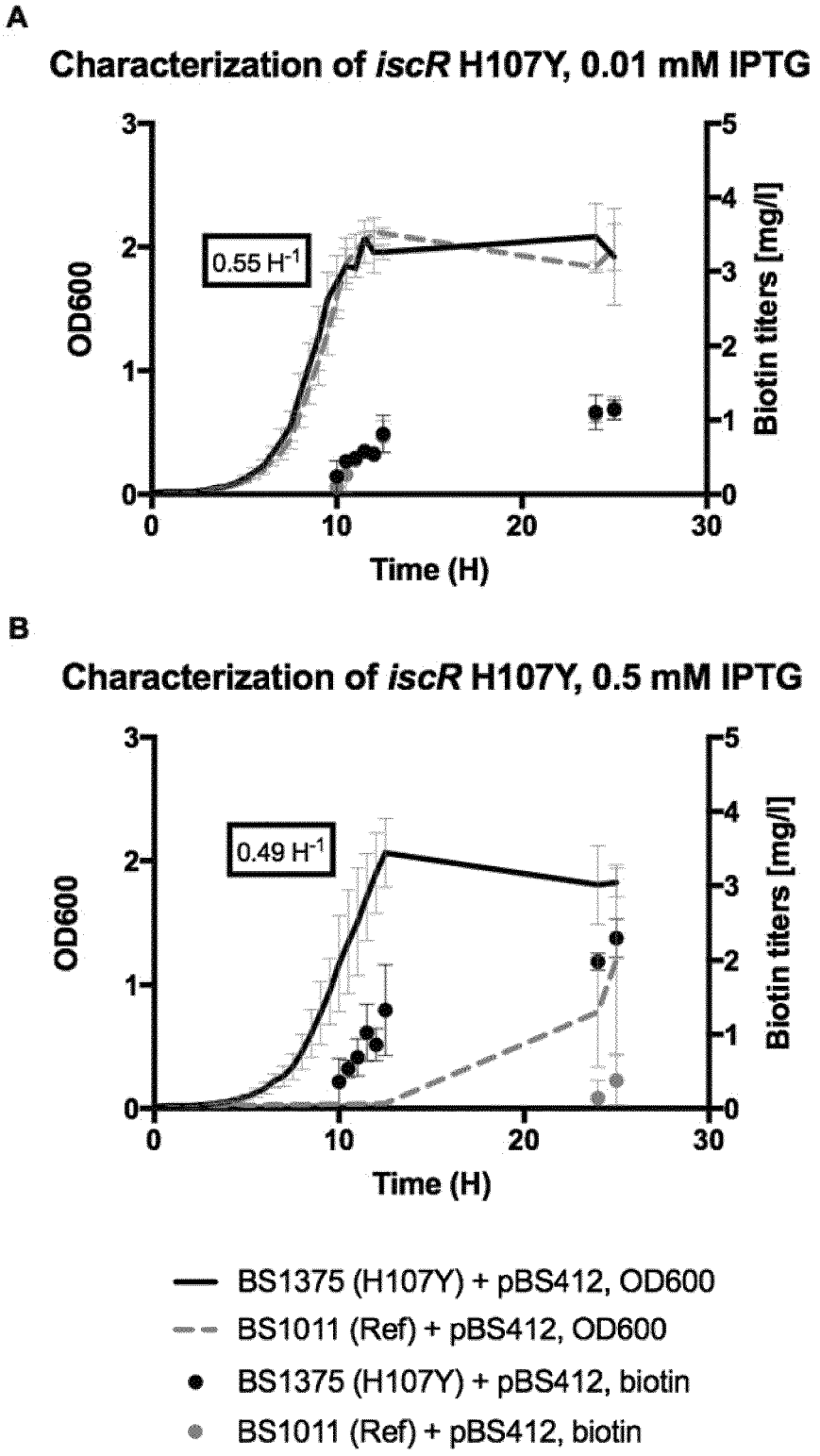
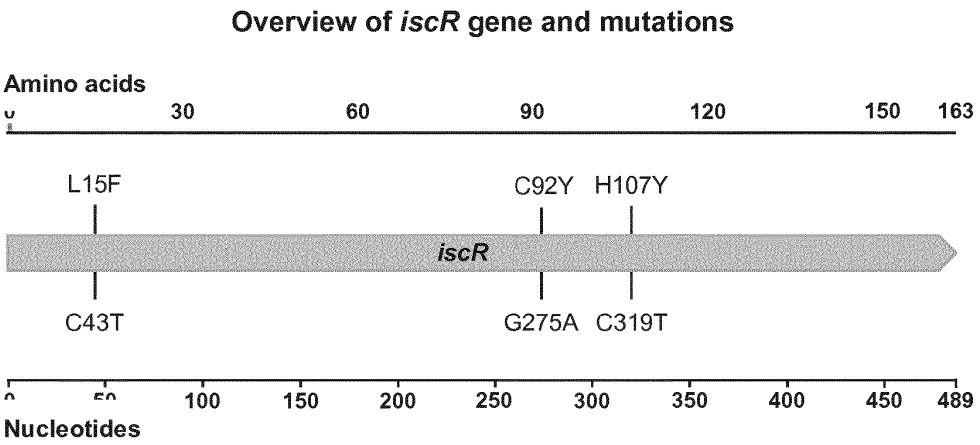

A
Characterization of iscR H107Y, 0.01 mM IPTG
OD600
Biotin titers [mg/l]
0.55 H-1
Time (H)
B
Characterization of iscR H107Y, 0.5 mM IPTG
OD600
Biotin titers [mg/l]
0.49 H-1
Time (H)
BS1375 (H107Y) + pBS412, OD600
BS1011 (Ref) + pBS412, OD600
BS1375 (H107Y) + pBS412, biotin
BS1011 (Ref) + pBS412, biotin

Figure 8



5

Figure 9

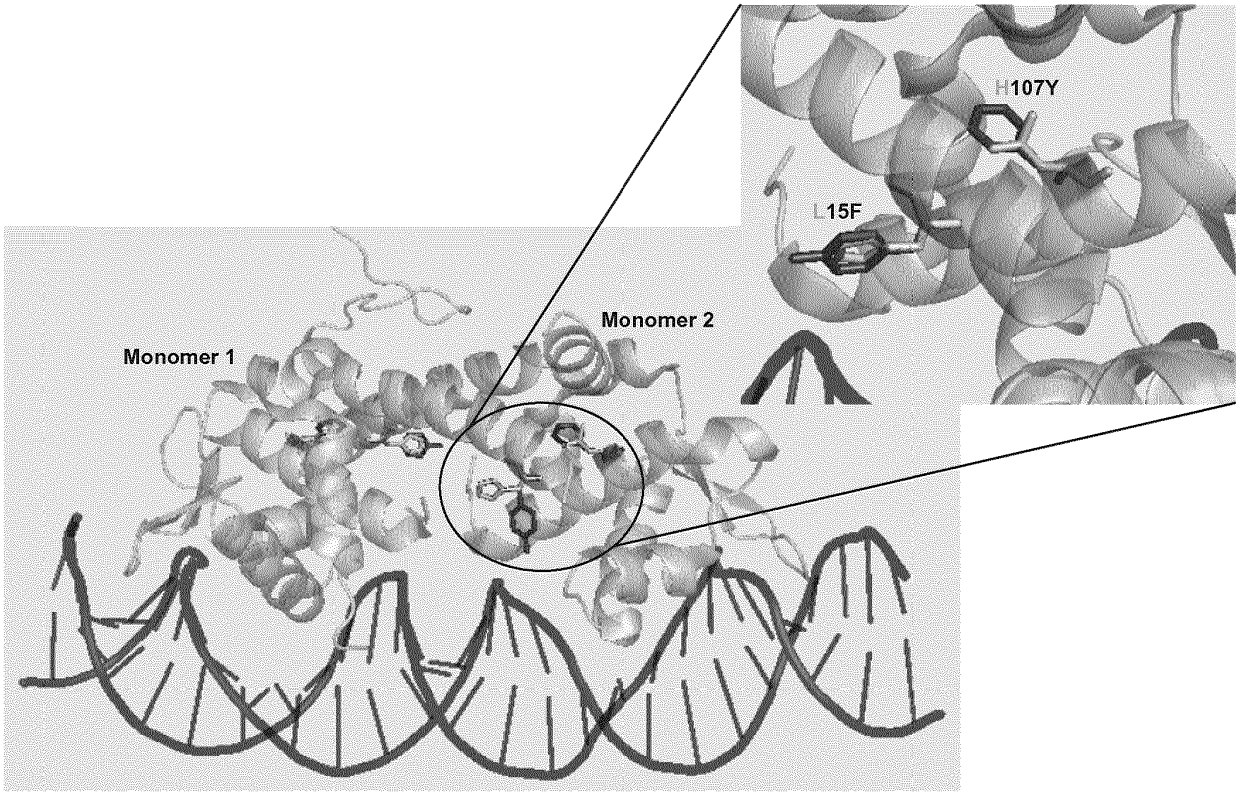
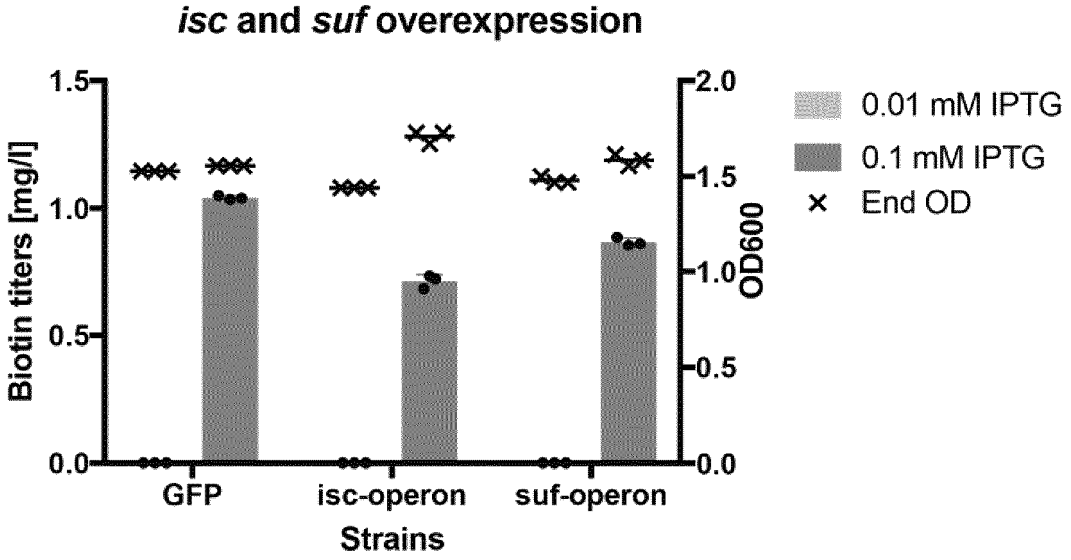
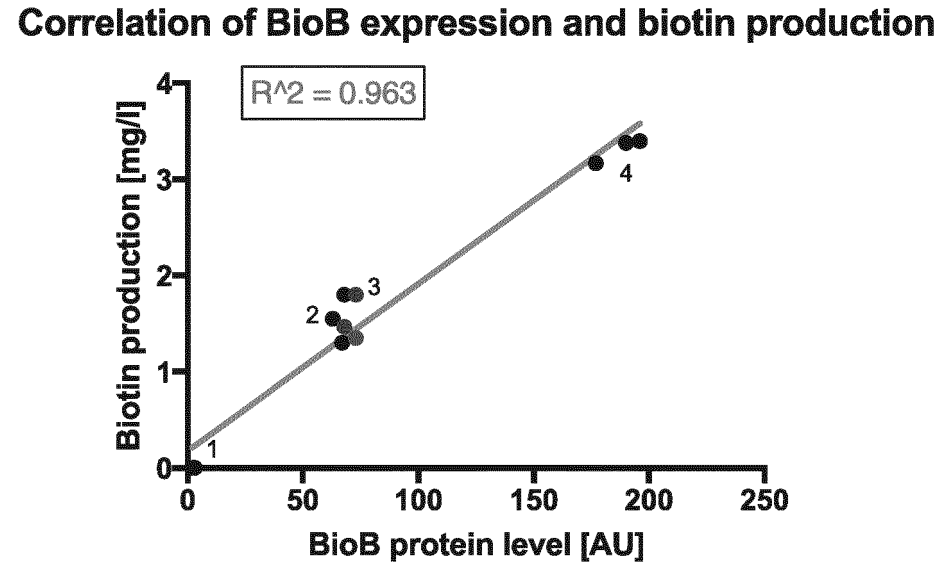


Figure 10



5

Figure 11



[1]BS1013+pBS430, 0.025 mM IPTG
[2]BS1011+pBS412, 0.025 mM IPTG
[3]BS1353+pBS412, 0.025 mM IPTG
[4]BS1353+pBS412, 1 mM IPTG

Figure 12

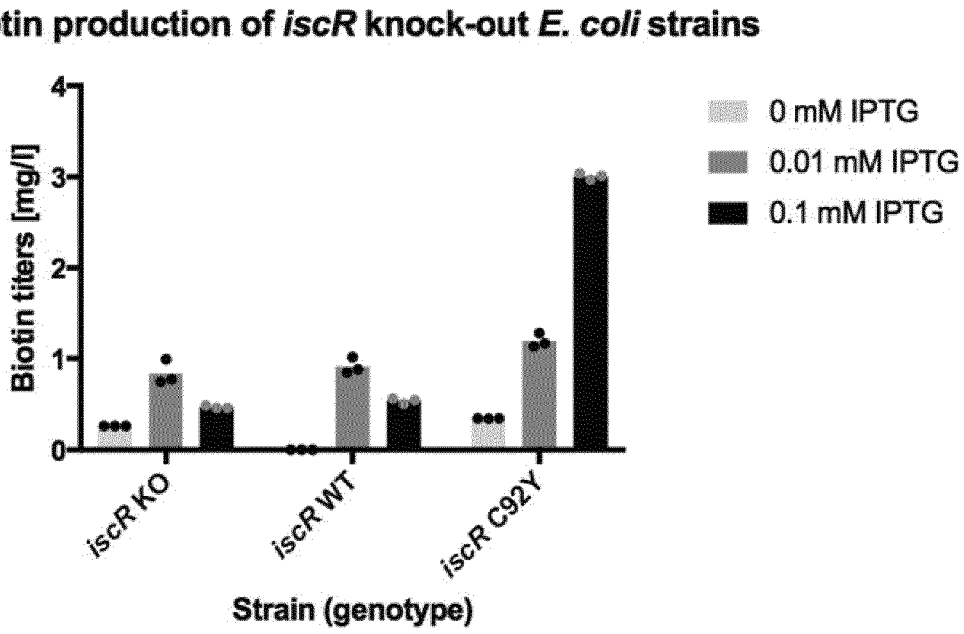


Figure 13

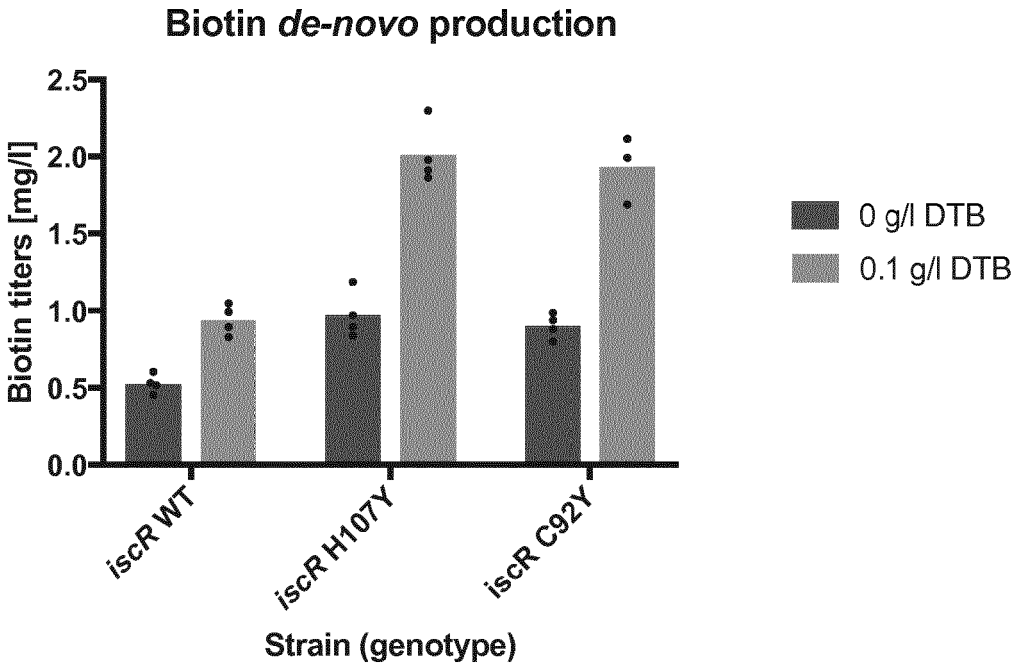


Figure 14

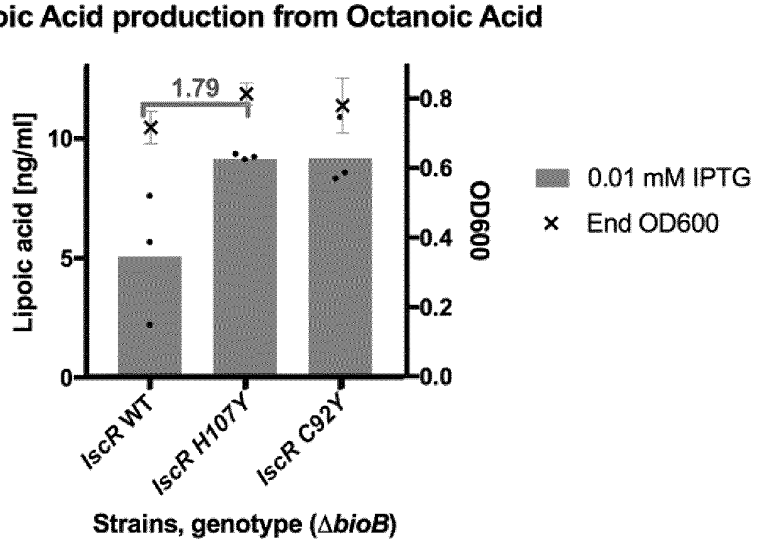


Figure 15

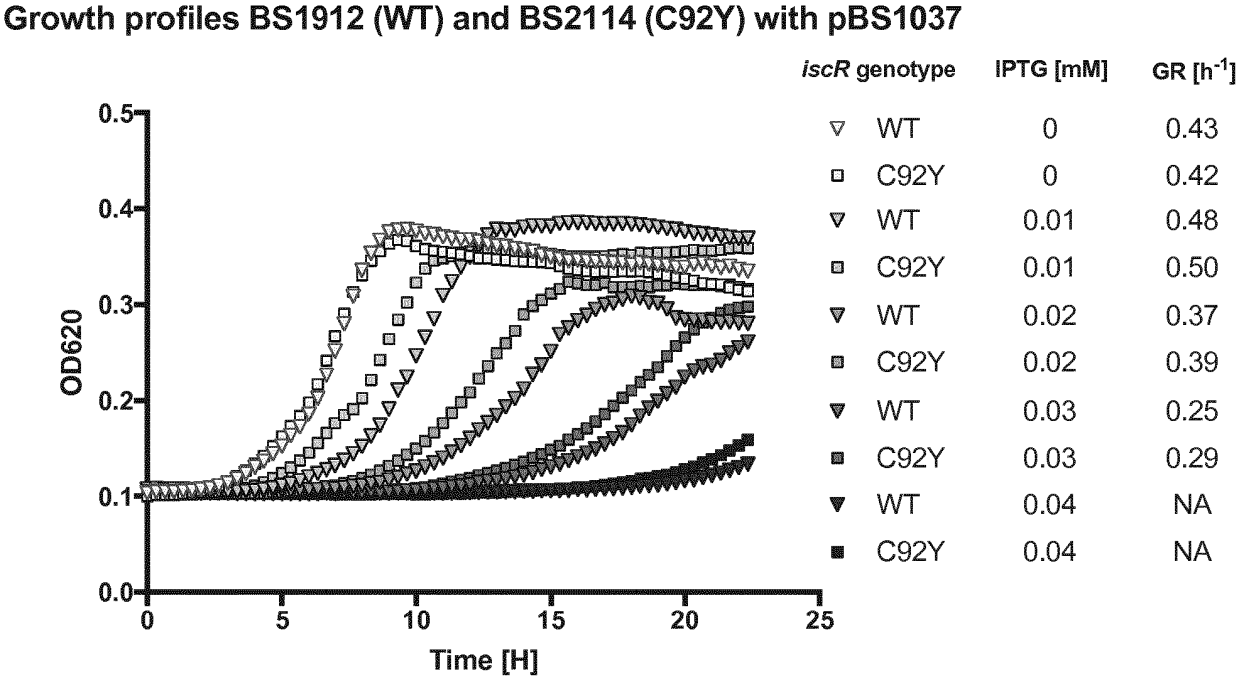


Figure 16

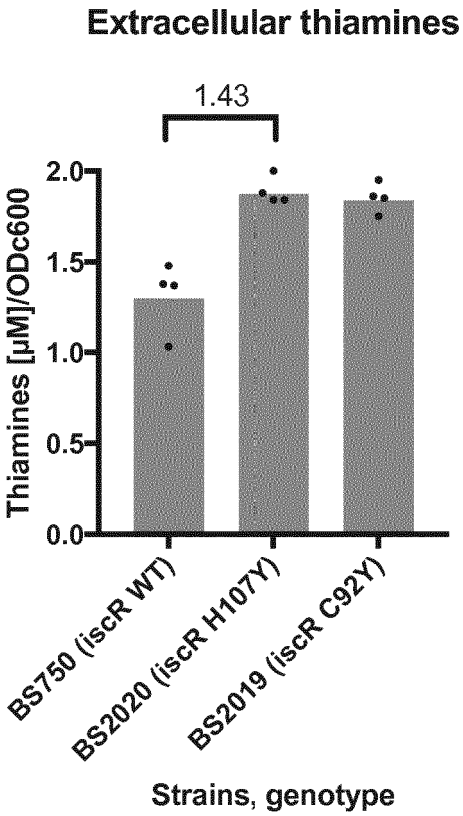


Figure 17

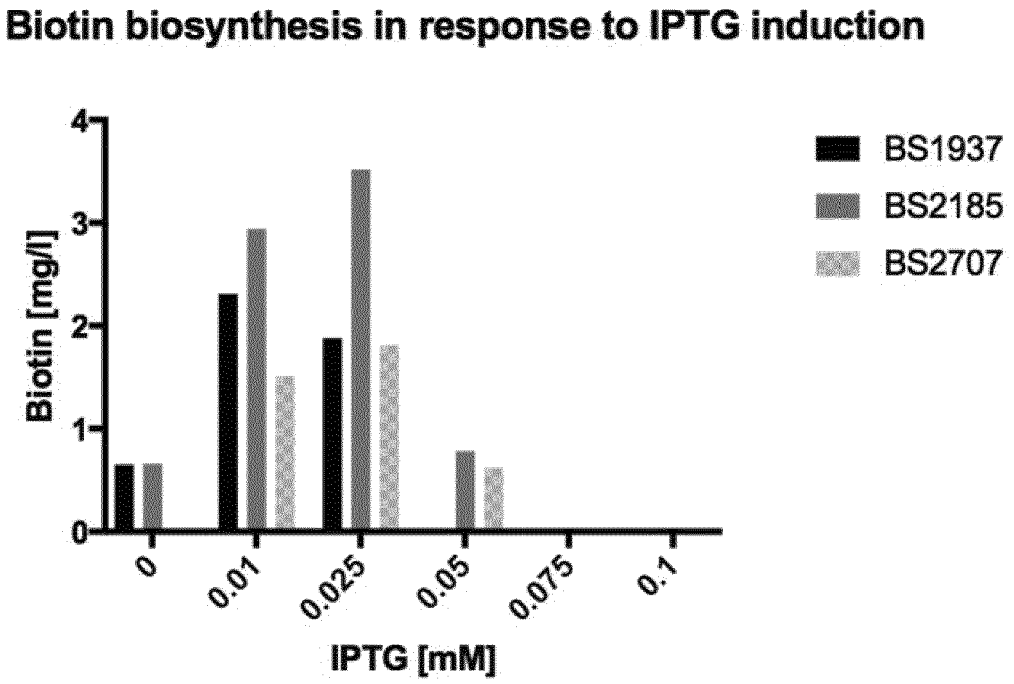
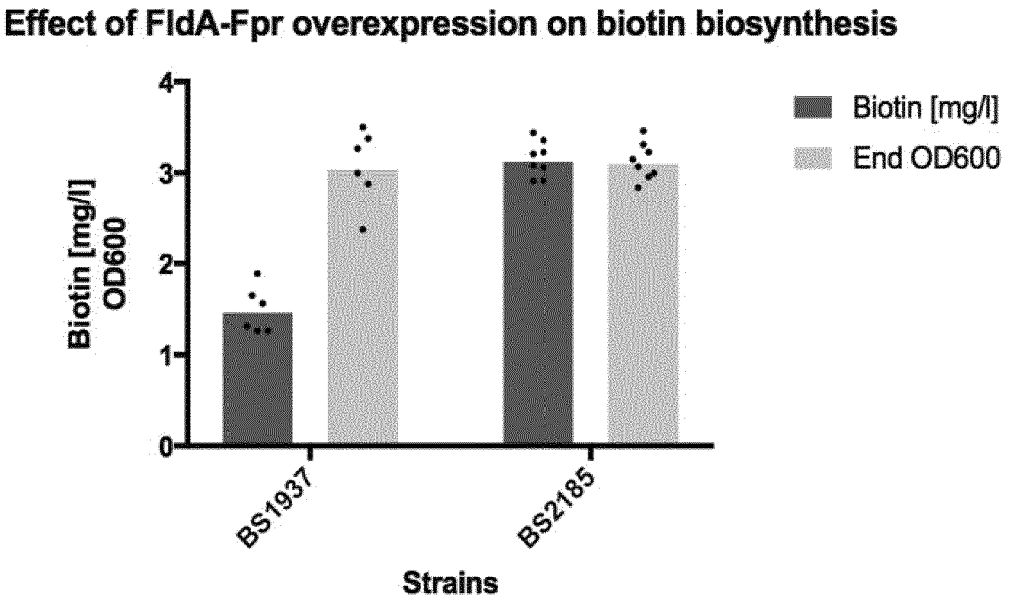


Figure 18


Effect of FldA-Fpr overexpression on biotin biosynthesis
Biotin [mg/l]
End OD600
Biotin [mg/l] OD600
0
1
2
3
4
BS1937
BS2185
Strains

REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- WO 02085293 A **[0011]**

Non-patent literature cited in the description

- **SAMBROOK et al.** Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, 1989 **[0083]**
- **DATSENKO KA ; WANNER BL.** One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc Natl Acad Sci U S A.,* 2000, vol. 97 (12), 6640-5 **[0140]**
- **FLEISCHHACKER, A. S. et al.** Characterization of the [2Fe-2S] cluster of Escherichia coli transcription factor IscR. *Biochemistry,* 2012, vol. 51, 4453-4462 **[0140]**
- **GIEL, J. L. ; RODIONOV, D. ; LIU, M. ; BLATTNER, F. R. ; KILEY, P. J.** IscR-dependent gene expression links iron-sulphur cluster assembly to the control of O2-regulated genes. *Escherichia coli. Mol. Microbiol.,* 2006, vol. 60, 1058-1075 **[0140]**
- **HERBERT AA ; GUEST JR.** Lipoic acid content of Escherichia coli and other microorganisms. *Arch Microbiol.,* 31 December 1975, vol. 106 (3), 259-66 **[0140]**
- **IFUKU, O. et al.** Sequencing analysis of mutation points in the biotin operon of biotin-overproducing Escherichia coli mutants. *Biosci Biotechnol Biochem,* 1993, vol. 57, 760-765 **[0140]**
- **IFUKU, O. et al.** Molecular analysis of growth inhibition caused by overexpression of the biotin operon in Escherichia coli. *Bioscience, biotechnology, and biochemistry,* 1995, vol. 59 (2), 184-189 **[0140]**
- **MARTIN, J. E. ; IMLAY, J. A.** *Replication during periods of iron starvation,* 2012, vol. 80, 319-334 **[0140]**
- **PY, B. ; BARRAS, F.** Building Fe-S proteins: bacterial strategies. *Nat. Rev. Microbiol.,* 2010, vol. 8, 436-446 **[0140]**
- **SCHMITT, A ; KOCHANOWSKI K. ; VEDELAAR S. ; AHRNÉ E. ; VOLKMER B. ; CALLIPO L. ; KNOOPS K. ; BAUER M. ; AEBERSOLD R. ; HEINEMANN M.** The quantitative and condition-dependent Escherichia coli proteome. *Nature Biotechnology 2015,* 2015 **[0140]**