



(51) International Patent Classification:

C12N 9/02 (2006.01) C12P 7/16 (2006.01)
C12P 7/06 (2006.01) C12P 7/18 (2006.01)

(21) International Application Number:

PCT/EP2016/067568

(22) International Filing Date:

22 July 2016 (22.07.2016)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

15306225.2 27 July 2015 (27.07.2015) EP

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: NEW POLYPEPTIDE HAVING FERREDOXIN-NADP+ REDUCTASE ACTIVITY, POLYNUCLEOTIDE ENCODING THE SAME AND USES THEREOF

(57) Abstract: The present invention relates to a novel polypeptide having the enzymatic activity of reduction of NADP+ using electrons from reduced ferredoxin (Ferredoxin-NADP+ reductase activity), a polynucleotide having a nucleotide sequence encoding such polypeptide and uses thereof. The invention relates to the modulation of the Ferredoxin NADP+ reductase activity in a microorganism by varying the expression level of the polynucleotide coding for such polypeptide. The invention also relates to the production of commodity chemicals, especially ethanol, n-butanol, 1,3-propanediol, 1,2-propanediol, isopropanol and acetone by fermenting microorganisms wherein their Ferredoxin NADP+ reductase activity is modulated.



**NEW POLYPEPTIDE HAVING FERREDOXIN-NADP+ REDUCTASE ACTIVITY,
POLYNUCLEOTIDE ENCODING THE SAME AND USES THEREOF**

FIELD OF THE INVENTION

5 The present invention relates to a novel polypeptide having the enzymatic activity of reduction of NADP⁺ using electrons from reduced ferredoxin (Ferredoxin-NADP⁺ reductase activity), a polynucleotide having a nucleotide sequence encoding such polypeptide and uses thereof.

10 More particularly, the invention relates to the modulation of the Ferredoxin-NADP⁺ reductase activity in a microorganism by varying the expression level of the polynucleotide coding for such polypeptide.

15 The present invention also relates to the production of commodity chemicals, especially ethanol, n-butanol, isopropanol, acetone, 1, 3-propanediol, 1,2-propanediol, by fermenting microorganisms wherein the Ferredoxin-NADP⁺ reductase activity is modulated.

PRIOR ART

1) N-butanol production:

20 N-butanol is naturally produced in slow-growing native hosts, that belong to genus *Clostridium*, as a mixture with acetone and ethanol in a biphasic fermentation, especially the most well-known *C. acetobutylicum* (Cornillot *et al.* 1997, Girbal and Soucaille 1998, Fontaine *et al.* 2002). Even if the acetone-butanol-ethanol (ABE) fermentation was one of the first large scale industrial fermentation and despite the long research history into the fermentation process, 25 obstacles still remain today. As *clostridia* are strict anaerobes, the growth condition must remain anaerobic and the growth rate is significantly slow compared to many of the aerobic microorganisms resulting in low butanol production rate. Moreover, the use of *Clostridium acetobutylicum* or *C. beijerinckii* strains (which are the most studied) in continuous process are be limited due to the risk of degeneration of the strains.

30 Advances in genetic manipulation of *clostridia* have recently led to large increases in titer and productivity (Papoutsakis 2008, Soucaille 2007, Jiang *et al.* 2009, Tracy 2012). Up to now, the best engineered *C. acetobutylicum* ATCC 824 strain for the production of n-butanol at high yield and titre was obtained using a combination of a disruption of particular genes involved in acids production with the overexpression of the AdhE1^{D485G} alcohol dehydrogenase variant. A maximal 35 concentration of 18.4 g/l n-butanol was produced from glucose in batch culture at a yield of 0.28 g/g glucose with low amounts of acetone, ethanol, acetate and butyrate (2 g/L, 3 g/L, 2 g/L and 4.3

g/L respectively) (Jang *et al.* 2012). As one of drawbacks of the *Clostridia* n-butanol production is the co-synthesis of acetone, which cannot be used as fuel source, several studies were focused on strategies to circumvent carbon lost to this product. One alternative was to overexpress the primary/secondary alcohol dehydrogenase from *C. beijerinckii* NRRL B-593 encoded by the *sadh_B* gene to reduce acetone into isopropanol. The engineered *C. acetobutylicum* 824 Δ *buk* strain overexpressing the *sadh* gene with the acetone pathway was able to produce, from glucose, 21 g/L of IBE mixture at a yield of 0.34 g/g, in controlled batch fermentations, without any acetone traces (Dusséaux *et al.* 2013). Another reported alternative was the engineering of *Clostridium tyrobutyricum* ATCC 25755, a non solventogenic acidogen, by expressing the AdhE2 alcohol dehydrogenase from *C. acetobutylicum*. The recombinant strain grown, in batch conditions at pH 6, on RCM medium with mannitol as carbon source, was able to produce 20.5 g/L of butanol at a yield of 0.33 g/g in presence of 6 g/L of butyric acid (Yu *et al.* 2012).

To overcome some of the problems associated with clostridial butanol production process, the clostridial butanol fermentation pathway was introduced into several heterologous organisms such as *E. coli* B or K12 (Atsumi *et al.* 2007, Inui *et al.* 2008, Garza *et al.* 2012) *Saccharomyces cerevisiae* (Steen *et al.* 2008), *Bacillus subtilis*, *Pseudomonas putida* (Nielsen *et al.* 2009) or *Lactobacillus brevis* (Berezina *et al.* 2010) to convert acetylCoA to butanol. Reported results showed that the seven *C. acetobutylicum* native genes of the pathway (*thl*, *hbd*, *crt*, *bcd*, *etfAB* and *adhE2*) are functional whatever the heterologous organism is, but the final butanol titer is rather low. Neither species could produce more than 1g/l of butanol.

Two bottlenecks limiting the 1-butanol production were identified: i) the pathway requires four moles of NADH to produce 1 mole of 1-butanol in the engineered *E. coli* strain, and balancing NADH needs an active pyruvate dehydrogenase complex under anaerobic conditions while this enzyme is normally inactive due to inhibition by NADH (Atsumi *et al.*, 2007, Garza *et al.* 2012) ii) the activity of the butyryl-CoA dehydrogenase (Bcd) catalysing the conversion of crotonyl-CoA to butyryl-CoA was low even if the *etfA* and *etfB* genes were expressed simultaneously with *bcd* (Inui M. *et al.* 2008, Berezina *et al.* 2010), and this enzyme complex produced reduced ferredoxin that has to be oxidized in *E. coli* for the reaction to proceed as previously described (Li *et al.* 2008). This last bottleneck can be overcome by using an alternative enzyme to replace Bcd, a NADH dependent trans-enoyl-CoA reductase (Ter) (Bond-Watts *et al.* 2011, Shen *et al.* 2011). The combination of the overexpression of the six following genes *atoB* (*E. coli*), *adhE2*, *crt*, *hbd* (*C. acetobutylicum*), *fdh* (*C. boidini*) and *Ter* (*T. denticola*) into the JCL 299 *E. coli* strain (Δ *ldhA*, Δ *adhE*, Δ *frdBC* Δ *pta*) led up to 15 g/L of butanol produced, after 75 hours of anaerobic culture in flasks containing 2% glucose terrific broth medium (Shen *et al.* 2011).

Another notable alternative to produce n-butanol was the engineering of the endogenous β -oxidation cycle in *E. coli* by introducing keys genetic modifications to eliminate the regulatory

mechanism that represses the genes involved in β -oxidation (*fadR* *atoC(c)* *crp***ΔarcA*), combined with the elimination of competitive native fermentative pathways (*ΔadhE*, *Δpta*, *ΔfrdA*, *ΔyqhD*, *ΔeutE*) and the overexpression of both YqeF (a predicted acyl transferase) and FucO (a L 1.2 propanediol oxidoreductase). Grown in bioreactor containing mineral medium supplemented with 5% glucose under micro-anaerobic conditions (5% dissolved oxygen), the engineered *E. coli* strain produced 14 g/l of n-butanol at a yield of 0.33g/g glucose) (Dellomonaco *et al.* 2011). However, it should be consider that this system level approach is limited by the ill-defined of the individual components of the engineered pathway.

10 2) 1.3 propanediol production:

1.3-propanediol (1.3Pdiol) is an important chemical widely used as a monomer in the production of polymers (polytrimethylene terephthalate just to cite an example). For a long time, 1.3Pdiol has been produced through chemical ways which generated huge cost and toxic products. Many microorganisms belonging to the genus *Klebsiella*, *Citrobacter*, *Lactobacilli* and *Clostridium* were found to naturally produce 1.3Pdiol from glycerol, an abundant waste metabolite produced in the biodiesel industry (Saxena *et al.* 2009). Most natural producers catabolize glycerol in two parallel pathways: i) the oxidation of glycerol into dihydroxyacetone then transformed into glyceraldehyde-3-phosphate and further to pyruvate, which in turn produces various byproducts and NADH₂ reducing equivalent and ii) the reduction of glycerol into 3-hydroxypropionaldehyde (HPA) by a glycerol dehydratase followed by the reduction of HPA into 1.3Pdiol by an NADH dependent 1.3 Pdiol dehydrogenase, and thereby regenerating NAD⁺. Reduced co-enzymes produced in the oxidative pathway are required for the reductive pathway maintaining the redox balance of the cell.

Depending of the microorganisms, the 1.3Pdiol production can be absolute anaerobic (*Clostridium butyricum*), microaerobic or aerobic (*Klebsiella* or *Citrobacter*) (Saint Amans *et al.* 2001, Saxena *et al.* 2009). Whatever the conditions, the methods and the natural producers are, the glycerol 1.3Pdiol conversion yield oscillated from 0.51 to 0.64 mol/mol, and a final 1.3Pdiol titer up to 102 g/L can be reached in fed-batch conditions (Xu *et al.* 2009, Almeida *et al.* 2012). Non-natural 1.3 propanediol producers like *E. coli* or *C. acetobutylicum* were also engineered by expressing the genes of the 1.3 Pdiol pathway. For example, the expression of the 1.3Pdiol pathway from *C. butyricum* into *C. acetobutylicum*, naturally unable to grow on glycerol as the sole carbon source, led to the production of 84 g/l of 1.3Pdiol at a yield of 0.65 mol/mol (Meynial-Salles *et al.* 2005). Finally, the combination of the overexpression of the B12 independent glycerol dehydratase from *C. butyricum* to YqhD (a NADPH 1.3 propanediol dehydrogenase from wild type *E. coli*) in *E. coli* (Tang *et al.* 2009) allowed the production of 104 g/l of 1.3 Pdiol in a two stages (aerobic and anaerobic) process, at a conversion yield of glycerol to 1.3Pdiol of 90.2 % (g/g) during the

anaerobic phase, a value that would mean that 1.12 mole of 1,3 propanediol are produced from 1 mole of glycerol which is impossible.

3) Propylene glycol production:

5 1.2 propanediol or propylene glycol is a three-carbon diol with stereogenic center at the central carbon, widely used as a commodity chemical. It is a constituent of unsaturated polyesters resins, non-ionic detergents, cooling, anti-freeze or de-icing fluid for aircraft. It can be used too as an additive in nutrition products and cosmetics. Produced up to now by a petrochemical way using a propylene oxide hydration process, this method has several drawbacks linked to the large
10 amounts of water consumed and the generation of waste streams containing environmental contaminants (Saxena et al. 2010).

As regards the biological way, wide ranges of microorganisms (both bacteria and yeasts) are known to produce 1.2 propanediol i) from common sugars such as glucose or xylose which are metabolized by the glycolysis pathway into dihydroxyacetone phosphate (DHAP) and, then, into
15 methyl glyoxal the precursor of 1.2 propanediol (Bennett and San 2001) or ii) from deoxysugars (fucose or rhamnose) like in *E. coli* which led to the synthesis of (S)-1.2 propanediol (Baldoma and Aguilar 1988).

Clostridium sphenoides (Tran-Din and Gottschalk 1985) and *Thermoanaerobacterium thermosaccharolyticum* were described as the best 1.2 propanediol natural producers, *T.*
20 *thermosaccharolyticum* being able to produce up to 9 g/l of (R)-1.2 propanediol from glucose, with a yield of 0.47 mol/mol glucose consumed (Sanchez-Riviera et al. 1987).

Metabolic engineering of the 1.2 propanediol pathway in *E. coli* was performed to force the cells to produce 1.2 propanediol from common sugars like glucose at the place of deoxysugars. A first strategy combining i) the overexpression of the key enzymes involved into DHAP to 1.2
25 propanediol conversion, ii) to the elimination of competing pathways and iii) to a glucose fed batch fermentation resulted in an engineered *E. coli* strain producing 4.5 g/l of (R)-1.2 propanediol, at a yield of 0.45 mol/mol glucose consumed (Altaras and Cameron 2000). A more recent study used the introduction of keys rational modifications of the central metabolism to link the growth and the glucose consumption to the 1.2 propanediol pathway to favour *in vivo* metabolic evolution
30 (Meynial-Salles *et al.* 2005). After adaptive evolution under appropriate culture conditions and reisolation of evolved clones from the population, the evolved engineered *E. coli* $\Delta tpiA$, $\Delta pflAB$, $\Delta adhE$, $\Delta ldhA$, $\Delta gloA$, $\Delta aldA$, $\Delta aldB$ Δedd strain, grown in batch conditions on glucose mineral medium, produced 1.8 g/l of 1.2 propanediol at a yield of 0.85 mol/mol.

Finally, *E. coli* was rationally engineered to produce 1.2 propanediol from glycerol. Grown
35 in controlled fermenters, the best resulting strain was capable of producing 5.6 g/l of 1.2 propanediol at a yield of 0.25 mol/mol glycerol consumed (Clomburg and Gonzalez 2011). The 1.2

propanediol production was also demonstrated on crude glycerol generated from the biodiesel industry with equivalent performance than on pure glycerol.

4) Ethanol production:

5 Several metabolic and evolutionary engineering techniques have been applied to develop efficient strains producing ethanol at a high yield and titer from a wide range of substrates. The most studied organisms are i) *Saccharomyces cerevisiae* and *Zymomonas mobilis* which naturally produce ethanol at high yield and efforts are concentrated on broadening substrate range and ii) *E. coli* in which efforts are concentrated on ethanol yield improvement (Girio et al. 2010). *E. coli* B strains producing anaerobically ethanol up to 45 g/L have already been obtained (Jarboe et al. 10 2007). These strains were constructed by either improving the endogenous pathway to ethanol (directed evolution performed on pyruvate dehydrogenase) or introducing a heterologous pathway composed of pyruvate decarboxylase and alcohol dehydrogenase (from *S. cerevisiae* or *Z. mobilis*). In best cases, ethanol was produced at yields of 90% predicted maximum, and these approaches 15 have already been patented (for instance Ingram et al. 1992 WO 9216615; Kim et al. 2007). The poor tolerance of *E. coli* to ethanol remains the major factor that limits final titers, and seems to be linked in particular to the ethanol inhibition of the pyruvate decarboxylase (Trinh et al. 2010).

In recent years, efforts were focused on developing strains i) able to co-utilize C6 (glucose, galactose) and C5 (xylose and arabinose) sugars, the components present in lignocellulosic biomass 20 hydrolysates or ii) growing on glycerol, a cheap and abundant carbon source. For example, the overexpression in *E. coli* K12 of both the glycerol dehydrogenase (glyDh) and the dihydroacetonekinase (DHAK), two enzymes specific of glycerol utilization, enabled the production of nearly 20 g/L of ethanol from crude glycerol during pH controlled anaerobic fermentation, at a yield of 0.92 mol ethanol produced per mole of glycerol consumed (Cintolesi et 25 al. 2012).

Finally, the fermentation performances of the three best engineered *E. coli*KO11, *Z. mobilis*AX101 and *S. cerevisiae*424A strains were compared on cellulosic hydrolysates (Lau et al. 2010). The results indicated that the metabolic pathway of *E. coli*KO101 and *Z. mobilis* was more effective to produce ethanol from consumed sugars relative to the *S. cerevisiae*424A pathway. 30 However, in both bacteria xylose consumption was strongly affected in comparison to *S. cerevisiae*424A, which was able to use both glucose and xylose from cellulosic hydrolysates.

DESCRIPTION OF THE INVENTION

In a first embodiment, the invention concerns a modified microorganism having modulated Ferredoxin NADP⁺ reductase enzymatic activity, wherein the activity of the polypeptide having at least 70 % identity with the sequence of SEQ ID N°1 is attenuated or enhanced.

5 More particularly, in such a first embodiment the modified microorganism having Ferredoxin NADP⁺ reductase enzymatic activity, is such that the activity of the polypeptide having at least 70 % identity with the sequence of SEQ ID N°1 is enhanced in comparison with the non-modified microorganism.

10 The modified microorganism contains or expresses a polypeptide having at least 70 % identity with the sequence of SEQ ID N°1 and is characterized in that the Ferredoxin NADP⁺ reductase enzymatic activity of such polypeptide is enhanced in comparison with the non-modified microorganism.

The polypeptide has at least 70%, particularly 75%, 80%, 81%, 82%, 83%, 84 %, 85 %, 86%, 87 %, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or even 100%
15 identity with the sequence of SEQ ID N°1.

In a second embodiment the invention concerns a microorganism according to the first embodiment with attenuated Ferredoxin NADP⁺ reductase enzymatic activity wherein the expression of the native gene coding for the polypeptide having at least 70 % identity with the
20 sequence of SEQ ID N°1 is attenuated.

In a third embodiment, the invention concerns a microorganism according to the second embodiment, wherein production of acetone and acetate is enhanced.

25 In a forth embodiment, the present invention relates to a microorganism according to first embodiment with enhanced Ferredoxin NADP⁺ reductase enzymatic activity, wherein a polynucleotide coding for the polypeptide having at least 70 % identity with the sequence of SEQ ID N°1 is overexpressed.

30 In a fifth embodiment, the invention is directed to a microorganism according to the forth embodiment wherein it contains at least one modification to produce ethanol.

In a sixth embodiment, the invention concerns a microorganism according to fifth embodiment, wherein the at least one modification to produce ethanol comprises:

- 35
- Enhancement of the expression of at least one of the following genes: *pfor*, *fdx*, *udhA*

- *and/or*
- Attenuation of the expression of at least one of the following genes: *aceE*, *aceF*, *lpd*, *pflA*, *pflB*, *poxB*, *frdABCD*, *ldhA*, *mgsA*, *ackA*, *pta* and *iscR*.

5 In a seventh embodiment, the present invention deals with a microorganism according to the forth embodiment wherein it contains at least one modification to produce n-butanol.

In an eighth embodiment, the invention concerns a microorganism according to seventh embodiment, wherein the at least one modification to produce n-butanol comprises:

- 10
- Enhancement of the expression of at least one of the following genes: *pfor*, *fdx*, *atoB*, *hbd*, *crt*, *bcd*, *etfA*, *etfB*, *adhE2*, *udhA*,
 - *and/or*
 - Attenuation of the expression of at least one of the following genes: *aceE*, *aceF*, *lpd*, *pflA*, *pflB*, *frdABCD*, *adhE*, *ldhA*, *mgsA*, *ackA*, *pta*, and *iscR*

15

In a ninth embodiment, the invention relates to a microorganism according to forth embodiment wherein it comprises at least one modification to produce 1, 3 propanediol from glycerol.

20 In a tenth embodiment, the invention concerns a microorganism according to ninth embodiment, wherein the at least one modification to produce 1, 3 propanediol comprises:

- Enhancement of the expression of at least one of the following genes : *pfor*, *fdx*, *pntAB*, *dhaB1*, *dhaB2*, *yqhD*
- *and/or*

- 25
- Attenuation of the expression of at least one of the following genes: *aceE*, *aceF*, *lpd*, *poxB*, *ndh*, *aldA*, *aldB*, *ldhA*, *pflA*, *pflB*, *adhE*, *iscR*, *glpA* and *glpD*.

According to a eleventh embodiment, the invention concerns a microorganism according to forth embodiment wherein it comprises at least one modification to produce 1, 2 propanediol.

30 In a twelfth embodiment, the invention relates to a microorganism according to eleventh embodiment, wherein the at least one modification to produce 1, 2 propanediol comprises:

- Attenuation of the expression of at least one of the following genes : *ptsG*, *ptsH*, *ptsI*, *crr*, *edd*, *eda*, *gloA*, *aceE*, *aceF*, *lpd*, *aldA*, *aldB*, *ldhA*, *pflA*, *pflB*, *adhE*, *tpiA*, *gapA*, *pykA*, *pykF*, *ackA*, *pta*, *poxB*, *arcA* and *ndh*,
- *And/or*
- 5 • Enhancement of the expression of at least one of the following genes : *pfor*, *fd*, *pntAB*, *gapN*, *galP*, *glk*, *ppsA*, *mgsA*, *sadh*, *yqhD*, *yafB*, *ydhF*, *ycdW*, *yqhE*, *yeaE*, *yghZ*, *yajO*, *tas*, *ydjG*, *ydbC*, *gldA*, *fucO*.

10 In a thirteenth embodiment, the present invention further relates to method for modulating the Ferredoxin NADP⁺ reductase enzymatic activity in a microorganism, wherein the activity of the polypeptide having at least 70 % identity with the sequence of SEQ ID N°1 is enhanced or attenuated in the said microorganism.

15 In a particular embodiment, the percentage of identity is of at least 75%, at least 80%, at least 85%, at least 90%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99 % with the sequence of SEQ IDN°1. In a further particular embodiment the polypeptide corresponds to SEQ ID N°1.

20 In a fourteenth embodiment, the invention relates to a method according to thirteenth embodiment, wherein the Ferredoxin NADP⁺ reductase enzymatic activity is enhanced by overexpressing a polynucleotide coding for the polypeptide having at least 70 % identity with the sequence of SEQ ID N°1.

25 In a particular embodiment, the percentage of identity is of at least 75%, at least 80%, at least 85%, at least 90%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99 % with the sequence of SEQ IDN°1. In a further particular embodiment the polypeptide corresponds to SEQ ID N°1.

30 The invention also relates to a fifteenth embodiment which relates to a method according to thirteenth embodiment, wherein the Ferredoxin NADP⁺ reductase enzymatic activity is attenuated by attenuating the expression of a polynucleotide coding for the polypeptide having at least 70 % identity with the sequence of SEQ ID N°1.

35 In a particular embodiment, the percentage of identity is of at least 75%, at least 80%, at least 85%, at least 90%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99 % with the sequence of SEQ IDN°1. In a further particular embodiment the polypeptide corresponds to SEQ ID N°1.

Also, the invention relates in a sixteenth embodiment to a method for preparing ethanol wherein the microorganism according to fifth or sixth embodiment is grown in an appropriate culture medium comprising a source of carbon and the ethanol is recovered.

5 In a seventeenth embodiment the invention relates to a method for preparing n-butanol wherein the microorganism according to seventh or eighth embodiment is grown in an appropriate culture medium comprising a source of carbon and the n-butanol is recovered.

10 In an eighteenth embodiment, the invention relates to a method for preparing 1, 3 propanediol wherein the microorganism according to ninth or tenth embodiment is grown in an appropriate culture medium comprising a source of carbon and the 1, 3-propanediol is recovered.

15 In a nineteenth embodiment, the present invention concerns a method for preparing 1, 2 propanediol wherein the microorganism according to eleventh or twelfth embodiment is grown in an appropriate culture medium comprising a source of carbon and the 1, 2-propanediol is recovered.

Also, in twentieth embodiment the present invention relates to the use of a microorganism according to fifth or sixth embodiment to produce ethanol.

20 In a twentyfirst embodiment, the present invention relates to the use of a microorganism according to seventh or eighth embodiment to produce n-butanol.

25 Also, in a twentysecond embodiment, the present invention discloses the use of microorganism according to ninth or tenth embodiment to produce 1,3 propanediol.

Eventually, the present invention, in a twentythird embodiment relates to the use of microorganism according to eleventh or twelfth embodiment to produce 1,2 propanediol.

30 The present invention concerns an isolated polypeptide having a Ferredoxin-NADP+ reductase enzymatic activity comprising the sequence of SEQ ID NO 1, a fragment or homologous sequence thereof.

The invention also related to a polypeptide having at least 70 % identity with the sequence of SEQ ID N°1 having Ferredoxin NADP+ reductase enzymatic activity.

35 The invention also relates to the use of a polypeptide having at least 70 % identity with the sequence of SEQ ID N°1 for the reduction of NADP+ using electrons from reduced ferredoxin in a microorganism or in the culture medium of a microorganism.

The invention also provides a polynucleotide comprising a sequence coding for said polypeptide. Inventors report the identification of a gene from *C. acetobutylicum* encoding a protein having a Ferredoxin-NADP⁺ reductase activity. This gene was previously known as the
5 *CA_C0764* gene annotated as coding for a potential small subunit of glutamate synthase (Nölling et al, 2001). Glutamate synthase (GOGAT) are heterodimeric proteins composed of a large alpha subunit and a small beta subunit that catalysed the NAD(P)H dependent conversion of alpha-ketoglutarate to glutamate. This protein has been purified from *Clostridium pasteurianum* (Singhal RK et al, 1989) and *Bacillus subtilis* (Matsuoka K, and Kimura K, 1986), and was shown to be a
10 complex iron-sulfur flavoprotein (Vanoni M and Curti B, 1999). No association of glutamate synthase with a Ferredoxin-NADP⁺ reductase activity has ever been reported in the literature.

The invention is furthermore related to an expression cassette comprising said polynucleotide under the control of regulatory elements functional in a host cell and to a transformation vector comprising said cassette or said polynucleotide.

15 The invention also provides a modified microorganism having modulated Ferredoxin-NADP⁺ reductase enzymatic activity wherein activity of the polynucleotide of the invention is attenuated or enhanced.

It is one object of the present invention to use the new knowledge of a gene encoding for Ferredoxin-NADP⁺ reductase protein from *C. acetobutylicum* to design microorganisms with
20 enhanced Ferredoxin-NADP⁺ reductase activity and new metabolic pathways for the production of either ethanol, n-butanol, 1,3 propanediol or 1, 2 propanediol. These microorganisms use new metabolic pathways to convert i) glucose to either ethanol, n-butanol, or 1, 2 propanediol, and ii) glycerol to 1,3 propanediol. In certain embodiments of the invention, microorganisms with enhanced Ferredoxin-NADP⁺ reductase activity are further modified to enhance i) the soluble
25 transhydrogenase activity for the production of either ethanol or n-butanol at high yield or ii) the membrane bound transhydrogenase for the production of either 1,3 propanediol or 1, 2 propanediol at high yield. Additionally, a method for preparing either ethanol, n-butanol, 1,3 propanediol or 1, 2 propanediol wherein said microorganisms are grown in appropriate growth medium and either ethanol, n-butanol, 1,3 propanediol or 1, 2 propanediol are recovered is provided.

30 It is also an object of the present invention to use the new knowledge of the gene encoding for the Ferredoxin-NADP⁺ reductase protein from *C. acetobutylicum* to design a microorganism with attenuated Ferredoxin-NADP⁺ reductase activity useful for the production of acetone. Attenuation of said gene coding for Ferredoxin-NADP⁺ reductase provides a *C. acetobutylicum* strain able to produce butanol at a lower yield and acetone at higher yield, therefore increasing the
35 acetone productivity of the process. A method for preparing acetone wherein said microorganisms are grown in appropriate growth medium and acetone is recovered is provided.

The invention is also related to a method for modulating the Ferredoxin-NADP⁺ reductase enzymatic activity in a microorganism, wherein activity of the polypeptide of the invention is enhanced or attenuated in said microorganism.

5 DETAILED DESCRIPTION OF THE INVENTION

The present invention is related to an isolated polypeptide having a Ferredoxin-NADP⁺ reductase enzymatic activity comprising the sequence of SEQ ID N°1, a fragment or homologous sequence thereof.

10 SEQ ID N°1:

MDNPNLLSEEANRCLLCKNPRCKANCPINTPIPEIISLYKEGKIMEAGEILFNNNPLSVICSL
 VCIHEDQCKGNCVRGIKSEPIKFHEIEEEISEKYLKEAKLKNVQKDKDRIAIVGGGPAGITV
 AFVLANKGYNVTIFEAHDKIGGVRLRYGIPEYRLTKKLVDKLEERLIEVGVKIRPNTVIGPVI
 SLDRLLSDSYKAVFIGTGVWNPKTLDVKGETLGNVHFAIDYLKSPESYRLGKKVAVIGAG
 15 NVAMDAARTAKRNGAEVTILYRKSFNEMPASKQEIRETKEDGVEFKLFRAPIEITEEGIKV
 AFTENVTD AEGKIRTKIIEGKEEFFECDSVVVAVSQAPKDNIVSNTTGLDTKWGLIVTDEK
 GNTTKKGT FACGDVVTGAKTVVEAAAQAKVVAETIDEYCKNN

20 As used herein, the following terms may be used for interpretation of the claims and specification.

According to the invention, the term “polypeptide” refers to peptide or protein which comprises a sequence of two or more amino-acids linked with peptide bonds.

The term “isolated” refers to a protein or DNA sequence that is removed from at least one component with which it is naturally associated.

25 The term “Ferredoxin-NADP⁺ reductase” refers to a polypeptide responsible for an enzyme activity that catalyzes the reduction of NADP⁺ using electrons from reduced ferredoxin. Such an enzyme activity was described in *C. acetobutylicum* by Vasconcelos et al (1994) and methods to measure this enzyme activity were provided.

30 The terms “enzyme activity “ and “enzymatic activity” are used interchangeably and refer to the ability of an enzyme to catalyse a specific chemical reaction, for example the reduction of NADP⁺ using electrons from reduced ferredoxin for Ferredoxin-NADP⁺ reductase enzyme activity.

The isolated polypeptide of the present invention can be obtained from microorganisms having Ferredoxin-NADP⁺ reductase activity, for example by using the purification procedure as
 35 described in the following examples. Microorganisms that can be used to isolate the polypeptide include, but are not limited to, *C. acetobutylicum*.

The term “comprising the sequence of SEQ ID N°1” means that the amino-acid sequence of the polypeptide may not be strictly limited to SEQ ID N°1 but may contain additional amino-acids. The term “a fragment of SEQ ID N°1” means that the sequence of the polypeptide may include less amino-acid than SEQ ID N°1 but still enough amino-acids to confer Ferredoxin-NADP+ reductase activity. It is well known in the art that a polypeptide can be modified by substitution, insertion, deletion and/or addition of one or more amino-acids while retaining its enzymatic activity. For example, substitutions of one amino-acid at a given position by a chemically equivalent amino-acid that do not affect the functional properties of a protein are common. For the purposes of the present invention, substitutions are defined as exchanges within one of the following groups:

- Small aliphatic, non-polar or slightly polar residues : Ala, Ser, Thr, Pro, Gly
- Polar, negatively charged residues and their amides : Asp, Asn, Glu, Gln
- Polar, positively charged residues : His, Arg, Lys
- Large aliphatic, non-polar residues : Met, Leu, Ile, Val, Cys
- Large aromatic residues : Phe, Tyr, Trp.

Thus, changes which result in substitution of one negatively charged residue for another (such as glutamic acid for aspartic acid) or one positively charged residue for another (such as lysine for arginine) can be expected to produce a functionally equivalent product.

The positions where the amino-acids are modified and the number of amino-acids subject to modification in the amino-acid sequence are not particularly limited. The man skilled in the art is able to recognize the modifications that can be introduced without affecting the activity of the protein. For example, modifications in the N- or C-terminal portion of a protein would not be expected to alter the activity of a protein.

The term “homologous” refers to polypeptides submitted to modifications such as defined above while still retaining the original enzymatic activity.

In a specific embodiment of the invention, the polypeptide of the present invention have at least 70% identity with the sequence of SEQ ID N°1, preferentially at least 80% identity and more preferentially at least 90% identity.

Methods for determination of the percentage of identity between two protein sequences are known from the man skilled in the art. For example, it can be made after alignment of the sequences by using the software CLUSTALW available on the website <http://www.ebi.ac.uk/clustalw/> with the default parameters indicated on the website. From the alignment, calculation of the percentage of identity can be made easily by recording the number of identical residues at the same position compared to the total number of residues. Alternatively, automatic calculation can be made by using for example the BLAST programs available on the

website <http://www.ncbi.nlm.nih.gov/BLAST/> with the default parameters indicated on the website.

In a specific embodiment of the invention, the polypeptide comprises at least 100 contiguous amino-acids from the sequence of SEQ ID N°1, preferentially at least 150, at least 200, 5 at least 250, at least 300, at least 350 or more preferentially at least 400 contiguous amino-acids of the sequence shown in SEQ ID N°1. In another embodiment of the invention, the polypeptide has a polypeptidic sequence strictly identical to the sequence of SEQ ID N°1.

The present invention is also related to a polynucleotide comprising a sequence coding for the polypeptide of the invention.

10 The term “polynucleotide” refer to a polymer of ribonucleotides (or RNA) or to a polymer of deoxyribonucleotides (or DNA), that is single or double-stranded, optionally containing synthetic, non-natural, or altered nucleotide bases. An isolated polynucleotide in the form of DNA may contain one or more segments of synthetic DNA, genomic DNA or cDNA.

The origin of the polynucleotide is not necessarily the organism where the enzymatic 15 activity is originally measured. Hybridization under different conditions of stringency with a probe that comprises the nucleotide sequence of SEQ ID N°2 can be used to screen a gene library for such polynucleotides by the man skilled in the art. Detailed protocols for hybridization are disclosed in Sambrook et al (1989).

The sequences of such polynucleotides can be extracted from the databases using for 20 example the BLAST programs defined above and searching for homology with the nucleotide sequence of SEQ ID N°2.

SEQ ID N°2:

ATGGATAACCCCTAATTTATTGTCAGAAGAGGCAAACAGATGCCTACTATGCAAAAACC
25 CTAGATGTAAAGCAAATTGCCCTATTAATACACCCCATACCAGAAATTATAAGCCTTTA
TAAAGAAGGAAAAATTATGGAAGCAGGAGAAATTTTATTTAATAACAATCCTCTTTCA
GTAATATGCTCATTGGTCTGTATTCATGAGGATCAATGTAAGGGAAATTGTGTAAGAG
GAATAAAAAGCGAGCCAATAAAATTTACGAGATAGAAGAAGAGATATCAGAGAAGT
ACTTAAAAGAGGCCAAGCTTAAGAATGTTTCAGAAGGACAAGGATAGAATTGCAATAG
30 TTGGAGGAGGTCCAGCAGGAATCACAGTTGCATTTGTAAGTGTCTAATAAAGGCTATAA
TGTTACCATTTTTGAGGCTCACGATAAAATAGGAGGAGTACTTAGATATGGTATTCCA
GAGTATAGATTGACTAAGAAATTAGTGGATAAGCTTGAAGAAAGACTTATAGAGGTTG
GAGTGAAAATTAGACCTAATACTGTTATTGGACCAGTTATTTCTTTGGACAGGTTACTT
GAAGATTCATATAAGGCAGTATTTATTGGAAGTGGAGTATGGAATCCAAAAACCTTAG
35 ATGTAAAGGGAGAACTTTAGGAAATGTTTCATTTTGCTATTGATTATTTAAATCTCCT
GAAAGCTATAGATTAGGAAAAAAGTAGCTGTAATAGGAGCAGGTAATGTTGCCATG

GATGCTGCAAGAACTGCTAAGAGAAATGGTGCTGAGGTAACAATACTTTATAGAAAA
 AGCTTTAATGAGATGCCAGCATCAAAACAAGAAATAAGAGAACTAAAGAAGATGGA
 GTAGAGTTTAAATTATTTAGAGCACCAATTGAAATAACAGAAGAGGGTATTAAAGTAG
 CATTTACAGAAAATGTTACAGATGCAGAAGGAAAAATAAGAATAAAATTATTGAAG
 5 GAAAAGAAGAGTTTTTTGAGTGTGATTTCAGTAGTTGTTGCCGTAAGTCAAGCACCTAA
 GGATAATATAGTATCTAATAACAACAGGCTTAGATACTAAATGGGGATTAATAGTAACA
 GATGAAAAAGGCAATACAATAAAAAAGGAACCTTTGCATGTGGAGATGTAGTTACA
 GGAGCAAAAAGTGTAGTTGAAGCTGCAGCACAAGCAAAAGTAGTTGCAGAACTATT
 GATGAGTATTGCAAGAATAATTAA.

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Preferred polynucleotides of the present invention are polynucleotides that are at least 80% identical to the nucleotide sequence of SEQ ID N°2. More preferred polynucleotides of the present invention are polynucleotides that are at least 90% identical to the nucleotide sequence of SEQ ID N°2. Even more preferred polynucleotides of the present invention are polynucleotides that are at
 15 least 95% identical to the nucleotide sequence of SEQ ID N°2.

In particular, the polynucleotide that comprises the nucleotide sequence of SEQ ID N°2 is included in the invention.

The terms “encoding” or “coding” refer to the process by which a polynucleotide, through the mechanisms of transcription and translation, produces an amino-acid sequence. This process is
 20 allowed by the genetic code, which is the relation between the sequence of bases in DNA and the sequence of amino-acids in proteins. One major feature of the genetic code is to be degenerate, meaning that one amino-acid can be coded by more than one triplet of bases (one “codon”). The direct consequence is that the same amino-acid sequence can be encoded by different polynucleotides. As an example, polynucleotide sequences derived from SEQ ID N°2 by
 25 degeneracy of the genetic code can also code for the polypeptide sequence of SEQ ID N°1 and are therefore contemplated by the present invention. It is well known from the man skilled in the art that the use of codons can vary according to the organisms. Among the codons coding for the same amino-acid, some can be used preferentially by a given microorganism. It can thus be of interest to design a polynucleotide adapted to the codon usage of a particular microorganism in order to
 30 optimize the expression of the corresponding protein in this organism.

The present invention is also related to an expression cassette comprising the polynucleotide of the invention under the control of regulatory elements functional in a host microorganism.

The term “expression” refers to the transcription and translation of a gene sequence leading
 35 to the generation of the corresponding protein, product of the gene.

The term “expression cassette” refers to a polynucleotide preferably linked with regulatory elements, such as promoters, enhancers, ribosome binding site or terminator allowing the expression of the gene contained in the polynucleotide inside a suitable host organism. Such regulatory elements can be the own regulatory elements of the gene, but also modified or synthetic elements, to allow a stronger expression of the gene. For example, stronger expression can be obtained by replacing the native promoter of the gene by stronger promoters. For *E. coli* these promoters are for example: lac promoter, tac promoter, trc promoter, lambda cI promoter and GI promoters (Meynial-Salles *et al.* 2005, Soucaille *et al.* 2012). For other organisms, the skilled artisan may be able to choose the more adapted promoter.

The term “host microorganism” refers to a microorganism able to receive foreign or heterologous genes or extra copies of its own genes and able to express those genes to produce an active protein product.

The present invention provides for a transformation vector comprising the polynucleotide or the cassette according to the invention.

The term “transformation” refers to the introduction of new genes or extra copies of existing genes into a host organism. The acquired genes may be incorporated into chromosomal DNA or introduced as extra-chromosomal elements. As an example, in *E. coli*, a method for transferring DNA into a host organism is electroporation.

The term “transformation vector” refers to any vehicle used to introduce a polynucleotide in a host organism. Such vehicle can be for example a plasmid, a phage or other elements known from the expert in the art according to the organism used. The transformation vector usually contains in addition to the polynucleotide or the expression cassette other elements to facilitate the transformation of a particular host cell. An expression vector comprises an expression cassette allowing the suitable expression of the gene borne by the cassette and additional elements allowing the replication of the vector into the host organism. An expression vector can be present at a single copy in the host organism or at multiple copies.

The present invention also provides for a modified microorganism having modulated Ferredoxin-NADP⁺ reductase activity, wherein activity of the polypeptide of the invention is attenuated or enhanced.

The term “attenuation of the activity of an enzyme” refers to a decrease of activity of the enzyme of interest, compared to the observed activity in the same microorganism before any modification. The man skilled in the art knows numerous means to obtain this result, and for example:

- Introduction of a mutation into the gene, decreasing the expression level of this gene, or the level of activity of the encoded protein.

- Replacement of the natural promoter of the gene by a low strength promoter, resulting in a lower expression.
- Use of elements destabilizing the corresponding messenger RNA or the protein.
- Deletion of the gene if no expression at all is needed.

5 An “increased enzymatic activity” or an “enhanced enzymatic activity” means that the activity is superior to the original activity measured in the same microorganism before any modification. The corresponding non-modified microorganism is a microorganism having the same characteristics of the modified microorganism except for the enzyme activity under consideration. Advantageously, the enzyme activity is increased by at least 50 %, preferably by at least 100%,
10 compared to the native activity of the corresponding non-modified microorganism. A method for measuring Ferredoxin-NADP⁺ reductase activity is given in Example 1 below.

 More particularly the expression “enhanced” or “Enhanced Enzyme Activity” means that enzyme activity is higher than that which naturally occurs in a microorganism that is non-modified or non-transformed (i. e. enzyme activity that occurs naturally in a microorganism in the absence of
15 direct or indirect manipulation of such activity/microorganism by man). This can apply to a non-modified microorganism that exhibits or not any enzyme activity prior to the modification.

 The expression “Enhanced activity” in relation with a modified strain that possesses Ferredoxin NADP⁺ reductase activity (naturally, prior to the the modification), means an increase in enzymatic activity that is larger than the margin of error inherent in the measurement technique,
20 preferably an increase by about 1.2-fold, 1.5 fold, particularly 2-fold or greater of the activity of the wild-type microorganism (ie activity of the enzyme of the non-modified microorganism), more preferably an increase by about 3-fold or greater, and most preferably an increase by about 5-fold or greater.

 In the case the non-modified microorganism does not contain any Ferredoxin NADP⁺
25 reductase activity prior to its modification, the “Enhanced activity” corresponds to the expression of such activity at a detectable level following the modification leading to obtaining the modified microorganism of the invention, such a detectable level being sufficient for orienting the metabolism of the modified microorganism.

 “Modification” or “Transformation” means a process for introducing heterologous DNA
30 into a bacterium cell. Transformed or Modified bacterium cells are understood to encompass not only the end product of a transformation process, but also transgenic or modified progeny thereof.

 “Modified” or “Transformed” or “recombinant” refer to a host organism such as a bacterium into which a heterologous nucleic acid molecule has been introduced.

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 The nucleic acid molecule can be stably integrated into the genome of the host or the

nucleic acid molecule can also be present as an extrachromosomal molecule. Such an extrachromosomal molecule can be auto-replicating.

A "non-transformed", "non-modified" or "non-recombinant" host cell refers to a wild-type organism, e. g. , a bacterium, which does not contain any heterologous nucleic acid molecule.

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Percent identity: the phrase "percent identical", in the context of two nucleic acid or protein sequences, refers to two or more sequences or subsequences that have for example 60%, preferably 70%, more preferably 80%, still more preferably 90%, even more preferably 95%, and most preferably at least 99% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using the following sequence comparison algorithms or by visual inspection.

Preferably, the percent identity exists over a region of the sequences that is at least about 50 residues in length, more preferably over a region of at least about 100 residues, and most preferably the percent identity exists over at least about 150 residues. In an especially preferred embodiment, the percent identity exists over the entire length of the coding regions.

For sequence comparison, typically one sequence acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence (s) relative to the reference sequence, based on the designated program parameters.

25

The microorganism according to the invention is selected among the group consisting of bacteria, yeast and fungi. Preferentially, the bacterium is selected among the group consisting of Clostridiaceae, Cyanobacteriaceae, Enterobacteriaceae, Bacillaceae, Streptomyetaceae, Pseudomonaceae and Corynebacteriaceae. More preferentially, the bacterium is selected among the group consisting of *Clostridium acetobutylicum*, *Thermoanaerobacterium saccharolyticum*, *Clostridium thermocellum*, *Synechococcus elongatus*, *Escherichia coli*, *Bacillus subtilis*, *Lactobacillus brevis*, *Pseudomonas putida* and *Corynebacterium glutamicum*. Preferentially, the yeast is *Saccharomyces cerevisiae*.

It is an object of the present invention to provide a microorganism with enhanced Ferredoxin-NADP⁺ reductase activity, wherein expression of the native gene coding for the

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polypeptide of the invention is enhanced. This is preferentially obtained by introducing a strong promoter upstream the coding sequence of the native gene.

5 Additionnally, other regulatory elements of the gene can be modified. For example, suitable mutations that can be selected by the expert in the field in the upstream region of the gene (start codon, ribosome binding site) can results in increased expression. In addition, an inducible promoter can be introduced in order to turn on/off the expression of the gene when desired.

In a prefered embodiment of the invention, a strong promoter is present upstream to the coding sequence of the native gene coding for the polypeptide according to the invention.

10 It is also an object of the present invention to provide a microorganism with enhanced Ferredoxin-NADP⁺ reductase activity, wherein the polynucleotide of the invention is overexpressed.

Preferentially, the overexpression is obtained by transforming the organism with the vector of the invention or by integrating the polynucleotide or the cassette of the invention into the chromosome of the organism.

15 One single copy or multiple copies of the gene borne by expression vectors or integrated into the chromosome can be introduced in order to modulate the overexpression. In addition, different kind of promoters inducing different level of expression of the gene can be used.

20 The adequate position on the chromosome for the insertion of the new gene can be selected by the expert in the art. This position (or locus) should not affect the essential functions of the host organism. Methods for integration of a gene into the chromosome of a host organism are disclosed for example in Sambrook et al (1989).

25 The present invention is also related to a microorganism with enhanced Ferredoxin-NADP⁺ reductase activity, which is further modified to produce ethanol, n-butanol, 1, 3 propanediol and 1, 2 propanediol by new metabolic pathways.

In a specific embodiment of the invention, modifications are introduced into the microorganism with enhanced Ferredoxin-NADP⁺ reductase activity specifically for the production of ethanol by a new metabolic pathway (figure 1).

30 Preferentially, the pyruvate ferredoxin oxidoreductase and the soluble transhydrogenase activities are increased. The preferred method is the overexpression of the *pfor* (*CA_C2229*), *fdx* (*CA_C0303*) genes coding for pyruvate ferredoxin oxidoreductase and ferredoxin respectively, and *udhA* gene coding for soluble transhydrogenase. Additionnally, the activity Fe-S cluster assembly proteins, IscS, IscU, and IscA are increase to improve [4Fe-4S] assembly in Pfor and Fdx to
35 increase the pyruvate ferredoxin oxidoreductase and the soluble transhydrogenase activities. This can be achieved by the attenuation of the *iscR* gene coding for a factor IscR, responsible for the

repression of the *isc* operon. Furthermore, one or several mutations can be introduced in the *pfor*, *fdx* or *udhA* genes to increase the pyruvate ferredoxin oxidoreductase and the soluble transhydrogenase activities under the culture conditions used.

The other gene whose expression may advantageously be enhanced is *adhE* coding for a bifunctional acetaldehyde and ethanol dehydrogenase.

In another embodiment of the invention, the methylglyoxal synthase enzyme activity, the first enzyme of the methylglyoxal bypass, encoded by the gene *mgsA* is attenuated.

Preferentially, in the microorganism according to the invention, some enzyme activities involved in by-product formation pathways are attenuated to increase the yield of ethanol:

- The pyruvate formate lyase activity, responsible for the synthesis of acetyl-CoA and formate from pyruvate, encoded by the genes *pflA* and *pflB*.
- The pyruvate dehydrogenase activity, responsible for the synthesis of acetyl-CoA and NADH from pyruvate, encoded by the genes *aceE*, *aceF* and *lpd*. The fumarate reductase activity, responsible for the synthesis of succinate from fumarate, encoded by the operon *frdABCD*.
- The lactate dehydrogenase activity, responsible for the synthesis of lactate from pyruvate, encoded by the gene *ldhA*.
- The phosphotransacetylase and acetate kinase activities, responsible for the synthesis of acetate in two steps from acetyl-CoA, encoded respectively by the genes *pta* and *ackA*.
- The pyruvate oxidase activity, responsible for the synthesis of acetate in one step from pyruvate, encoded by the gene *poxB*.

Preferentially, the attenuation of activity is obtained by the attenuation of at least one of these genes.

Preferentially the microorganism designed to produce ethanol by this new metabolic pathway is selected among bacteria, yeasts or fungi. More preferentially, the microorganism is selected among Enterobacteriaceae, Cyanobacteriaceae, Bacillaceae, Clostridiaceae, Streptomyetaceae, Pseudomonaceae and Corynebacteriaceae. Even more preferentially, the microorganism is either from the species *Escherichia coli*, *Synechococcus elongatus*, *Bacillus subtilis*, *Lactobacillus brevis*, *Pseudomonas putida*, *Clostridium acetobutylicum*, *Thermoanaerobacterium saccharolyticum*, *Clostridium thermocellum*, or *Corynebacterium glutamicum*. Preferentially, the yeast is *Saccharomyces cerevisiae*.

In another specific embodiment of the invention, modifications are introduced into the microorganism with enhanced Ferredoxin-NADP⁺ reductase activity specifically for the production of n-butanol by a new metabolic pathway (Figure 2).

Preferentially, the pyruvate ferredoxin oxidoreductase, the thiolase, the β -hydroxybutyryl-CoA dehydrogenase, the crotonase, the butyryl-coA dehydrogenase, the butyraldehyde dehydrogenase, the butanol dehydrogenase and the soluble transhydrogenase activities are increased. The preferred method is the overexpression of the *pfor* (CA_C2229), *fdx* (CA_C0303) genes coding for pyruvate ferredoxin oxidoreductase and ferredoxin respectively, *atoB* gene coding for thiolase, *hbd* (CA_C2708) gene coding for β -hydroxybutyryl-CoA dehydrogenase, *crt* (CA_C2712) gene coding for crotonase, *bcd* (CA_C2711), *etfA* (CA_C2709) and *etfB* (CA_C2710) genes coding for butyrylCoA dehydrogenase complex, *adhE2* (*cap035*) gene coding for bifunctional butyraldehyde and butanol dehydrogenase and *udhA* gene coding for soluble transhydrogenase. Additionally, the activity Fe-S cluster assembly proteins, IscS, IscU, and IscA are increase to improve [4Fe-4S] assembly in Pfor and Fdx to increase the pyruvate ferredoxin oxidoreductase and the soluble transhydrogenase activities. This can be achieved by the attenuation of the *iscR* gene coding for a factor IscR, responsible for the repression of the *isc* operon. Furthermore,, one or several mutations can be introduced in the *pfor*, *fdx*, *atoB*, *hbd*, *crt*, *bcd*, *etfA*, *etfB*, *adhE2* or *udhA* genes to increase the pyruvate ferredoxin oxidoreductase, the thiolase, the β -hydroxybutyryl-CoA dehydrogenase, the crotonase, the butyryl-coA dehydrogenase, the butyraldehyde dehydrogenase, the butanol dehydrogenase or the soluble transhydrogenase activities under the culture conditions used.

In another embodiment of the invention, the methylglyoxal synthase enzyme activity, the first enzyme of the methylglyoxal bypass, encoded by the gene *mgsA* is attenuated.

Preferentially, in the microorganism according to the invention, some enzyme activities involved in by-product formation pathways are attenuated in order to increase the yield of n-butanol:

- The pyruvate formate lyase activity, responsible for the synthesis of acetyl-CoA and formate from pyruvate, encoded by the genes *pflA* and *pflB*.
- The pyruvate dehydrogenase activity, responsible for the synthesis of acetyl-CoA and NADH from pyruvate, encoded by the genes *aceE*, *aceF* and *lpd*. The fumarate reductase activity, responsible for the synthesis of succinate from fumarate, encoded by the operon *frdABCD*.
- The lactate dehydrogenase activity, responsible for the synthesis of lactate from pyruvate, encoded by the gene *ldhA*.
- The phosphotransacetylase and acetate kinase activities, responsible for the synthesis of acetate in two steps from acetyl-CoA, encoded respectively by the genes *pta* and *ackA*.
- The pyruvate oxidase activity, responsible for the synthesis of acetate in one step from pyruvate, encoded by the gene *poxB*.
- The alcohol-aldehyde dehydrogenase activity, responsible for the synthesis of ethanol from acetyl-CoA, encoded by the gene *adhE*.

Preferentially, the attenuation of activity is obtained by the attenuation of at least one of these genes.

Preferentially the microorganism designed to produce n-butanol by this new metabolic pathway is selected among bacteria, yeasts or fungi. More preferentially, the microorganism is selected among Enterobacteriaceae, Cyanobacteriaceae, Bacillaceae, Clostridiaceae, Streptomycetaceae, Pseudomonaceae and Corynebacteriaceae. Even more preferentially, the microorganism is either from the species *Escherichia coli*, *Synechococcus elongatus*, *Lactobacillus brevis*, *Pseudomonas putida*, *Bacillus subtilis*, *Clostridium acetobutylicum*, *Thermoanaerobacterium saccharolyticum*, *Clostridium thermocellum* or *Corynebacterium glutamicum*. Preferentially, the yeast is *Saccharomyces cerevisiae*.

In another specific embodiment of the invention, modifications are introduced into the microorganism with enhanced Ferredoxin-NADP⁺ reductase activity specifically for the production of 1, 3 propanediol from glycerol by a new metabolic pathway (Figure 3).

Preferentially, the pyruvate ferredoxin oxidoreductase, the B12-independent glycerol dehydratase, the NADP⁺ dependent 1, 3 propanediol dehydrogenase and the membrane bound transhydrogenase activities are increased. The preferred method is the overexpression of the *pfor* (*CA_C2229*) and *fdx* (*CA_C0303*) genes coding for pyruvate ferredoxin oxidoreductase and ferredoxin, *dhaB1*, *dhaB2* genes coding for glycerol dehydratase and glycerol dehydratase activating enzyme (Sarcabal et al, 1999), *yqhD* gene coding for 1, 3 propanediol dehydrogenase and *pntAB* genes coding for membrane bound transhydrogenase. Additionally, the activity of Fe-S cluster assembly proteins, IscS, IscU, and IscA are increased to improve [4Fe-4S] assembly in Pfor and Fdx to increase the pyruvate ferredoxin oxidoreductase activity. This can be achieved by the attenuation of the *iscR* gene coding for a factor IscR, responsible for the repression of the *isc* operon. Furthermore, one or several mutations can be introduced in the *pfor*, *fdx*, *dhaB1*, *dhaB2*, *yqhD* and *pntAB* genes to increase the pyruvate ferredoxin oxidoreductase, the glycerol dehydratase, the NADP⁺ dependent 1, 3 propanediol dehydrogenase and the membrane bound transhydrogenase activities under the culture conditions used.

Other genes whose expression may advantageously be enhanced are the following: *ack* and *pta* coding respectively for acetate kinase and phosphotransacetylase.

In another embodiment of the invention, the soluble transhydrogenase enzyme involved in NADPH oxidation to produce NADH encoded by the gene *udhA* is attenuated.

Preferentially, in the microorganism according to the invention, some enzyme activities involved in by-product formation pathways are attenuated in to increase the yield of 1, 3-propanediol:

- The pyruvate formate lyase activity, responsible for the synthesis of acetyl-CoA and formate from pyruvate, encoded by the genes *pflA* and *pflB*.
- The pyruvate dehydrogenase activity, responsible for the synthesis of acetyl-CoA and NADH from pyruvate, encoded by the genes *aceE*, *aceF* and *lpd*. The fumarate reductase activity, responsible for the synthesis of succinate from fumarate, encoded by the operon *frdABCD*.
- The lactate dehydrogenase activity, responsible for the synthesis of lactate from pyruvate, encoded by the gene *ldhA*.
- The pyruvate oxidase activity, responsible for the synthesis of acetate in one step from pyruvate, encoded by the gene *poxB*
- The alcohol-aldehyde dehydrogenase activity, responsible for the synthesis of ethanol from acetyl-CoA, encoded by the gene *adhE*.
- The aldehyde dehydrogenase activities, responsible of the synthesis of hydroxypropionate from hydroxypropionaldehyde encoded by the *aldA* and *aldB* genes
- The anaerobic glycerol-3-phosphate dehydrogenase, responsible of the synthesis of dihydroxyacetone phosphate from glycerol-3-phosphate encoded by the gene *glpA*
- The aerobic glycerol-3-phosphate dehydrogenase, responsible of the synthesis of dihydroxyacetone phosphate from glycerol-3-phosphate encoded by the gene *glpD*

Preferentially, the attenuation of activity is obtained by the attenuation of at least one of these genes.

Preferentially the microorganism designed to heterologously produce 1, 3 propanediol from glycerol by this new metabolic pathway is selected among bacteria, yeasts or fungi. More preferentially, the microorganism is selected among Enterobacteriaceae, Cyanobacteriaceae, Bacillaceae, Clostridiaceae, Streptomyetaceae, Pseudomonaceae and Corynebacteriaceae. Even more preferentially, the microorganism is either from the species *Escherichia coli*, *Synechococcus elongates*, *Lactobacillus brevis*, *Bacillus subtilis*, *Pseudomonas putida*, *Clostridium acetobutylicum*, *Thermoanaerobacterium saccharolyticum*, *Clostridium thermocellum* or *Corynebacterium glutamicum*. Preferentially, the yeast is *Saccharomyces cerevisiae*.

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In one embodiment of the invention, the microorganism with enhanced Ferredoxin-NADP+ reductase activity activity is further modified to produce 1,2-propanediol from a source of carbon by a new metabolic pathway.

Preferentially, the pyruvate ferredoxin oxidoreductase, methylglyoxal synthase, methylglyoxal reductase, 1,2-propanediol dehydrogenase and membrane bound transhydrogenase.

To obtain the increase of the specific enzymatic activities, the genes coding for these activities are preferentially overexpressed : the *pfor* (CA_C2229), and *fdx* (CA_C0303) coding for pyruvate ferredoxin oxidoreductase and ferredoxin, *mgsA* gene, coding for methylglyoxal synthase, *yqhD*, *yafB*, *ydH*, *yedW*, *yqhE*, *yeaE*, *yghZ*, *yajO*, *tas*, *ydgG*, and *ydbC*, all coding for methylglyoxal reductases, *gldA*, *fucO* or *sadh* from *C. beijerinckii* coding for 1,2-propanediol dehydrogenase and *pntAB* coding for membrane bound transhydrogenase.

The combination of the overexpression of the *pfor*, *fdx*, *mgsA*, *yqhD* and *gldA* genes or *pfor*, *fdx*, *mgsA*, *yqhD* and *sadh* and *pntAB* are preferentially used.

Additionally, the activity of Fe-S cluster assembly proteins, IscS, IscU, and IscA, are increase to improve [4Fe-4S] assembly in Pfor and Fdx to increase the pyruvate ferredoxin oxidoreductase and the soluble transhydrogenase activities. This can be achieved by the attenuation of the *iscR* gene coding for a factor IscR, responsible for the repression of the *isc* operon. Furthermore, one or several mutation can be introduced in the *pfor*, *fdx*, *mgsA*, *yqhD* and *gldA* to increase the pyruvate ferredoxin oxidoreductase, methylglyoxal synthase, methylglyoxal reductase and 1,2-propanediol dehydrogenase activities under the culture conditions used.

- Attenuation of the enzymatic activities involved in the Entner-Doudoroff pathway, encoded by the genes *edd* and *eda*. The Entner-Doudoroff pathway provides an alternative way to degrade glucose to glyceraldehyde-3-phosphate and pyruvate besides glycolysis. The attenuation of the Entner-Doudoroff pathway assures that most or at best all glucose is degraded via glycolysis and is utilized for the production of 1,2-propanediol.

- Attenuation of enzymes involved in the conversion of methylglyoxal into lactate: glyoxalase I and II, encoded respectively by the *gloA* and *gloB* genes, catalysing the synthesis of lactoyl glutathione from methylglyoxal and lactaldehyde dehydrogenases encoded by the *aldA* and *aldB* genes, catalysing the synthesis of (S) lactate from (S) lactaldehyde and glyoxalase III encoded by *yedU* gene (also known as *hchA*) catalysing the reduction of methylglyoxal to (S) lactate. Attenuation of these enzymes is intended to spare the precursor methylglyoxal for the synthesis of the desired products.

- Attenuation of enzymes involved in the synthesis of by-products such as lactate, ethanol and formate: lactate dehydrogenase, encoded by the gene *ldhA*, catalysing the synthesis of lactate from pyruvate, alcohol-aldehyde dehydrogenase, encoded by the gene *adhE*, catalysing the synthesis of ethanol from acetyl-CoA and pyruvate formate lyase, encoded by the genes *pflA* and *pflB*, catalysing the synthesis of acetyl-CoA and formate from pyruvate.

Preferentially, at least one of these genes is attenuated.

In another specific embodiment of the invention, the triose phosphate isomerase activity is attenuated. Preferentially, this result is achieved by attenuating the expression of the *tpiA* gene. More preferentially, the *tpiA* gene is deleted. The *tpiA* gene encodes the enzyme 'triose phosphate

isomerase', which catalyses the conversion of DHAP into glyceraldehyde 3-phosphate. The attenuation of the expression of this gene ensures that half of the glucose metabolized is converted to 1,2-propanediol.

5 In another specific embodiment of the invention, the glyceraldehyde 3 phosphate dehydrogenase activity is attenuated. The glyceraldehyde 3-phosphate dehydrogenase, also called GAPDH, is one of the key enzymes involved in the glycolytic conversion of glucose to pyruvic acid. The attenuation of the enzyme resulted in the redirection of part of the GA3P toward the synthesis of 1,2-propanediol. The yield of 1,2-propanediol over glucose can then be greater than 1 mole / mole.

10 In another embodiment of the invention, in the microorganism according to the invention, the efficiency of the sugar import is increased. A strong attenuation of the expression of the *gapA* gene resulting in a decrease of the carbon flux in the GAPDH reaction by more than 50% result in the synthesis of less than 1 mole of PEP per mole of glucose imported. PEP is required by the sugar-phosphotransferase system (PTS) normally used for the import of simple sugars into the cell, 15 since import is coupled to a phospho-transfer from PEP to glucose yielding glucose-6-phosphate. Thus reducing the amount of PEP will negatively impact on sugar import.

In a specific embodiment of the invention, the sugar might be imported into the microorganism by a sugar import system independent of phosphoenolpyruvate. The galactase-proton symporter encoded by the gene *galP* that does not involve phosphorylation can be utilized. 20 In this case, the imported glucose has to be phosphorylated by the glucose kinase activity encoded by the *glk* gene. To promote this pathway, the expression of at least one gene selected among *galP* and *glk* is increased.

In another specific embodiment of the invention, the efficiency of the sugar-phosphotransferase system (PTS) is increased by increasing the availability of the metabolite phosphoenolpyruvate by increasing the activity of the phosphoenolpyruvate synthase enzyme. This 25 enzyme is encoded by the *ppsA* gene. Therefore, preferentially in the microorganism, the expression of the *ppsA* gene is preferentially increased.

In another specific embodiment of the invention, the synthesis of the by-product acetate is prevented by attenuating at least one enzyme involved in its synthesis. It is preferable to avoid such 30 acetate synthesis to optimize the production of 1,2-propanediol.

To prevent the production of acetate, advantageously at least one gene selected among *ackA*, *pta* is attenuated. These genes all encode enzymes involved in the different acetate biosynthesis pathways.

35 To obtain an overexpression of a gene of interest, the man skilled in the art knows different methods, and for example:

1- Replacement of the native promoter of the gene with a promoter inducing a stronger level of expression of the gene of interest.

2- Introduction into the microorganism of an expression vector carrying and expressing said gene of interest.

5 3- Introduction of additional copies of the gene of interest into the chromosome of the microorganism.

Another way to obtain an increased enzymatic activity is to introduce into the gene of interest a specific mutation allowing the translation of a gene product presenting a higher activity than the native protein.

10 Under anaerobic or microaerobic conditions, availability of NADH for the reduction of the precursors into 1,2-propanediol is advantageously increased. This is obtained by alleviating the repression on the tricarboxylic acid cycle mediated by the global regulator ArcA (encoded by the *arcA* gene).

Preferentially the microorganism designed to produce mainly 1,2-propanediol is selected
15 among bacteria, yeasts or fungi. More preferentially, the microorganism is selected among Enterobacteriaceae, Cyanobacteriaceae, Bacillaceae, Clostridiaceae, Pseudomonaceae Streptomycetaceae and Corynebacteriaceae. Even more preferentially, the microorganism is either from the species *Escherichia coli*, *Synechococcus elongatus*, *Lactobacillus brevis*, *Bacillus subtilis*, *Pseudomonas putida*, *Clostridium acetobutylicum*, *Thermoanaerobacterium*
20 *saccharolyticum*, *Clostridium thermocellum* or *Corynebacterium glutamicum*. Preferentially, the yeast is *Saccharomyces cerevisiae*.

The term “attenuation of the expression of a gene” according to the invention denotes the partial or complete suppression of the expression of a gene, which is then said to be “attenuated”.
25 This suppression of expression can be either an inhibition of the expression of the gene, a deletion of all or part of the promoter region necessary for the gene expression, or a deletion in the coding region of the gene. Preferentially, the attenuation of a gene is essentially the complete deletion of that gene, which gene can be replaced by a selection marker gene that facilitates the identification, isolation and purification of the strains according to the invention. A gene is inactivated
30 preferentially by the technique of homologous recombination (Datsenko, K.A. & Wanner, B.L., 2000).

The present invention provides for a method for modulating the Ferredoxin-NADP+ reductase activity enzymatic activity in a microorganism, wherein activity of the polypeptide of the invention is enhanced or attenuated in said microorganism.

35 Preferentially, in said method, the Ferredoxin-NADP+ reductase activity enzymatic activity is enhanced by overexpressing the polynucleotide of the invention.

Preferentially, in said method, the Ferredoxin-NADP⁺ reductase activity enzymatic activity is attenuated by attenuating the expression of the polynucleotide of the invention.

The invention is also related to a method for preparing ethanol, wherein a microorganism
5 according to the invention is grown in an appropriate culture medium comprising a source of carbon, and the produced ethanol is recovered. The production of ethanol is performed under microaerobic or anaerobic conditions, preferentially under anaerobic conditions.

The term "carbon substrate" or "source of carbon" means any carbon source capable of being metabolized by a microorganism wherein the substrate contains at least one carbon atom.
10 Authors refer particularly to renewable, inexpensive and fermentable carbon source such as monosaccharides, oligosaccharides, polysaccharides, single-carbon substrates, and polyols such as glycerol. Saccharides of the formula $(CH_2O)_n$ are also called oses or "simple sugars"; monosaccharides include fructose, glucose, galactose and mannose. Other carbon sources are disaccharides, trisaccharides, oligosaccharides and polysaccharides. Disaccharides include
15 saccharose (sucrose), lactose and maltose. Starch and hemicellulose are polysaccharides, also known as "complex sugars".

Advantageously the recovered ethanol is furthermore purified.

The invention is also related to a method for preparing n-butanol, wherein a microorganism
20 according to the invention is grown in an appropriate growth medium containing a carbon source, and the n-butanol is recovered. The production of n-butanol is performed under microaerobic or anaerobic conditions, preferentially under anaerobic conditions.

Advantageously, the recovered n-butanol is furthermore purified.

25 The invention is also related to a method for preparing 1,3-propanediol, wherein a microorganism according to the invention is grown in an appropriate growth medium containing glycerol as a carbon source, and the 1,3-propanediol is recovered. The production of 1,3-propanediol is performed under microaerobic or anaerobic conditions, preferentially under anaerobic conditions.

30 Advantageously, the recovered 1,3-propanediol is furthermore purified.

The invention is also related to a method for preparing 1,2-propanediol, wherein a microorganism according to the invention is grown in an appropriate growth medium containing a carbon source, and the 1,2-propanediol is recovered. The production of 1,2-propanediol is
35 performed under microaerobic or anaerobic conditions, preferentially under anaerobic conditions.

Advantageously, the recovered 1,2-propanediol is furthermore purified.

The culture conditions for fermentation processes can be readily defined by those skilled in the art. In particular, bacteria are fermented at temperatures between 20°C and 55°C, preferably between 25°C and 40°C, and preferably at about 35°C for *C. acetobutylicum* and at about 37°C for
5 *E. coli* and *K. pneumoniae*.

This process can be carried out either in a batch process, in a fed-batch process or in a continuous process.

Micro-aerobic conditions are defined as culture conditions wherein low percentages of oxygen (e.g. using a mixture of gas containing between 0.1 and 10% of oxygen, completed to
10 100% with nitrogen), is dissolved into the liquid phase.

Anaerobic conditions are defined as culture conditions wherein no oxygen is provided to the culture medium. Strictly anaerobic conditions are obtained by sparging an inert gas like nitrogen into the culture medium to remove traces of other gas. Nitrate can be used as an electron acceptor to improve ATP production by the strain and improve its metabolism.

15 The term “appropriate growth medium” according to the invention denotes a medium of known molecular composition adapted to the growth of the micro-organism. For example, a mineral culture medium of known set composition adapted to the bacteria used, containing at least one carbon source. In particular, the mineral growth medium for *E. coli* or *K. pneumoniae* can thus be of identical or similar composition to M9 medium (Anderson, 1946, *Proc. Natl. Acad. Sci. USA*
20 32:120-128), M63 medium (Miller, 1992; A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York) or a medium such as that defined by Schaefer *et al.* (1999, *Anal. Biochem.* 270: 88-96).

The carbon source used for the culture of *E. coli* or *C. acetobutylicum* is preferentially a
25 simple carbon source and can be arabinose, fructose, galactose, glucose, lactose, maltose sucrose, xylose or glycerol. An especially preferred simple carbon source is glucose.

The invention is described above, below and in the Examples with respect to *C. acetobutylicum* and *E. coli*. Thus the genes that can be attenuated, deleted or over-expressed for the initial and evolved strains according to the invention are defined using the denomination of the
30 genes from *E. coli* or *C. acetobutylicum*. However, this designation has a more general meaning according to the invention, and covers the corresponding genes in other micro-organisms. Using the GenBank references of the genes from *E. coli* or *C. acetobutylicum*, those skilled in the art can determine equivalent genes in other organisms than *E. coli* or *C. acetobutylicum*.

The means of identification of the homologous sequences and their percentage homologies
35 are well-known to those skilled in the art, and include in particular the BLAST programmes that can be used on the website <http://www.ncbi.nlm.nih.gov/BLAST/> with the default parameters

indicated on that website. The sequences obtained can be exploited (aligned) using for example the programmes CLUSTALW (<http://www.ebi.ac.uk/clustalw/>), with the default parameters indicated on these websites.

5 The PFAM database (protein families database of alignments and hidden Markov models <http://www.sanger.ac.uk/Software/Pfam/>) is a large collection of alignments of protein sequences. Each PFAM makes it possible to visualise multiple alignments, view protein domains, evaluate distributions among organisms, gain access to other databases and visualise known protein structures.

10 COGs (clusters of orthologous groups of proteins <http://www.ncbi.nlm.nih.gov/COG/>) are obtained by comparing protein sequences derived from 66 fully sequenced unicellular genomes representing 14 major phylogenetic lines. Each COG is defined from at least three lines, making it possible to identify ancient conserved domains.

15

DESCRIPTION OF DRAWING

Figure 1: New metabolic pathway for ethanol production

Figure 2: New metabolic pathway for butanol production

Figure 3: New metabolic pathway for 1.3 propanediol production

20 Figure 4: New metabolic pathway for 1.2 propanediol production

Figure 5: Another New metabolic pathway for 1.2 propanediol production

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EXAMPLES

EXAMPLE 1: Purification of Ferredoxin NADP⁺ activity in *C. acetobutylicum* and identification of the encoding gene

35 **1.1 – Ferredoxin NADP⁺ reductase activity and NADPH ferredoxin reductase activity assays:**

All enzyme assays were performed in the anaerobic workstation under a nitrogen atmosphere. All reagent solutions were prepared in the assay buffer (previously boiled and degassed with nitrogen) and kept under a nitrogen atmosphere. Specific activities were determined in a range where linearity with protein concentration was established. Each enzyme assay was done at least in duplicate. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the conversion of 1 μ mol of substrate per min. The concentrations of components in the reaction mixtures (1 ml total volume) are given below.

In vitro ferredoxin NADP⁺ reductase activity was assayed by measuring the reduction of NADP⁺ using electrons from reduced methyl viologen or reduced ferredoxin (CA_C0303) with H₂ as the reductant of the methyl viologen or ferredoxin (CA_C 0303) (Demuez et al. 2007) in the presence of the Fe-Fe Hydrogenase from *Clostridium acetobutylicum* (CA_C0028) (Vasconcelos et al. 1994, Girbal et al. 2005). The reaction was performed anaerobically at 37°C, in 100 mM Tris-HCl buffer (pH 7) with 2 mM DTT, 25 μ M FAD, 150 μ M methyl viologen, 1,6 mM NADP⁺, 6 U (or more) of purified hydrogenase HydA from *C. acetobutylicum* and crude extract (or purified protein) and followed by monitoring the increase in A₃₄₀ nm as an indication of the apparition of NADPH using a spectrophotometer (Hewlett Packard 8453). After a gentle stream with hydrogen in the quartz cuvette cells, assays were initiated by the addition of methyl-viologen and then, after the reduction of methyl viologen followed at a wavelength of 560 nm, by the addition of NADP⁺. In all reactions, non-enzymatic rates were subtracted from the observed initial reaction rates.

In vitro NADPH ferredoxin reductase activity was assayed by monitoring the increase of A₅₆₀ nm as an indication of the reduction of methyl viologen using a spectrophotometer (Hewlett Packard 8453). The reaction was carried out anaerobically at 37°C in quartz cuvette cells in 100 mM Tris-HCl buffer (pH 7,6) with 2 mM DTT, 10 μ M FAD, 250 μ M NADPH, ethanol 3% vol/vol, 45 U Adh (*S. cerevisiae*), 10 mM methyl viologen, 250 μ M NADPH, and crude extract or purified protein. Assays were initiated by the addition of methyl viologen. In all reactions, non-enzymatic rates were subtracted from the observed initial reaction rates.

The extinction coefficient of methyl viologen at 560 nm and NADPH at 340 were 7.71 mM⁻¹ cm⁻¹ and 6.29 mM⁻¹ cm⁻¹ respectively. The total protein concentration of the cell-free extract or purified fraction was determined using the Bradford method (Biorad reagent) (Bradford 1976) with bovine serum albumin as the standard.

1.2 – Purification of Ferredoxin NADP⁺ reductase in *C. acetobutylicum* under solventogenic conditions:

C. acetobutylicum ATCC 824 strain was kept in spore form at -20°C in the synthetic medium (MS), as previously described (Meynial-Salles et al. 2005). The flask cultures of *C. acetobutylicum* strains were grown anaerobically in synthetic medium (MS) and were inoculated with spore stock at 10% (v/v) and heat-shocked at 80°C for 15 min. Cells were grown up at 37°C to

an OD₆₂₀ nm of approximately 2.0 and the pH was maintained by buffering the culture medium with calcium carbonate, prior to inoculation of the bioreactor at 10% (v/v).

The pH-controlled batch fermentations were performed in synthetic medium. A 2 L Biostat B bioreactor (Sartorius, Aubagne, France) was used with a working volume of 1.3 L. After
5 sterilization, the medium was sparged with O₂-free nitrogen for 30 min. During the course of the experiment, the medium was maintained under a slight nitrogen overpressure to avoid O₂ entry into the reactor. All tubing was made of butyl rubber, and the reactor gas outlet was protected with a pyrogallol arrangement.

Cultures were stirred at 300 rpm, the temperature was set at 35°C, and the pH was
10 maintained at 4.8 with automatic addition of NH₄OH (3N). The cell concentration was measured turbidimetrically by monitoring the optical density (OD) at 620 nm (Biochrom libra S11) and products formation were measured in duplicate using High Performance Liquid Chromatography (HPLC) analysis (Agilent 1200 series, Massy, France) (Dusséaux et al. 2013).

When the OD₆₂₀ nm reached an approximatively value of 16, after the switch from
15 acidogenic to solventogenic phase, cells were harvested under Hydrogen pressure and transferred into anaerobic chamber. Cells were washed and concentrated 20 times in 100 mM Tris-HCl 2 mM DTT 10% glycerol (pH 7.6) buffer and frozen at -80°C.

The entire purification procedure was performed under anaerobic conditions. All
purification buffers were preliminary degassed and 10 µM FAD and 2 mM DTT were added to
20 prevent non-reversible activity losses.

Later, the frozen cells from solventogenic batch cultures of *C. acetobutylicum* ATCC 824 were thawed and broken by sonication using an ultrasonic disintegrator (vibracell 72434, Bioblock) at 4°C in four cycles of 30 sec with 2-min intervals. Debris were removed by centrifugation at 8600 g for 10 min, 4°C (Sigma centrifuge 2-16K). Nucleic acids were precipitated by addition of
25 streptomycin sulfate (200 mg/ml) in the supernatant and removed by centrifugation as before. The recovered extract was then diluted 5 times in 100mM Tris-HCL buffer (pH 8) before loaded on a 5mL HiTrap Capto-DEAE matrix (GE Healthcare, ref. 28-9165-40) connected to an AKTA purifier (GE Healthcare, Sweden). Active fractions were screened with the NADPH Ferredoxin Reductase assay using methyl viologen as previously described. The column was equilibrate in 100 mM Tris-
30 HCL buffer (pH 8) and elution was performed with a 3 steps gradient of 100mM Tris-HCL + 1M NaCl buffer (pH 8): 1CV 0-4%, 20CV 4-16% (target elution) and 5CV 16-100% ; 2 mL fractions were collected. The most active fraction from Capto-DEAE column was concentrated on Vivaspinn 15/10000MW (Sartorius Stedim, ref. VS1502) to reduce the sample volume at 150 µL by centrifugation at 3000g, 15 min. For the last purification step, the 150 µL sample volume was
35 applied on a Superose 12, 10/300 GL column (GE Healthcare, ref. 17-5173-01) preliminary equilibrated in 100 mM Tris-HCl + 150 mM NaCl buffer (pH7,6) and fractions of 400µL were

collected. Finally, the total protein concentration of the cell-free extract or purified fraction was determined using the Bradford method (Biorad reagent) (Bradford 1976) with bovine serum albumin as the standard.

Yields and purification factor of each step were calculated. The purity factor of the separate active fractions was also evaluated using a SDS electrophoresis in 40 mL polyacrylamide gels.

Both in vitro ferredoxin NADP⁺ reductase and NADPH ferredoxin reductase activities were evaluated on the recovered pure fraction. According to table 1, the purified enzyme is strictly NADPH/NADP⁺ dependent:

Table 1: Ferredoxin NADP⁺ reductase and NADPH ferredoxin reductase specific activities on the final purified fraction using methyl viologen as a substrate:

Activities	Specific activity U/mg protein			
	Ferredoxin NAD ⁺ reductase	Ferredoxin NADP ⁺ reductase	NADH ferredoxin reductase	NADPH ferredoxin reductase
Purified fraction	ND*	0.43 +/- 0.064 9.5 +/- 1.41 [#]	ND*	56.5 +/- 2.03

*ND: non-detectable; # using reduced ferredoxin (CA_C0303) instead of methyl viologen as the substrate.

1.3 – Identification of the gene coding for Ferredoxin NADP⁺ reductase activity:

The region of the gel corresponding to the protein at 45 kDa was cut off using a sterile pipette tip. This gel plug was then used for identification of proteins by mass spectroscopy.

The sample was subjected to trypsin digestion and analyzed by nano LC/MS/MS on a CapLC-Q-TOF2 (Waters) and by MALDI on MALDI MX (Waters). The candidate proteins were identified with the softwares ProteinLynx Global Server (Waters) and Mascot (Matrix Science) using the protein data bank of *C. acetobutylicum*. For both analyses, there was only one protein identified with a significant score (77% sequence coverage). This 45 kDa protein was shown to be encoded by CA_C0764 a gene annotated to encode for a small subunit of glutamate synthase.

EXAMPLE 2: Modulation of Ferredoxin NADP⁺ reductase activity in *C. acetobutylicum*:

2.1 – Disruption of *CA_C0764* gene in *C. acetobutylicum*, validation of loss of Ferredoxin NADP⁺ reductase activity and enhanced selectivity for acetone:

To investigate the involvement of *CA_C0764* in the in vivo butanol metabolic pathway in *C. acetobutylicum*, the group II intron-based Clostron technology (Heap J et al . 2007) was used to inactivate the *CA_C0764* gene into the *C. acetobutylicum* Δ *cac15* (Soucaille et al. 2006). This technology uses the insertion of a group II intron into a genomic target site coupled to a retrotransposition-activated marker (erythromycin resistance) allowing a stable gene inactivation.

2.1.1: Construction of the *C. acetobutylicum* Δ *cac15cac0764-408s::CT* mutant:

The intron target site was identified at the 408/409s bp (from the start of the orf) on the sense strand and intron retargeting PCR primers were designed using a computer algorithm (Perutka et al. 2004):

1-408/409s-IBS consisting of 53 bases (SEQ ID NO 3)

AAAAAAGCTTATAATTATCCTTAGGCTACAATGTTGTGCGCCCAGATAGGGTG

2. 408/409s-EBS1d consisting of 60 bases (SEQ ID NO 4)

CAGATTGTACAAATGTGGTGATAACAGATAAGTCAATGTTACTAACTTACCTTTCTTTG
T

3. 408/409s-EBS2 consisting of 49 bases (SEQ ID NO 5)

TGAACGCAAGTTTCTAATTTTCGATTTAGCCTCGATAGAGGAAAGTGTCT

The three 408/409-IBS, 408/409-IEBS1d, 408/409-EBS2 and the EBS universal primers were used in a single-tube reaction with the pMTL007 plasmid (Heap J. et al. 2007) to mutate the intron at several positions spanning a 350 bp region. The PCR reaction, which re-targets the intron by primer-mediated mutation, was performed according to the Targetron Gene Knockout System kit Protocol (<http://www.sigmaaldrich.com/life-science/functional-genomics-and-rnai/targetron.html>). The 350 bp PCR fragment was purified and then cloned into the pMTL007 plasmid at the HindIII and BsrGI sites, to replace the original intron fragment. The ligation product was then introduced into Top10 chemically competent *E. coli* cells (InvitrogenTM). Some single colonies were then grown in LB liquid culture supplemented with ampicillin (100 µg/ml), overnight at 37°C, to finally carry out a DNA plasmid extraction (GenElute HP plasmid miniprep kit, Sigma) and check for the presence of the pMTL007:Ca-*cac0764-408s* plasmid.

The retargeted plasmid pMTL007:Ca-*cac0764-408s* was finally controlled by restriction and by DNA sequencing using the 408/409-IBS and 408/409-IEBS1d primers.

C. acetobutylicum Δ *cac15* was then electroporated as previously described (Mermelstein et al.,1992) using unmethylated retargeted pMTL007:Ca-*cac0764-408s* plasmid, because the

CA_C1502 gene encoding the type II restriction endonuclease Cac824I was deleted (Soucaille et al. 2006). After 5 hours of recovery, cells were plated on RCA (Clostridium Nutrient Medium with 15 g/l agar, Fluka (Saint-Quentin Fallavier, France, n°27546) medium supplemented with thiamphenicol (10 µg/ml). Single colonies were chosen from the plate and streaked separately on a

RCA plate with erythromycin (40 µg/ml) to select integrants.

The insertion mutants were screened via colony-PCR using the two following primers flanking the target site:

cac0764del_for (SEQ ID NO 6): (homologous to the sequence 886263 to 886287)

cgagccaataaaatttcacgagata

cac0764del_rev (SEQ ID NO 7): (homologous to the sequence 886512 to 886541)

ccaacctctataagtcttttcaagctta

PCR products were purified and confirmed by DNA sequencing.

One of the colonies was selected to cure the pMTL007:Ca-cac0764-408s plasmid and generate the *C. acetobutylicum* Δ cac15cac0764408s::CT.

This clone was inoculated into Clostridium Growth Medium (CGM) medium supplemented with erythromycin (40 µg/mL) for successive subcultures, as previously described (Dusséaux et al. 2013). 100 µL of fully grown culture was inoculated into 1 mL fresh CGM medium supplemented with erythromycin (40 µg/mL), grown anaerobically at 37°C for at least 12 h until full growth achieved. This transfer process was repeated at least 3 times. The last culture was then plated onto solid RCA plate supplemented with erythromycin (40 µg/mL). Colonies were re-streaked successively onto RCA plates supplemented with thiamphenicol (10 µg/mL) and then onto RCA plates supplemented with erythromycin (40 µg/mL). One erythromycin-resistant and thiamphenicol-sensitive clone was selected and inoculated into 3 mL of synthetic medium supplemented with erythromycin (40 µg/mL), grown anaerobically at 37 °C for at least 24h and transferred in 30 mL synthetic medium supplemented with erythromycin (40 µg/mL). The culture was grown anaerobically at 37°C for 7 days until sporulation and then the spore suspension was stored at – 20°C.

2.1.2: Phenotypic analysis of the *C. acetobutylicum* Δ cac15cac0764408s::CT mutant:

C. acetobutylicum Δ cac15cac0764408s::CT strain was kept in spore form at -20°C in the synthetic medium (MS), as previously described (Meynial-Salles et al. 2005). The flask cultures of *C. acetobutylicum* strains were grown anaerobically in synthetic medium (MS) and were inoculated with spore stock at 10% (v/v) and heat-shocked at 80°C for 15 min. Cells were grown up at 37°C during several days, and the pH was maintained by buffering the culture medium with calcium carbonate. As a control, the *C. acetobutylicum* $\square$ cac15 was grown in the same conditions.

The comparative phenotypic analysis was performed by measuring both glucose consumption as well as the concentration of fermentation products as is shown in table 2:

	Products yield (% g/g glucose consumed)						
	Ethanol	Acetone	Butanol	Acetic acid	Lactate	Acetoin	Butyric acid
<i>C. acetobutylicum</i> <i>Δcac15</i>	1.90	9.70	23	0	6	3.15	1.37
<i>C. acetobutylicum</i> <i>Δcac15cac0764408s::CT</i>	1.80	13.88	16.10	0	5.59	5.18	1.58

- 5 The *C. acetobutylicum* *Δcac15cac0764408s::CT* strain produces more acetone and less butanol than the control strain.

2.1.3: Ferredoxin NADP⁺ reductase activity determination in *C. acetobutylicum* *Δcac15cac0764408s::CT* mutant:

10

C. acetobutylicum *Δcac15cac0764408s::CT* strain was grown anaerobically in synthetic medium (MS) at 37°C, inoculated with spore stock at 10% (v/v) and heat-shocked at 80°C for 15 min. Cells were grown up to an approximatively OD_{620nm} of 2, and the pH was maintained by buffering the culture medium with calcium carbonate. As a control, the *C. acetobutylicum* *Δcac15* was grown in the same conditions. Cells were transferred into anaerobic chamber, harvested, washed and 20 times concentrated in 100 mM Tris-HCl 2 mM DTT 10% glycerol (pH 7.6) buffer, frozen at -80°C or immediately used. Cells were further broken by sonication using an ultrasonic disintegrator (vibracell 72434, Bioblock) at 4°C in four cycles of 30 sec with 2-min intervals. Debris were removed by centrifugation at 8600 g for 10 min, 4°C (Sigma centrifuge 2-16K) and the acellular crude extract was loaded onto a SephadexG25 column to eliminate salts and metabolites, before the ferredoxin NADP⁺ reductase activity determination using ferredoxin as a substrate and following the procedure already described in 1.1 (table A).

20

Strains	Ferredoxin NADP ⁺ reductase specific activity U/mg protein
<i>C. acetobutylicum</i> <i>Δcac15</i>	0.64
<i>C. acetobutylicum</i> <i>Δcac15cac0764408s::CT</i>	0

Table A : Ferredoxin NADP⁺ reductase specific activities determined on crude extracts using ferredoxin as a substrate:

- 5 No ferredoxin NADP⁺ reductase activity is detected in the acellular crude extract prepared from the *C. acetobutylicum* $\Delta cac15cac0764408s::CT$ mutant cells in which the *CA_C0764* encoding gene is inactivated.

10

2 2– Overexpression of *CA_C0764* gene in *C. acetobutylicum*, validation of Ferredoxin NADP⁺ reductase activity increase and enhanced selectivity for butanol :

2.2.1 : Construction of the *pCLF 0764* for *CA_C 0764* overexpression:

- 15 The *CA_C0764* gene was amplified from the genomic DNA of *C. acetobutylicum* ATCC824 using primers Ocac0764l and Ocac0764r. The primers were designed to introduce a RBS region along with the *CA_C0764* gene, as well as placing BamHI and SfoI restriction sites upstream and downstream respectively:

Ocac0764f (SEQ ID NO 8):

- 20 AGGATCCATCAAAATTTAGGAGGTTAGTTA

Ocac0764r (SEQ ID NO 9):

GGCGCCTTAATTATTCTTGCAATACTCATCAATAGTTTC

- 25 The amplified PCR fragment was then subcloned into a Zero Blunt TOPO vector (Invitrogen, Saint Aubin, France) to yield the Zero Blunt TOPO–cac0764 plasmid and sequenced using universal primers T7P and T3P to assure that no mutations were introduced. The fragment containing the *CA_C0764* gene was purified on an agarose gel after digestion of the Zero Blunt TOPO–cac0764 vector with BamHI and SfoI. The 7 kb pSOS94 vector (Dusséaux et al. 2013) was also digested with BamHI and SfoI and ligated to the BamHI-SfoI digested *sadh* gene, yielding the
- 30 6.25 kb pSOS94-cac0764 vector. The pSOS94-cac0764 vector was digested with Sall, and the operon-containing fragments from each vector were purified on an agarose gel. The 4.9 kb pCLF1 vector (Soucaille et al. 2006) was digested with Sall, treated with Antarctic phosphatase and ligated with the previously purified fragment to yield the pCLFcac0764.

- 35 *C. acetobutylicum* $\Delta cac15$ was then electroporated as previously described (Mermelstein et al., 1992) using unmethylated pCLFcac0764 plasmid. After 5 hours of recovery, cells were plated on RCA (Clostridium Nutrient Medium with 15 g/l agar, Fluka (Saint-Quentin Fallavier, France,

n°27546) medium supplemented with thiamphenicol (10 µg/ml). Single colonies were chosen from the plate and streaked separately on a RCA plate with thiamphenicol (10 µg/ml). The transformants were screened using PCR amplification of the synthetic operon expressing CA_C0764. The cells were then transferred and grown on a MS agar plate with thiamphenicol (10 µg/ml) before inoculation into liquid MS medium. One selected clone was grown anaerobically at 37°C for 7 days until sporulation and then the spore suspension was stored at – 20°C.

2. 2.2: Phenotypic analysis of the *C. acetobutylicum* Δ *cac15* pCLF 0764 mutant:

C. acetobutylicum Δ *cac15*pCLF 0764 mutant was kept in spore form at -20°C in the synthetic medium (MS), as previously described (Meynial-Salles *et al.* 2005). The flask cultures of *C. acetobutylicum* strains were grown anaerobically in synthetic medium (MS) (at the exception that cystein was omitted) and were inoculated with spore stock at 10% (v/v) and heat-shocked at 80°C for 15 min. Cells were grown up at 37°C during several days, and the pH was maintained by buffering the culture medium with calcium carbonate. As a control, the *C. acetobutylicum* Δ *cac15* was grown in the same conditions.

The comparative phenotypic analysis was performed by measuring both glucose consumption as well as the concentration of fermentation products as is shown in table 3:

	Products yield (% g/g glucose consumed)						
	Ethanol	Acetone	Butanol	Acetic acid	Lactate	Acetoin	Butyric acid
<i>C. acetobutylicum</i> Δ <i>cac15</i>	1.96	9	20.9	-3.47	1.15	3.75	5.34
<i>C. acetobutylicum</i> Δ <i>cac15</i> pCLF <i>cac0764</i>	8.69	4.69	25.72	-3.14	0.63	1.39	3.53

The *C. acetobutylicum* Δ *cac15* pCLF*cac0764* strain produces less acetone and more butanol and ethanol than the control strain.

2.2.3. Ferredoxin NADP⁺ reductase activity determination in *C. acetobutylicum* Δ *cac15* pCLF 0764 mutant:

C. acetobutylicum $\Delta cac15$ pCLF 0764 mutant was grown anaerobically in synthetic medium (MS), at 37°C, inoculated with spore stock at 10% (v/v) and heat-shocked at 80°C for 15 min. Cells were grown up to an approximatively OD_{620nm} of 2, and the pH was maintained by buffering the culture medium with calcium carbonate. As a control, the *C. acetobutylicum* $\Delta cac15$ was grown in the same conditions. Cells were transferred into anaerobic chamber, harvested, washed and 20 times concentrated in 100 mM Tris-HCl 2 mM DTT 10% glycerol (pH 7.6) buffer, frozen at -80°C or immediately used. Cells were further broken by sonication using an ultrasonic disintegrator (vibracell 72434, Bioblock) at 4°C in four cycles of 30 sec with 2-min intervals. Debris were removed by centrifugation at 8600 g for 10 min, 4°C (Sigma centrifuge 2-16K) and the acellular crude extract was loaded onto a SephadexG25 column to eliminate salts and metabolites, before the ferredoxin NADP⁺ reductase activity determination using ferredoxin as a substrate and following the procedure already described in 1.1 (table B).

Table B: Ferredoxin NADP⁺ reductase specific activities determined on crude extracts using ferredoxin as a substrate:

Strains	Ferredoxin NADP ⁺ reductase specific activity U/mg protein
<i>C. acetobutylicum</i> $\Delta cac15$	0.64
<i>C. acetobutylicum</i> $\Delta cac15$ pCLFCA_C0764	2

A 3-fold increase of the ferredoxin NADP⁺ reductase activity is measured in the acellular crude extract prepared from the *C. acetobutylicum* $\Delta cac15$ pCLFCA_C0764 mutant cells in which the CA_C0764 encoding gene is overexpressed from a plasmid.

EXAMPLE 3: Heterologous production of ethanol by a new metabolic pathway in an *E. coli* strain with an enhanced Ferredoxin NADP⁺ reductase activity:

25

3.1: construction of an *E. coli* MG1655 $\Delta ldhA::FRT$, $\Delta pflAB::FRT$, $\Delta ack-pta::FRT$, $\Delta iscR::FRT$, $\Delta frdABCD::FRT$ $\Delta ace::FRT$, $\Delta mgsA$ modified strain

3.1.1 Construction of an *E. coli* MG1655 $\Delta ldhA::FRT$ -cm- FRT modified strain

The *ldhA* gene was replaced with a chloramphenicol antibiotic resistance cassette flanked by F₁p recognition target (FRT) that is generated by PCR by using primers with 80-nt homology

30

extensions, deleting most of the gene concerned. The used technique was described by Datsenko and Wanner in 2000.

Two oligonucleotides were designed and used to replace the *ldhA* gene:

1-DldhAr, consisting of 101 bases (SEQ ID NO 10):

5 ttaaaccagttcgttcgggcaggtttcgctttttccagattgcttaagtttgcagcgtagctctgagaaatactggctagCATATGAATAT
CCTCCTTAG

With a region (lower-case letters) homologous to the sequence (1439878-1439958) of the *ldhA* gene (sequence 1439878 to 1440867), a reference sequence on the website <http://ecocyc.org/> (Keseler *et al.* 2005) and a region (upper-case letters) for the amplification of the chloramphenicol
10 resistance cassette of the pKD3 plasmid (Datsenko and Wanner 2000).

2. DldhAf, consisting of 100 bases (SEQ ID NO 11):

gaaactcgccgtttatagcacaacacagtagcacaagaagtagctgcaacaggtgaacgagtcctttggccttgagctggTGTAGGCTG
GAGCTGCTTCG

With a region (lower-case letters) homologous to the sequence (1440786-1440865) of the *ldhA*
15 gene (sequence 1439878 to 1440867), a reference sequence on the website <http://ecocyc.org/> and a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette carried by the pKD3 plasmid.

Both oligonucleotides DldhAr and DldhAf were used to amplify the chloramphenicol resistance cassette from the pKD3plasmid. The amplified PCR fragment was then introduced by
20 electroporation into the *MG1655 E. coli* strain carrying the RED plasmid helper (pKD46) expressing the system α , β and *exo* genes encoding Gam, Bet and Exo recombinases to promote homologous recombination. The antibiotic-resistant transformants were then selected and the replacement of the *ldhA* gene by the chloramphenicol cassette was checked by PCR analysis using both hslJC and ldhAC2 oligonucleotides.

25 3-hslJC (SEQ ID NO 12):

gccatcagcaggcttagcgc (homologous to the sequence 1439724 to 1439743)

4-ldhAC2 (SEQ ID NO 13):

gggtattgtggcatgtttaaccg (homologous to the sequence 1441007 to 1441029).

The chloramphenicol resistance cassette was then eliminated by using the Flp helper pCP20
30 plasmid (Cheperanov and Wagnagel 1995). Cm^R mutants were transformed with pCP20, and ampicillin-resistant transformants were selected at 30°C, after which a few were colony purified at 42°C and then tested for loss of the antibiotic resistance by PCR analysis using oligonucleotides previously described (SEQ ID N°12 and 13).

3.1.2 Construction of a *E. coli* MG1655 $\Delta pflAB::FRT\text{-}cm\text{-}FRT$ modified strain:

The *pflAB* genes were replaced with a chloramphenicol antibiotic resistance cassette flanked by F₁p recognition target (FRT) that is generated by PCR by using primers with 80-nt homology extensions deleting most of the concerned gene. The used technique was described by Datsenko and Wanner in 2000.

Two oligonucleotides were designed and used to replace the *pflAB* genes:

1. DpflBr, consisting of 100 bases (SEQ ID NO 14):

ccggacatcctgcgttgccgtaaatctggtgttctgaccggtctgccagatgcataatggccgtggccgtatcatcggtgaCATATGAAT
ATCCTCCTTAG

With a region (lower-case letters) homologous to the sequence (952236 -952315) of the *pflB* gene (sequence 950495-952777), a reference sequence on the website <http://ecocyc.org/> and a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette of the pKD3 plasmid (Datsenko and wanner 2000).

2. DpflAf, consisting of 100 bases (SEQ ID NO 15):

gatgcactataagatgtgttaaaaacgctgtagcagaatgaagcgcggaataaaaaagcggcaactcaataaagttgccgTGTAGGCT
GGAGCTGCTTCG

With a region (lower-case letters) homologous to the sequence (949470-949549) located upstream the *pflA* gene (sequence 949563-950303), a reference sequence on the website <http://ecocyc.org/> and a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette carried by the pKD3 plasmid.

Both oligonucleotides DpflBr and DpflAf were used to amplify the chloramphenicol resistance cassette from the pKD3 plasmid. The amplified PCR fragment was then introduced by electroporation into the MG1655 *E. coli* strain carrying the RED plasmid helper (pKD46) expressing the system α , β and *exo* genes encoding Gam, Bet and Exo recombinases to promote homologous recombination. The antibiotic-resistant transformants were then selected and the replacement of the *pflAB* genes by the chloramphenicol cassette was checked by PCR analysis using both *pflAB1* and *pflAB2* oligonucleotides.

3. *pflAB1* (SEQ ID NO 16): agacattaaaaatatacgtgcagctaccg (homologous to the sequence 948462 to 948491)

4. pflAB2 (SEQ ID NO 17): gtgaaagctgacaacccttttgatctttta (homologous to the sequence 953660 to 953689).

3.1.3 Construction of an *E. coli* MG1655 $\Delta ldhA::FRT \Delta pflAB::cm$ modified strain.

Both *pflA* and *pflB* genes were then replaced with a chloramphenicol resistance cassette
5 into the MG1655 $\Delta ldhA::FRT$ strain and the deletion was achieved by generalized P1 phage transduction from the single mutant lysate (Miller 1972).

The Cm^R transductants were selected on plate and the replacement of the *pflAB* genes by the chloramphenicol cassette into the *MG1655* $\Delta ldhA::FRT$ was checked by PCR analysis using
10 both pflAB1 and pflAB2 oligonucleotides. Finally, both hslJC and ldhAC2 primers were also used in PCR analysis to confirm the deletion of *ldhA* the gene in the strain $\Delta pflAB::cm$. The resulting strain was named MG1655 $\Delta ldhA::FRT \Delta pflAB::cm$.

The chloramphenicol-resistance cassette was then eliminated by using the Flp helper pCP20 plasmid (Cheperanov and Wagnagel 1995). Cm^R mutants were transformed with pCP20,
15 and ampicillin-resistant transformants were selected at 30°C, after which a few were colony purified at 42°C and then tested for loss of the antibiotic resistance by PCR analysis using oligonucleotides previously described (SEQ ID N°16 and 17). The new strain was named MG1655 $\Delta ldhA::FRT \Delta pflAB::FRT$.

3.1.4 Construction of an MG1655 $\Delta ackA-pta::cm$ *E. coli* modified strain:

20 The *ackA-pta* genes were replaced with a chloramphenicol antibiotic resistance cassette flanked by Flp recognition target (FRT) that is generated by PCR by using primers with 80-nt homology extensions deleting most of the concerned gene. The used technique was described by Datsenko and Wanner in 2000.

25 Two oligonucleotides were designed and used to replace the *ackA-pta* genes:

1. DackAf, consisting of 100 bases (SEQ ID NO 18):

gtcgagtaagtagtactggttctgaactcgcggtagttcttactgaaatttgccatcatcgatgcagtaaatggtgaagTG TAGGCTGG
AGCTGCTTCG

30

With a region (lower-case letters) homologous to the sequence (2411494-2411573) of the *ackA* gene (sequence 2411492 to 2412694), a reference sequence on the website <http://ecocyc.org/> and a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette of the pKD3 plasmid (Datsenko and Wanner 2000).

2. Dptar, consisting of 97 bases (SEQ ID NO 19):

tgctgtgcagactgaatcgagtcagcgcatggtgttagacgatatcgtaaccagtgcgccacgggacaggtcgttCATATGAATA
TCCTCCTTAG

5

With a region (lower-case letters) homologous to the sequence (2414906-2414830) of the *pta* gene (sequence 2412769 to 2414913), a reference sequence on the website <http://ecocyc.org/> and a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette carried by the pKD3 plasmid.

10 Both DackAf and Dptar oligonucleotides were used to amplify the chloramphenicol resistance cassette from the pKD3 plasmid. The amplified PCR fragment was then introduced by electroporation into the *MG1655 E. coli* strain carrying the RED plasmid helper (pKD46) expressing the system α , β and *exo* genes encoding Gam, Bet and Exo recombinases to promote homologous recombination. The antibiotic-resistant transformants were then selected and the
15 replacement of the *ackA-pta* genes by the chloramphenicol cassette was checked by PCR analysis using both B2295f and YfcCR oligonucleotides.

3. B2295f (SEQ ID NO 20):

gcatgggtaaacttaaggcg (homologous to the sequence 2410902 to 2410921)

20

4. YfcCr (SEQ ID NO 21):

taatcaccaacgtatcgggc (homologous to the sequence 2415147 to 2415166).

3.1.5: Construction of an *E. coli* MG1655 Δ *ldhA*::FRT Δ *pflAB*::FRT *ΔackA-pta*::FRT modified strain

25

Both *ackA* and *pta* genes were then replaced with a chloramphenicol resistance cassette flanked by Flp recognition target (FRT) into the MG1655 Δ *ldhA*::FRT Δ *pflAB*::FRT strain and the deletion was achieved by generalized P1 phage transduction from the single mutant lysate (Miller
30 1972).

The Cm^R transductants were selected on plate and the replacement of the *ackA-pta* genes by the chloramphenicol cassette into the *MG1655ΔldhA::FRT ΔpflAB::FRT* was checked by PCR analysis using both B2295f and YfcCr oligonucleotides. Finally, both i) *hslJC* and *ldhAC2* and *pFIAB1* and *pflAB2* couple of primers were also used in PCR analysis to confirm the deletion of

ldhA and *pflAB* genes in the strain $\Delta ackA-pta::cm$. The resulting strain was named MG1655 $\Delta ldhA::FRT \Delta pflAB::FRT \Delta ackA-pta::cm$.

The chloramphenicol-resistance cassette was then eliminated by using the Flp helper pCP20 plasmid (Cheperanov and Wagnagel 1995). Cm^R mutants were transformed with pCP20, and ampicillin-resistant transformants were selected at 30°C, after which a few were colony purified at 42°C and then tested for loss of the antibiotic resistance by PCR analysis using oligonucleotides previously described (SEQ ID N°20 and 21). The new strain was named MG1655 $\Delta ldhA::FRT \Delta pflAB::FRT \Delta ackA-pta::FRT$.

3.1.7. Construction of an *E. coli* MG1655 $\Delta iscR::km$ modified strain

The *iscR* gene was replaced with a kanamycin antibiotic resistance cassette flanked by Flp recognition target (FRT) that is generated by PCR by using primers with 50-nt homology extensions, deleting most of the concerned gene. The used technique was described by Datsenko and Wanner in 2000.

Two oligonucleotides were designed and used to replace the *iscR* gene:

1. DiscRf, consisting of 70 bases (SEQ ID NO 22):

tacaataaaaaccccgggcagggcgagtttgaggtgaagtaagacatgATTCCGGGGATCCGTCGACC

With a region (lower-case letters) homologous to the sequence (2660151-2660200) corresponding to the upstream chromosomal sequence of the *iscR* gene (sequence 2659665 to 2660153), a reference sequence on the website <http://ecocyc.org/>, including the *iscR* gene initiation codon, and a region (upper-case letters) for the amplification of the kanamycin resistance cassette carried by the pKD13 plasmid.

25

2. DiscRr, consisting of 70 bases (SEQ ID NO 23):

cactccggcctgattctgaattcttttattaagcgcgtaacttaacgtcTG TAGGCTGGAGCTGCTTCG

With a region (lower-case letters) homologous to the sequence (2659636-2659685) corresponding to the downstream chromosomal sequence of the *iscR* gene (sequence 2659665 to 2660153), a reference sequence on the website <http://ecocyc.org/>, including the codons for the six C-terminal residues and the stop codon of the *iscR* gene, and a region (upper-case letters) for the amplification of the kanamycin resistance cassette of the pKD13 plasmid Datsenko and Wanner in 2000.

Both DiscRf and DiscRr oligonucleotides were used to amplify the kanamycin resistance cassette from the pKD13 plasmid. The amplified PCR fragment was then introduced by electroporation into

the *MG1655 E. coli* strain carrying the RED plasmid helper (pKD46) expressing the system α , β and *exo* genes encoding Gam, Bet and Exo recombinases to promote homologous recombination. The antibiotic-resistant transformants were then selected and the replacement of the *iscR* gene by the chloramphenicol cassette was checked by PCR analysis using both *iscr1s* and *iscr1rv* oligonucleotides.

3. *iscr1s* (SEQ ID NO 24): cgccgcacccgacaacagg (homologous to the sequence 2660325 to 2660343)
4. *iscr1rv* (SEQ ID NO 25): tgctggtgatgatgtgcttcct (homologous to the sequence 2659253 to 2659275).

3.1.8. Construction of an *E. coli* *MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ ackA-pta::FRT Δ iscR::FRT modified strain:*

The *iscR* gene was then replaced with a kanamycin resistance cassette into the *MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ ackA-pta::FRT* strain and the deletion was achieved by generalized P1 phage transduction from the single mutant lysate (Miller 1972).

The Km^R transductants were selected on plate and the replacement of the *iscR* gene by the kanamycin cassette into the *MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ ackA-pta::FRT* was checked by PCR analysis using both *iscs* and *iscr1rv* oligonucleotides. Finally, i) *hslJC* and *ldhAC2*, ii) *pFLAB1* and *pflAB2* and iii) *B2295f* and *YfcCR* couple of primers were also used in PCR analysis to confirm the deletion of *ldhA*, *pflAB* and *ackA-pta* genes respectively in the strain *Δ iscR::km*. The resulting strain was named *MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ ackA-pta::FRT Δ iscR::km*.

The kanamycin-resistance cassette was then eliminated by using the *Flp* helper pCP20 plasmid (Cheperanov and Wagnernagel 1995). Km^R mutants were transformed with pCP20, and ampicillin-resistant transformants were selected at 30°C, after which a few were colony purified at 42°C and then tested for loss of the antibiotic resistance by PCR analysis using oligonucleotides previously described (SEQ ID N°24 and 25). The new strain was named *MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ ackA-pta::FRT Δ iscR::FRT*.

3.1.9 Construction of a *E. coli* *MG1655 Δ frdABCD::cm* modified strain:

The *frdA*, *frdB*, *frdC* and *frdD* genes were replaced with a chloramphenicol antibiotic resistance cassette flanked by *Flp* recognition target (FRT) that is generated by PCR by using primers with 50-nt homology extensions, deleting most of the concerned genes. The used technique was described by Datsenko and Wanner in 2000.

Two oligonucleotides were designed and used to replace the *frdA*, *frdB*, *frdC* and *frdD* genes:

- 5 1. DfrdAf, consisting of 70 bases (SEQ ID N° 26):

accctgaagtacgtggctgtgggataaaaacaatctggaggaatgtcgtgTGTAGGCTGGAGCTGCTTCG

With a region (lower-case letters) homologous to the sequence (4380339-4380388) corresponding to the upstream chromosomal sequence of the *frdA* gene (sequence 4378533 to 4380341), a reference sequence on the website <http://ecocyc.org/>, including the *frdA* gene initiation codon, and
10 a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette of the plasmid pKD3 (Datsenko and Wanner in 2000).

2. DfrdDr, consisting of 70 bases (SEQ ID N° 27):

15 agcgccggccggatttacattggcgatgcgtagattgtaacgacaccaatCATATGAATATCCTCCTTAG

With a region (lower-case letters) homologous to the sequence (4377001-4377050) corresponding to the downstream chromosomal sequence of the *frdD* gene (sequence 4377030 to 4377389), a reference sequence on the website <http://ecocyc.org/>, including the codons for the six C-terminal
20 residues and the stop codon of the *frdD* gene, and a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette carried by the plasmid pKD3.

Both DfrdAf and DfrdDr oligonucleotides were used to amplify the chloramphenicol resistance cassette from the pKD3 plasmid. The amplified PCR fragment was then introduced by
25 electroporation into the *MG1655 E. coli* strain carrying the RED plasmid helper (pKD46) expressing the system α , β and *exo* genes encoding Gam, Bet and Exo recombinases to promote homologous recombination. The antibiotic-resistant transformants were then selected and the replacement of the *frdABCD* genes by the chloramphenicol cassette was checked by PCR analysis using both *frdABCD*1s and *frdABCD*1rv oligonucleotides.

30

3. *frdABCD*1s (SEQ ID NO 28):

ctggctcatacaaggcgtctcc (homologous to the sequence 4380779 to 4380800)

4. *frdABCD*1rv (SEQ ID NO 29):

tccattccactgttttagcggtta (homologous to the sequence 4376610 to 4376632).

35

3.1.10 Construction of an *E. coli* MG1655 $\Delta dhA::FRT \Delta pflAB::FRT \Delta ackA-pta::FRT \Delta iscR::FRT \Delta frdABCD::FRT$ modified strain:

The *frdABCD* genes were then replaced with a chloramphenicol resistance cassette into the MG1655 $\Delta dhA::FRT \Delta pflAB::FRT \Delta ackA-pta::FRT \Delta iscR::FRT$ strain and the deletion was achieved by generalized P1 phage transduction from the single mutant lysate (Miller 1972).

The Cm^R transductants were selected on plate and the replacement of the *frdABCD* genes by the chloramphenicol cassette into the MG1655 $\Delta dhA::FRT \Delta pflAB::FRT \Delta ackA-pta::FRT \Delta iscR::FRT$ was checked by PCR analysis using both *frdABCD*1s and *frdABCD*1rv oligonucleotides. Finally, i) hslJC and *ldhAC2*, ii) *pFLAB1* and *pFLAB2* iii) B2295f and *YfcCR* and iv) *iscr1s* and *iscr1rv* couple of primers were also used in PCR analysis to confirm the deletion of *ldhA*, *pflAB*, *ackA-pta* and *iscR* genes respectively, in the strain $\Delta iscR::km$. The resulting strain was named MG1655 $\Delta dhA::FRT \Delta pflAB::FRT \Delta ackA-pta::FRT \Delta iscR::FRT \Delta frdABCD::cm$.

The chloramphenicol-resistance cassette was then eliminated by using the Flp helper pCP20 plasmid (Cheperanov and Wagnernagel 1995). Cm^R mutants were transformed with pCP20, and ampicillin-resistant transformants were selected at 30°C, after which a few were colony purified at 42°C and then tested for loss of the antibiotic resistance by PCR analysis using oligonucleotides previously described (SEQ ID N°28 and 29). The new strain was named MG1655 $\Delta dhA::FRT \Delta pflAB::FRT \Delta ackA-pta::FRT \Delta iscR::FRT \Delta frdABCD::FRT$.

3.1.11 Construction of an *E. coli* MG1655 $\Delta aceE::km$ modified strain:

The *aceE* gene was replaced with a kanamycin antibiotic resistance cassette flanked by Flp recognition target (FRT) that is generated by PCR by using primers with 50-nt homology extensions deleting most of the concerned gene. The used technique was described by Datsenko and Wanner in 2000.

Two oligonucleotides were designed and used to replace the *aceE* gene:

1. DaceEf, consisting of 70 bases (SEQ ID NO 30):

acaggttcagaaaaactcaacgttattagatagataaggaataacccatgATTCCGGGGATCCGTCGACC

With a region (lower-case letters) homologous to the sequence (122970-123019) corresponding to the upstream chromosomal sequence of the *aceE* gene (sequence 123017 to 125680), a reference sequence on the website <http://ecocyc.org/>, including the *aceE* gene initiation codon, and a region (upper-case letters) for the amplification of the kanamycin resistance cassette carried by the pKD13 plasmid (Datsenko and Wanner 2000).

2. DaceEr, consisting of 70 bases (SEQ ID NO 31):

gatttcgatagccattattcttttacctcttacgccagacgcgggttaacTGTAGGCTGGAGCTGCTTCG

- 5 With a region (lower-case letters) homologous to the sequence (125660-125709) corresponding to the downstream chromosomal sequence of the *aceE* gene (sequence 123017 to 125680), a reference sequence on the website <http://ecocyc.org/>, including the codons for the six C-terminal residues and the stop codon of the *aceE* gene and a region (upper-case letters) for the amplification of the kanamycin resistance cassette of the pKD13 plasmid (Datsenko and Wanner 2000).
- 10 Both DaceEf and DaceEr oligonucleotides were used to amplify the kanamycin resistance cassette from the pKD13 plasmid. The amplified PCR fragment was then introduced by electroporation into the MG1655 *E. coli* strain carrying the RED plasmid helper (pKD46) expressing the system α , β and *exo* genes encoding Gam, Bet and Exo recombinases to promote homologous recombination. The antibiotic-resistant transformants were then selected and the replacement of the *aceE* gene by
- 15 the kanamycin cassette was checked by PCR analysis using both *aceEs* and *aceErv* oligonucleotides.

3. *aceEs* (SEQ ID NO 32): gagagccgccgtgagcgttc (homologous to the sequence 122806 to 122825)

4. *aceErv* (SEQ ID NO 33): ctgcaccgtcggcggaatcg (homologous to the sequence 125916 to
- 20 125935).

3.1.12. Construction of an *E. coli* MG1655 Δ *ldhA::FRT* Δ *pflAB::FRT* Δ *ackA-pta::FRT* Δ *iscR::FRT* Δ *frdABCD::FRT* Δ *aceE::FRT* modified strain

- The *aceE* gene was then replaced with a kanamycin resistance cassette into the MG1655 Δ *ldhA::FRT* Δ *pflAB::FRT* Δ *ackA-pta::FRT* Δ *iscR::FRT* Δ *frdABCD::FRT* strain and the deletion was
- 25 achieved by generalized P1 phage transduction from the single mutant lysate (Miller 1972).

- The km^R transductants were selected on plate and the replacement of the *aceE* gene by the kanamycin cassette into the MG1655 Δ *ldhA::FRT* Δ *pflAB::FRT* Δ *ackA-pta::FRT* Δ *iscR::FRT* Δ *frdABCD::FRT* was checked by PCR analysis using both *aceEs* and *aceErv* oligonucleotides. Finally, both i) *hsIJC* and *ldhAC2*, ii) *pFLAB1* and *pflAB2* iii) *B2295f* and
- 30 *YfcCR*, iv) *iscr1s* and *iscr1rv* and v) *frdABCD1s* and *frdABCD1rv* primers were also used in PCR analysis to confirm the deletion of *ldhA*, *pflAB*, *ackA-pta*, *iscR* and *frdABCD* genes respectively in the strain Δ *aceE::kan*. The resulting strain was named MG1655 Δ *ldhA::FRT* Δ *pflAB::FRT* Δ *ackA-pta::FRT* Δ *iscR::FRT* Δ *aceE::km*.

- The kanamycin-resistance cassette was then eliminated by using the Flp helper pCP20
- 35 plasmid (Cheperanov and Wagnernagel 1995). Km^R mutants were transformed with pCP20, and

ampicillin-resistant transformants were selected at 30°C, after which a few were colony purified at 42°C and then tested for loss of the antibiotic resistance by PCR analysis using oligonucleotides previously described (SEQ ID N°32 and 33). The new strain was named MG1655 Δ dhA::FRT Δ pflAB::FRT Δ ackA-pta::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT.

5 3.1.13. Construction of an *E. coli* MG1655 Δ mgsA::cm modified strain:

The *mgsA* gene was replaced with a chloramphenicol antibiotic resistance cassette flanked by Flp recognition target (FRT) that is generated by PCR by using primers with 50-nt homology extensions deleting most of the concerned gene. The used technique was described by Datsenko and Wanner in 2000.

10

Two oligonucleotides were designed and used to replace the *mgsA* gene:

1. DmgsAf, consisting of 70 bases (SEQ ID NO 34):

taagtgcttacagtaatctgtagaaagttaactacggatgtacattatgTGTAAGCTGGAGCTGCTTCG

15

With a region (lower-case letters) homologous to the sequence (1026236-1026285) corresponding to the upstream chromosomal sequence of the *mgsA* gene (sequence 1025780 to 1026238), a reference sequence on the website <http://ecocyc.org/>, including the *mgsA* gene initiation codon, and a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette of the pKD3 plasmid.

20

2. DmgsAr, consisting of 70 bases (SEQ ID NO 35):

aacaggtggcggttccacctgtgcaatattacttcagacggtcgcgagCATATGAATATCCTCCTTAG

25 With a region (lower-case letters) homologous to the sequence (1025751-1025800) corresponding to the downstream chromosomal sequence of the *mgsA* gene (sequence 1025780 to 1026238), a reference sequence on the website <http://ecocyc.org/>, including the codons for the six C-terminal residues and the stop codon of the *mgsA* gene and a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette carried by the pKD3 plasmid.

30

Both DmgsAf and DmgsAr oligonucleotides were used to amplify the chloramphenicol resistance cassette from the pKD3 plasmid. The amplified PCR fragment was then introduced by electroporation into the MG1655 *E. coli* strain carrying the RED plasmid helper (pKD46) expressing the system α , β and *exo* genes encoding Gam, Bet and Exo recombinases to promote
35 homologous recombination. The antibiotic-resistant transformants were then selected and the

replacement of the *mgsA* gene by the chloramphenicol cassette was checked by PCR analysis using both *mgsAs* and *mgsArv* oligonucleotides.

3. *mgsAs* (SEQ ID NO 36): cccagctcatcaaccaggtc (homologous to the sequence 1026715 to 1026734)

4. *mgsArv* (SEQ ID NO 37): ggagtcgattatggaagagcg (homologous to the sequence 1025559 to 1025580).

3.1.14. Construction of an *E. coli* MG1655 Δ *ldhA*::*FRT* Δ *pflAB*::*FRT* Δ *ackA-pta*::*FRT* Δ *iscR*::*FRT* Δ *frdABCD*::*FRT* Δ *aceE*::*FRT* Δ *mgsA*::*FRT* modified strain.

The *mgsA* gene was then replaced with a chloramphenicol resistance cassette into the MG1655 Δ *ldhA*::*FRT* Δ *pflAB*::*FRT* Δ *ackA-pta*::*FRT* Δ *iscR*::*FRT* Δ *frdABCD*::*FRT* Δ *aceE*::*FRT* strain and the deletion was achieved by generalized P1 phage transduction from the single mutant lysate (Miller 1972).

The Cm^R transductants were selected on plate and the replacement of the *mgsA* gene by the chloramphenicol cassette into the MG1655 Δ *ldhA*::*FRT* Δ *pflAB*::*FRT* Δ *ackA-pta*::*FRT* Δ *iscR*::*FRT* Δ *frdABCD*::*FRT* Δ *aceE*::*FRT* was checked by PCR analysis using both *mgsAs* and *mgsArv* oligonucleotides. Finally, i) *hslJC* and *ldhAC2*, ii) *pFLAB1* and *pflAB2* iii) *B2295f* and *YfcCR*, iv) *iscr1s* and *iscr1rv* v) *frdABCD1s* and *frdABCD1rv* and vi) *aceEs* and *aceEr* primers were also used in PCR analysis to confirm the deletion of *ldhA*, *pflAB*, *ackA-pta*, *iscR*, *frdABCD* and *aceE* genes respectively in the strain Δ *mgsA*::*cm*. The resulting strain was named MG1655 Δ *ldhA*::*FRT* Δ *pflAB*::*FRT* Δ *ackA-pta*::*FRT* Δ *iscR*::*FRT* Δ *frdABCD*::*FRT* Δ *aceE*::*FRT* Δ *mgsA*::*cm*. The chloramphenicol-resistance cassette was then eliminated by using the Flp helper pCP20 plasmid (Cheperanov and Wagnernagel 1995). Cm^R mutants were transformed with pCP20, and ampicillin-resistant transformants were selected at 30°C, after which a few were colony purified at 42°C and then tested for loss of the antibiotic resistance by PCR analysis using oligonucleotides previously described (SEQ ID N°36 and 37). The new strain was named MG1655 Δ *ldhA*::*FRT* Δ *pflAB*::*FRT* Δ *ackA-pta*::*FRT* Δ *iscR*::*FRT* Δ *frdABCD*::*FRT* Δ *aceE*::*FRT* Δ *mgsA*::*FRT*.

3.2. Construction of the *pSC101-pGII.6-cac2229-cac0764-cac0303* expression vector

Three *CA_C2229*, *CA_C0764*, *CA_C0303* synthetic genes encoding the pyruvate ferredoxin oxidoreductase, the ferredoxin NADP⁺ reductase and the ferredoxin respectively, from *Clostridium acetobutylicum* were firstly designed using the method termed codon harmonization (Angov *et al.* 2008). Synonymous codons from *E. coli* that match as closely as possible the codon usage frequency used in the native genes from *C. acetobutylicum* were selected. Based on the

generated sequences, the synthetic genes were further synthesized by Life Technologies (ThermoFisher Scientific, Saint aubin, France) introducing the native RBS region along with each gene, as well as placing two unique restriction sites upstream and downstream each gene. Each synthetic gene with its RBS region flanked by two unique restriction sites were then subcloned into the pCR4TOPO vector (Invitrogen, Saint Aubin, France), before to be cloned as an operon into a low-copy pSC101-derived plasmid (Bernardi and Bernardi 1984) under the control of the GI 1.6 promoter (Meynial-Salles *et al.* 2005, Soucaille *et al.* 2012) and upstream the *adc* terminator (from *C. acetobutylicum*) to yield the 9.54 kb *pSC101-pGI-cac2229-cac0764-cac0303* plasmid.

10

15 **3.3. Introduction of the *pSC101-PGI-cac2229-cac0764-cac0303* expression vector into the MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ ackA-pta::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ mgsA::FRT *E. coli* strain.**

The *pSC101-PGI-cac2229-cac0764-cac0303* expression vector was used to transform the MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ ackA-pta::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ mgsA::FRT by electroporation (Sambrook and Russel 2001).

20 The transformants were selected on LB agar plates supplemented with spectinomycin (70 μ g/ml) at 37°C. Some transformants were then grown in LB liquid culture supplemented with spectinomycin (100 μ g/ml) overnight at 37°C to carry out a DNA plasmid extraction (GenElute HP plasmid miniprep kit, Sigma) and check for the presence of the *pSC101-PGI-cac2229-cac0764-cac0303* plasmid. The *pSC101-pGI-cac2229-cac0764-cac0303* plasmid was finally controlled by restriction profile.

25 The final *E. coli* MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ ackA-pta::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ mgsA::FRT (*pSC101-PGI-cac2229-cac0764-cac0303*) was grown in LB liquid medium supplemented with spectinomycin (100 μ g/ml) and kept in 20% glycerol solution at -80°C.

30

3.4. : Physiological characterization of the MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ ackA-pta::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ mgsA::FRT (*pSC101-pGI1.6-cac2229-cac0764-cac0303*) *E. coli* strain:

The MG1655 $\Delta ldhA::FRT\Delta pflAB::FRT\Delta ackA-pta::FRT\Delta iscR::FRT\Delta frdABCD::FRT\Delta aceE::FRT\Delta mgsA::FRT$ (*pSC101-pGI1.6-cac2229-cac0764-cac0303*) *E. coli* strain was grown anaerobically on a 20 g/L glucose mineral medium containing minerals salts as previously described (Meynial-salles *et al.* 2005) supplemented with 4 g/l of yeast extract, 5 mM sodium nitrate and spectinomycin (100 µg/ml) and inoculated with 100 µl of a LB overnight culture. Cells were grown up at 37°C during several days, and the pH was maintained by buffering the culture medium with MOPS. As controls, both the MG1655 $\Delta ldhA::FRT\Delta pflAB::FRT\Delta ackApta::FRT\Delta iscR::FRT\Delta frdABCD::FRT\Delta aceE::FRT\Delta mgsA::FRT$ *E.coli* (without plasmid) and the MG1655 $\Delta ldhA::FRT\Delta pflAB::FRT\Delta ackApta::FRT\Delta iscR::FRT\Delta frdABCD::FRT\Delta aceE::FRT\Delta mgsA::FRT$ (*pSC101-pGI1.6-cac2229-cac0303*) (without the *cac0764* gene) were grown in the same conditions.

The comparative phenotypic analysis was performed by measuring glucose consumption as well as the concentration of fermentation products for the culture of each strain as is shown in table 3:

Strains	Glucose consumption (g/L)	Products yield (% g/g glucose consumed)						
		Pyruvate	Succinate	Lactate	Formate	Acetate	Ethanol	CO ₂
MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ ackApta::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ mgsA::FRT	2.27	87.7	1.54	0.66	1.45	4.84	17	19.86
MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ ackApta::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ mgsA::FRT (pSC101-pGII.6-cac2229-cac0303)	3.14	37.90	3.18	3.82	2.23	1.27	23.89	23.71
MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ ackApta::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ mgsA::FRT (pSC101-pGII.6-cac2229-cac0764-cac0303)	20.23	2.37	0.84	6.13	0.00	1.43	42.46	41.31

As shown in table 3, the expression of the complete synthetic *CA_C2229-CA_C0764-CA_C0303* operon into the MG1655 *ΔldhA::FRTΔpflAB::FRTΔackApta::FRTΔiscR::FRTΔfrdABCD::FRTΔaceE::FRTΔmgsA::FRT* *E. coli* led to the production of ethanol as the main fermentation product from glucose at a yield of 42.5 g/g glucose consumed corresponding to 83 % of the theoretical yield. Moreover, the expression of the complete synthetic *CA_C2229-CA_C0764-CA_C0303* operon favoured the growth which was strongly hampered in both control strains as the strains were unable to balance the redox.

3.5. Ferredoxin NADP⁺ reductase activity determination in the MG1655

ΔldhA::FRTΔpflAB::FRTΔackA-pta::FRTΔiscR::FRTΔfrdABCD::FRT

ΔaceE::FRTΔmgsA::FRT (pSC101-pGII.6-cac2229-cac0764-cac0303) E. coli strain:

The MG1655 $\Delta ldhA::FRT\Delta pflAB::FRT\Delta ackA-pta::FRT\Delta iscR::FRT\Delta frdABCD::FRT\Delta aceE::FRT\Delta mgsA::FRT$ (pSC101-pGI1.6-cac2229-cac0764-cac0303) *E. coli* strain was grown anaerobically on a 20 g/L glucose mineral medium containing minerals salts as previously described (Meynial-salles *et al.* 2005) supplemented with 4 g/l of yeast extract, 5 mM sodium nitrate and spectinomycin (100 μ g/ml) and inoculated with 100 μ l of a LB overnight culture. Cells were grown up at 37°C up to an approximatively OD_{550 nm} of 2, and the pH was maintained by buffering the culture medium with MOPS. As controls, both the MG1655 $\Delta ldhA::FRT\Delta pflAB::FRT\Delta ackApta::FRT\Delta iscR::FRT\Delta frdABCD::FRT\Delta aceE::FRT\Delta mgsA::FRT$ (pSC101-pGI1.6-cac2229-cac0303) (without the *cac0764* gene) were grown in the same conditions. Cells were transferred into anaerobic chamber, harvested, washed and 20 times concentrated in 100 mM Tris-HCl 2 mM DTT 10% glycerol (pH 7.6) buffer, frozen at -80°C or immediately used. Cells were further broken by sonication using an ultrasonic disintegrator (vibracell 72434, Bioblock) at 4°C in four cycles of 30 sec with 2-min intervals. Debris were removed by centrifugation at 8600 g for 10 min, 4°C (Sigma centrifuge 2-16K) and the acellular crude extract was loaded onto a SephadexG25 column to eliminate salts and metabolites, before the ferredoxin NADP⁺ reductase activity determination using ferredoxin as a substrate and following the procedure already described in 1.1 (table C).

Table C: Ferredoxin NADP⁺ reductase specific activities determined on crude extracts using ferredoxin as a substrate:

Strains	Ferredoxin NADP ⁺ reductase specific activity U/mg protein
MG1655 $\Delta ldhA::FRT\Delta pflAB::FRT\Delta ackApta::FRT\Delta iscR::FRT\Delta frdABCD::FRT\Delta aceE::FRT\Delta mgsA::FRT$ (pSC101-pGI1.6-cac2229-cac0303)	0
MG1655 $\Delta ldhA::FRT\Delta pflAB::FRT\Delta ackApta::FRT\Delta iscR::FRT\Delta frdABCD::FRT\Delta aceE::FRT\Delta mgsA::FRT$ (pSC101-pGI1.6-cac2229-cac0764-cac0303)	0.08

As shown in table C, a ferredoxin NADP⁺ reductase activity is only detected into the *E. coli* modified strain expressing the *CA_C0764* encoding gene.

EXAMPLE 4: Heterologous production of n-butanol by a new metabolic pathway in an *E. coli* strain with an enhanced Ferredoxin NADP⁺ reductase activity.

5

4.1: construction of an *E. coli* MG1655 $\Delta dhA::FRT$, $\Delta pflAB::FRT$, $\Delta ack-pta::FRT$, $\Delta iscR::FRT$, $\Delta frdABCD::FRT$ $\Delta ace::FRT$, $\Delta mgsA::FRT$ $\Delta adhE::FRT$ $\Delta melB::TT02-Ptrc01/RBS01*2-crt-hbd-TT07-FRT$ ptrc30/RBS01*2-*atoB*-FRT modified strain

4.1.1: Construction of an *E. coli* MG1655 $\Delta adhE::FRT$ -*cm*-FRT modified strain:

10

The *adhE* gene was replaced with a chloramphenicol antibiotic resistance cassette flanked by Flp recognition target (FRT) that is generated by PCR by using primers with 80-nt homology extensions deleting most of the concerned gene. The used technique was described by Datsenko and Wanner in 2000.

15

Two oligonucleotides were designed and used to replace the *adhE* gene:

DadhE r de 100 bases (SEQ ID N° 38):

atggctgttactaatgtcgtgaaacttaacgcactcgtagagcgtgtaaaaaagcccagcgtgaatatgccagtttactCATATGAAT
ATCCTCCTTAG

20

With a region (lower-case letters) homologous to the sequence (1297264 -1297344) including the *adhE* gene initiation codon of the *adhE* gene (sequence 1294669 to 1297344), a reference sequence on the website <http://ecocyc.org/>, and a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette of the pKD3 plasmid.

25

DadhEf de 100 bases (SEQ ID NO 39):

ttaagcggatttttcgctttttctcagcttagccggagcagcttcttctcgctgcagttcaccttctacataat
TG TAGGCTGGAGCTGCTTCG

30

With a region (lower-case letters) homologous to the sequence (1294693-1294748) corresponding to the downstream chromosomal sequence of the *adhE* gene (sequence 1294669 to 1297344), a reference sequence on the website <http://ecocyc.org/> and a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette carried by the pKD3 plasmid.

Both DadhEf and DadhEr oligonucleotides were used to amplify the chloramphenicol resistance cassette from the pKD3 plasmid. The amplified PCR fragment was then introduced by electroporation into the *MG1655 E. coli* strain carrying the RED plasmid helper (pKD46) expressing the system α , β and *exo* genes encoding Gam, Bet and Exo recombinases to promote homologous recombination. The antibiotic-resistant transformants were then selected and the replacement of the *adhE* gene by the chloramphenicol cassette was checked by PCR analysis using both ychGf and adhECr oligonucleotides.

- 10 ychGf (SEQ ID NO 40) : ggctcattgcaccaccatccag (homologous to the sequence 1294357 to 1294378)
 adhECr (SEQ ID NO 41) : gaaaagacgcgctgacaatacgcc (homologous to the sequence 1297749 to 1297772).

15 **4.1.2. Construction of an *E. coli* *MG1655* Δ *ldhA::FRT* Δ *pflAB::FRT* Δ *ackA-pta::FRT* Δ *iscR::FRT* Δ *frdABCD::FRT* Δ *aceE::FRT* Δ *mgsA::FRT* Δ *adhE::FRT* modified strain.**

The *adhE* gene was then replaced with a chloramphenicol resistance cassette into the *MG1655* Δ *ldhA::FRT* Δ *pflAB::FRT* Δ *ackA-pta::FRT* Δ *iscR::FRT* Δ *frdABCD::FRT* Δ *aceE::FRT* Δ *mgsA::FRT* strain and the deletion was achieved by generalized P1 phage transduction from the single mutant lysate (Miller 1972).

The Cm^R transductants were selected on plate and the replacement of the *adhE* gene by the chloramphenicol cassette into the *MG1655* Δ *ldhA::FRT* Δ *pflAB::FRT* Δ *ackA-pta::FRT* Δ *iscR::FRT* Δ *frdABCD::FRT* Δ *aceE::FRT* Δ *mgsA::FRT* was checked by PCR analysis using both ychGf and adhECr oligonucleotides. Finally, i) hslJC and ldhAC2, ii) pFLAB1 and pflAB2 iii) B2295f and YfcCR, iv) *iscR1s* and *iscR1rv* v) *frdABCD1s* and *frdABCD1rv* vi) *aceEs* and *aceErv* and vii) *mgsAs* and *mgsArv* couple of primers were also used in PCR analysis to confirm the deletion of *ldhA*, *pflAB*, *ackA-pta*, *iscR*, *frdABCD*, *aceE*, *mgsA* genes respectively in the strain Δ *adhE::cm*. The resulting strain was named *MG1655* Δ *ldhA::FRT* Δ *pflAB::FRT* Δ *ackA-pta::FRT* Δ *iscR::FRT* Δ *frdABCD::FRT* Δ *aceE::FRT* Δ *mgsA::FRT* Δ *adhE::cm*.

30 The chloramphenicol-resistance cassette was then eliminated by using the Flp helper pCP20 plasmid (Cheperanov and Wagnernagel 1995). Cm^R mutants were transformed with pCP20, and ampicillin-resistant transformants were selected at 30°C, after which a few were colony purified at 42°C and then tested for loss of the antibiotic resistance by PCR analysis using oligonucleotides previously described (SEQ ID N°40 and 41). The new strain was named *MG1655*

ΔldhA::FRTΔpflAB::FRTΔackA-pta::FRTΔiscR::FRTΔfrdABCD::FRT
ΔaceE::FRTΔmgsA::FRTΔadhE::FRT.

5 4.1.3. Construction of an *E. coli* MG1655Δ*melB*::TT02-ptrc01/RBS01*2-*crt-hbd*-TT07-Km modified strain

Both *CA_C2712* and *CA_C2708* synthetic genes encoding the crotonase and the β-hydroxy-butyrylCoA dehydrogenase respectively, from *Clostridium acetobutylicum* were firstly designed using the method termed codon harmonization (Angov *et al.* 2008). Synonymous codons
 10 from *E. coli* that match as closely as possible the codon usage frequency used in the native genes from *C. acetobutylicum* were selected. Based on the generated sequences, the synthetic genes were further synthesized in operon by Life Technologies (ThermoFisher Scientific, Saint aubin, France) introducing the RBS01 region along with each gene, as well as placing two unique restriction sites upstream and downstream each gene. The synthetic *crt-hbd* operon was then cloned under the
 15 control of ptrc01 promoter into the pUC18-TTadc-CI*0-PlambdaR*(-35)-Δ*melB*::Km (Norlander *et al.* 1983) carrying two homologous region of the *melB* locus and the kanamycin resistant cassette. The plasmid was digested with *SmaI*-*BamHI* and ligated with the *SmaI*-*crt-hbd*-*BamHI* synthetic operon to yield the pUC18-TTadc-CI*0-PlambdaR*(-35)-Δ*melB*::TT02-ptrc01/RBS01*2-*crt-hbd*-TT07-Km plasmid.

20 Both ome1847 and ome1850 primers were then used in a single tube PCR reaction with the pUC18-TTadc-CI*0-PlambdaR*(-35)-Δ*melB*::TT02-Ptrc01/RBS01*2-*crt-hbd*-TT07-Km plasmid to amplify the TT02-ptrc01/RBS01*2-*crt-hbd*-TT07::Km region.

Ome1847 (SEQ ID N°42)

25 TTCGTCACGGAATCGTCAGAAC

Ome1850 (SEQ ID N°43)

CCTGATTTATACCGGCATTTTCGG

The amplified PCR fragment was then introduced by electroporation into the MG1655 *E.*
 30 *coli* strain carrying the RED plasmid helper (pKD46) expressing the system α, β and exo genes encoding Gam, Bet and Exo recombinases to promote homologous recombination. The antibiotic-resistant transformants were then selected and the replacement of the *melB* gene by the TT02-ptrc01/RBS01*2-*crt-hbd*-TT07-Km cassette was checked by PCR analysis using both ome1845 and 1846rv oligonucleotides. The final strain was named MG1655 Δ*melB*::TT02-ptrc01/RBS01*2-*crt-hbd*-TT07-Km.
 35

ome1845s (SEQ ID N°44)

gccgatttgcgtgtgggc (homologous to the sequence from 4340168 to 4340187)

ome1846rv (SEQ ID N°45)

5 gccggttatccatcaggttcac (homologous to the sequence from 4344065 to 4344044)

4.1.4 Construction of an *E. coli* MG1655 $\Delta ldhA::FRT\Delta pflAB::FRT\Delta ackA-pta::FRT\Delta iscR::FRT\Delta frdABCD::FRT\Delta aceE::FRT\Delta mgsA::FRT\Delta adhE::FRT\Delta melB::TT02-ptrc01/RBS01*2-crt-hbd-TT07-FRT$ modified strain

The *melB* locus into the MG1655 $\Delta ldhA::FRT\Delta pflAB::FRT\Delta ackA-pta::FRT\Delta iscR::FRT\Delta frdABCD::FRT\Delta aceE::FRT\Delta mgsA::FRT\Delta adhE::FRT$ *E. coli* strain
10 was then replaced by the *ptrc01/RBS01*2-crt-hbd-TT07::Km* synthetic operon and the replacement was achieved by generalized P1 phage transduction from the single mutant lysate (Miller 1972). The Km^R transductants were selected on plate and the replacement of the *melB* gene by the *ptrc01/RBS01*2-crt-hbd-TT07-Km* synthetic operon into the MG1655 $\Delta ldhA::FRT\Delta pflAB::FRT\Delta ackA-pta::FRT\Delta iscR::FRT\Delta frdABCD::FRT\Delta aceE::FRT\Delta mgsA::FRT\Delta adhE::FRT$ was
15 checked by PCR analysis using both ome1845 and 1846rv oligonucleotides. Finally, i) *hslJC* and *ldhAC2*, ii) *pFLAB1* and *pFLAB2* iii) *B2295f* and *YfcCR*, iv) *iscr1s* and *iscr1rv* v) *frdABCD1s* and *frdABCD1rv* vi) *aceEs* and *aceErv* vii) *mgsAs* and *mgsArv* and viii) *ychGf* and *adhECr* couple of primers were also used in PCR analysis to confirm the deletion of *ldhA*, *pflAB*, *ackA-pta*, *iscR*,
20 *frdABCD*, *aceE*, *mgsA* and *adhE* genes respectively in the strain $\Delta melB::TT02-ptrc01/RBS01*2-crt-hbd-TT07-Km$. The resulting strain was named MG1655 $\Delta ldhA::FRT\Delta pflAB::FRT\Delta ackA-pta::FRT\Delta iscR::FRT\Delta frdABCD::FRT\Delta aceE::FRT\Delta mgsA::FRT\Delta adhE::FRT\Delta melB::TT02-ptrc01/RBS01*2-crt-hbd-TT07-Km$.

The kanamycin-resistance cassette was then eliminated by using the Flp helper pCP20
25 plasmid (Cheperanov and Wagnagel 1995). Km^R mutants were transformed with pCP20, and ampicillin-resistant transformants were selected at 30°C, after which a few were colony purified at 42°C and then tested for loss of the antibiotic resistance by PCR analysis using oligonucleotides previously described (SEQ ID N°44 and 45). The new strain was named MG1655 $\Delta ldhA::FRT\Delta pflAB::FRT\Delta ackA-pta::FRT\Delta iscR::FRT\Delta frdABCD::FRT\Delta aceE::FRT\Delta mgsA::FRT\Delta adhE::FRT\Delta melB::TT02-ptrc01/RBS01*2-crt-hbd-TT07-FRT$.
30

4.1.5. Construction of an *E. coli* MG1655 *ptrc30/RBS01*2-atoB-cm* modified strain:

Both Ome1246 and Odi0247 oligonucleotides were used to amplify the chloramphenicol resistance cassette from the pKD3 plasmid and introduced *ptrc30* and *RBS01* regions upstream the
35 *atoB* gene and replace the natural promoter region.

Ome1246_3'D r (SEQ ID 46)

TGTAGGCTGGAGCTGCTTCG

Homologous to a region for the amplification of the chloramphenicol resistance cassette of the
5 pKD3 plasmid

Odi 0247_ P_{trc30}/RBS01*2-atoB R r (SEQ ID 47)

CAATTTTTCAT*ttataacctcctta****TTCCACACAGTATACGAGCCGGATGATTAATCGTCAACAGCT***
CCATGGTCCATATGAATATCCTCCTTA

With a region (bold letters) homologous to the sequence 2324141-2324131 of the *atoB*
10 gene (sequence 2 324 131 to 2 325 315) a reference sequence on the website <http://ecocyc.org/>, a
region (lower case letters) homologous to the RBS01*2 consensus sequence containing the *PsiI*
restriction site, a region (italic letters) containing the artificial *ptrc30* promoter and a region
(underlined letters) for the amplification of the chloramphenicol resistance cassette of the pKD3
plasmid .

15 The PCR product was then purified and used for a second PCR using both Odi0242 and
0246 oligonucleotides to increase the size of homologous regions and, finally, favour *in vivo*
homologous recombination.

Odi 0242 (SEQ ID N°48)

GCATCACTGCCCTGCTCTTCTCCGGTGTCAATTTTCGTCATTGGTTTAACGCTGTTCTGAC
20 GGCACCCCTACAAACAGAAGGAATATAAACTGGCTCACCTTCGGGTGGGCCTTTCTGCT
GTAGGCTGGAGCTGCTTC

With a region (bold letters) homologous to the sequence 2324042-2324130 upstream of the
atoB gene (sequence 2 324 131 to 2 325 315) a reference sequence on the website
<http://ecocyc.org/>, a region (italic letters) corresponding to the transcriptional terminator T7T of the
25 T7 phage (Harrington *et al.* 2001) and a region (underlined letters) for the amplification of the
chloramphenicol resistance cassette of the pKD3 plasmid.

Odi 0246 (SEQ ID N°49)

TTACTGTCGCCCCCAGGTCGATGGCGCTGGTGGGAAGCGAGTGAACCGTTAAAAC
30 ***TACCGATAGCAGTACGTACCGCACTGACGATGACACAATTTTTCATTTATAACCTC***
CTTA

With a region (bold letters) homologous to the sequence 2324230-2324131 of the *atoB*
gene (sequence 2 324 131 to 2 325 315) a reference sequence on the website <http://ecocyc.org/> and

a region (underlined letters) homologous to the RBS01*2 consensus sequence containing the PstI restriction site.

The second amplified PCR fragment was then introduced by electroporation into the *MG1655 E. coli* strain carrying the RED plasmid helper (pKD46) expressing the system α , β and *exo* genes encoding Gam, Bet and Exo recombinases to promote homologous recombination. The antibiotic-resistant transformants were then selected and the replacement of the natural promoter region of the *atoB* gene by the ptrc01/RBS01*2-cm cassette was checked by PCR analysis using both *atoBs* and *atoBrv* oligonucleotides. The final strain was named *MG1655 ptrc30/RBS01*2-atoB-cm*.

10 *atoBs* (SEQ ID N°50)

gcgcggcagcagcagctac (homologous to the sequence 2323667 to 2323685)

atoBrv (SEQ ID N°51)

15 cgcacatcaggccatcgcc (homologous to the sequence 2324584 to 2324565)

4.1.6. Construction of an *E. coli* *MG1655* Δ *ldhA::FRT* Δ *pflAB::FRT* Δ *ackA-pta::FRT* Δ *iscR::FRT* Δ *frdABCD::FRT* Δ *aceE::FRT* Δ *mgsA::FRT* Δ *adhE::FRT* Δ *melB::TT02-Ptrc01/RBS01*2-crt-hbd-TT07::FRT* *ptrc30/RBS01*2-atoB::FRT* modified strain

20 The natural promoter region of the *atoB* gene was then replaced by the artificial ptrc01/RBS01*2 region into the *MG1655* Δ *ldhA::FRT* Δ *pflAB::FRT* Δ *ackA-pta::FRT* Δ *iscR::FRT* Δ *frdABCD::FRT* Δ *aceE::FRT* Δ *mgsA::FRT* Δ *adhE::FRT* Δ *melB::TT02-Ptrc01/RBS01*2-crt-hbd-TT07-FRT* and the replacement was achieved by generalized P1 phage transduction from the single mutant lysate (Miller 1972).

25 The Cm^R transductants were selected on plate and the replacement of the natural promoter region by the ptrc01/RBS01*2 artificial region into the *MG1655* Δ *ldhA::FRT* Δ *pflAB::FRT* Δ *ackA-pta::FRT* Δ *iscR::FRT* Δ *frdABCD::FRT* Δ *aceE::FRT* Δ *mgsA::FRT* Δ *adhE::FRT* Δ *melB::TT02-Ptrc01/RBS01*2-crt-hbd-TT07-FRT* was checked by PCR analysis using both *atoBs* and *atoBrv* oligonucleotides. Finally, i) *hsIJC* and *ldhAC2*, ii) *pFLAB1* and *pFLAB2* iii) *B2295f* and *YfcCR*,
30 iv) *iscr1s* and *iscr1rv* v) *frdABCD1s* and *frdABCD1rv* vi) *aceEs* and *aceErv* vii) *mgsAs* and *mgsArv* viii) *ychGf* and *adhECr* and ix) *ome1845* and *1846rv* couple of primers were also used in PCR analysis to confirm the deletion of *ldhA*, *pflAB*, *ackA-pta*, *iscR*, *frdABCD*, *aceE*, *mgsA* and *adhE* genes respectively and the *crt-hbd* expression in the *ptrc30/RBS01*2-atoB-cm* strain. The resulting strain was named *MG1655* Δ *ldhA::FRT* Δ *pflAB::FRT* Δ *ackA-*

*pta::FRTΔiscR::FRTΔfrdABCD::FRT ΔaceE::FRTΔmgsA::FRTΔadhE::FRT ΔmelB::TT02-
ptrc01/RBS01*2-crt-hbd-TT07-FRT ptrc30/RBS01*2-atoB-cm.*

The chloramphenicol-resistance cassette was then eliminated by using the Flp helper pCP20 plasmid (Cheperanov and Wagnagel 1995). Cm^R mutants were transformed with pCP20,
5 and ampicillin-resistant transformants were selected at 30°C, after which a few were colony purified at 42°C and then tested for loss of the antibiotic resistance by PCR analysis using oligonucleotides previously described (SEQ ID N°50 and 51). The new strain was named MG1655
*ΔldhA::FRTΔpflAB::FRTΔackA-pta::FRTΔiscR::FRTΔfrdABCD::FRT
ΔaceE::FRTΔmgsA::FRTΔadhE::FRT ΔmelB::TT02-ptrc01/RBS01*2-crt-hbd-TT07-FRT
10 ptrc30/RBS01*2-atoB-FRT.*

4.2. Construction of pSC101_pGI-cac2229-cac0764-cac0303_pthl-cap0035-cac2711-cac2710-cac2709 expression vector:

Four *CA_P0035*, *CA_C2711*, *CA_C2710*, *CA_C2701* synthetic genes encoding the
15 bifunctional butyraldehyde and butanol dehydrogenase NADH dependent AdhE2, the butyrylCoA dehydrogenase, the electron transfer flavoproteins B and A respectively, from *Clostridium acetobutylicum* were firstly designed using the method termed codon harmonization (Angov *et al.* 2008). Synonymous codons from *E. coli* that match as closely as possible the codon usage frequency used in the native genes from *C. acetobutylicum* were selected. Based on the generated
20 sequences, the synthetic genes were further synthesized by Life Technologies (ThermoFisher Scientific, Saint aubin, France) introducing the RBS region along with each gene, as well as placing two unique restriction sites upstream and downstream each gene. The *CA_C2711*, *CA_C2710* and *CA_C2701* were synthesized as an operon and the *adhE2* was synthesized independently. The synthetic *adhE2* gene was firstly inserted into the pSOS95 plasmid (Genbank
25 accession number AY187686.1) between the BamHI site located downstream the thl promoter and the SfoI site, located upstream the adc terminator. The *bcd-etfB-etfA* synthetic artificial operon was then introduced into the pSOS95-*adhE2* between the XhoI site located downstream the *adhE2* gene and the SfoI site, located upstream the adc terminator to yield the *pSOS95 adhE2-bcd-etfB-etfA* plasmid. The *pSOS95 adhE2-bcd-etfB-etfA* plasmid was then digested with Sall and the operon
30 containing fragment was purified on agarose gel before to be ligated to the pSC101_pGI-cac2229-cac0764-cac0303 plasmid pre-digested with Sall, yielding the 15.4 kb *pSC101-pGI-cac2229-cac0764-cac0303-pthl-cap0035-cac2711-cac2710-cac2709* plasmid.

4.3. Construction of pSC101_pGI-cac2229-cac0764-cac0303-udhA_pthl-cap0035-cac2711-cac2710-cac2709 expression vector:

The *udhA* gene was amplified from the genomic DNA of *E. coli* MG1655 using primers *udhA*-NheI and *udhA*-XmaI. The primers were designed to introduce a RBS region along with the *udhA* gene, as well as placing NheI and XmaI restriction sites upstream and downstream respectively:

udhA-NheI: (SEQ ID N° 52):

10 AATTGCTAGCATTATATACAAGGAGGAAACAGCTATGCCACATTCCTACGATTACGA
TG

udhA-XmaI: (SEQ ID N° 53)

AATTCCTGGGATAATTTTAAAACAGGCGGTTTAAACCGTTTAAC

15 The amplified PCR fragment was then subcloned into a Zero Blunt TOPO vector (Invitrogen, Saint Aubin, France) to yield the Zero Blunt TOPO-*udhA* plasmid and sequenced using universal primers T7P and T3P to assure that no mutations were introduced. The fragment containing the *udhA* gene was purified on an agarose gel after digestion of the Zero Blunt TOPO-*udhA* vector with NheI and XmaI. The 15.4 kb *pSC101_pGI-cac2229-cac0764-cac0303_pThl-cap0035-cac2711-cac2710-cac2709* expression vector was also digested with NheI and XmaI and
20 ligated to the NheI-XmaI digested *udhA* fragment, yielding the 16.8 kb *pSC101pGI-cac2229-cac0764-cac0303-udhA_pthl-cap0035-cac2711-cac2710-cac2709* vector.

25 **4.4. Introduction of the *pSC101_pGI-cac2229-cac0764-cac0303-udhA_pthl-cap0035-cac2711-cac2710-cac2709* expression vector into the *E. coli* MG1655**
ΔldhA::FRTΔpflAB::FRTΔackA-pta::FRTΔiscR::FRTΔfrdABCD::FRT
ΔaceE::FRTΔmgsA::FRTΔadhE::FRT ΔmelB::TT02-Ptrc01/RBS01*2-crt-hbd-
***TT07::FRT ptrc30/RBS01*2-atoB::FRT* modified strain.**

The *pSC101pGI-cac2229-cac0764-cac0303-udhA_pthl-cap0035-cac2711-cac2710-*
30 *cac2709* expression vector was used to transform the *E. coli* MG1655
ΔldhA::FRTΔpflAB::FRTΔackA-pta::FRTΔiscR::FRTΔfrdABCD::FRT ΔaceE::FRTΔmgsA::FRT
*ΔadhE::FRT ΔmelB::TT02-ptrc01/RBS01*2-crt-hbd-TT07::FRT ptrc30/RBS01*2-atoB::FRT*
modified strain by electroporation (Sambrook and Russel 2001).

The transformants were selected on LB agar plates supplemented with spectinomycin (70
35 μg/ml) at 37°C. Some transformants were then grown in LB liquid culture supplemented with

spectinomycin (100 µg/ml) overnight at 37°C to carry out a DNA plasmid extraction (GenElute HP plasmid miniprep kit, Sigma) and check for the presence of the *pSC101_pGI-cac2229-cac0764-cac0303-udhA_pthl-cap0035-cac2711-cac2710-cac2709* plasmid. The *pSC101_pGI-cac2229-cac0764-cac0303-udhA_pthl-cap0035-cac2711-cac2710-cac2709* plasmid was finally controlled by restriction profile.

The final *E. coli* MG1655 *ΔldhA::FRTΔpflAB::FRTΔackA-pta::FRTΔiscR::FRTΔfrdABCD::FRT ΔaceE::FRTΔmgsA::FRTΔadhE::FRT ΔmelB::TT02-ptrc01/RBS01*2-crt-hbd-TT07::FRT ptrc30/RBS01*2-atoB::FRT* (*pSC101_pGI-cac2229-cac0764-cac0303-udhA_pthl-cap0035-cac2711-cac2710-cac2709*) was grown in LB liquid medium supplemented with spectinomycin (100 µg/ml) and kept in 20% glycerol solution at -80°C.

4.5. : Physiological characterization of the *E. coli* MG1655 *ΔldhA::FRTΔpflAB::FRTΔackA-pta::FRTΔiscR::FRTΔfrdABCD::FRT ΔaceE::FRTΔmgsA::FRTΔadhE::FRT ΔmelB::TT02-ptrc01/RBS01*2-crt-hbd-TT07::FRT ptrc30/RBS01*2-atoB::FRT* pSC101_PGI-cac2229-cac0764-cac0303-udhA_PThl-cap0035-cac2711-cac2710-cac2709 modified strain.

The *E. coli* MG1655 *ΔldhA::FRTΔpflAB::FRTΔackA-pta::FRTΔiscR::FRTΔfrdABCD::FRT ΔaceE::FRTΔmgsA::FRTΔadhE::FRT ΔmelB::TT02-Ptrc01/RBS01*2-crt-hbd-TT07::Km ptrc30/RBS01*2-atoB::cm* *pSC101_pGI-cac2229-cac0764-cac0303-udhA_pthl-cap0035-cac2711-cac2710-cac2709* strain was grown anaerobically on a 20 g/L glucose mineral medium containing minerals salts as previously described (Meynial-salles *et al.* 2005) supplemented with 4 g/l of yeast extract, 5 mM nitrate and spectinomycin (100 µg/ml) and inoculated with 100 µl of a LB overnight culture. Cells were grown up at 37°C during several days, and the pH was maintained by buffering the culture medium with MOPS. As a control, the *E. coli* MG1655 *ΔldhA::FRTΔpflAB::FRTΔackApta::FRTΔiscR::FRTΔfrdABCD::FRTΔaceE::FRTΔmgsA::FRTΔadhE::FRT ΔmelB::TT02-ptrc01/RBS01*2-crt-hbd-TT07::FRT ptrc30/RBS01*2-atoB::FRT* strain was grown in the same conditions.

The comparative phenotypic analysis was performed by measuring both glucose consumption as well as the concentration of fermentation products for the culture of each strain as is shown in table 4:

Strains	Glucose consumption (g/l)	Products yield (% g/g glucose consumed)							
		Butanol	Succinate	Lactate	Butyrate	Acetate	Ethanol	Pyruvate	CO ₂
MG1655 Δ ldhA: :FRT Δ pflAB::F RT Δ ackApta::F RT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ m gsA::FRT Δ adhE ::FR T Δ melB::T T02- Ptrc01/RBS01* 2-crt-hbd- TT07ptrc30/RB S01*2-atoB	2.53	0	5.30	0	10.27	0	12.25	62	16.9
MG1655 Δ ldhA: :FRT Δ pflAB::F RT Δ ackA- pta::FRT Δ iscR: :FRT Δ frdABCD::FR T Δ aceE::FRT Δ mgsA::FRT Δ ad hE::FRT Δ melB: :TT02- Ptrc01/RBS01* 2-crt-hbd-TT07 ptrc30/RBS01* 2-atoB pSC101_PGI- cac2229- cac0764- cac0303- udhA_pthl- cap0035- cac2711- cac2710- cac2709	32	29.54	0.65	0.4	4.71	0.46	2.88	0.37	7

As shown in table 4, the expression of the both synthetic *CA_C2229-CA_C0764-CA_C0303* and *CA_P0035-CA_C2711-CA_C2710-CA_C2709* operons into the *MG1655ΔldhA::FRTΔpflAB::FRTΔackApta::FRTΔiscR::FRTΔfrdABCD::FRTΔaceE::FRTΔmgsA::FRTΔadhE::FRTΔmelB::TT02-Ptrc01/RBS01*2-crt-hbd-TT07ptrc30/RBS01*2-atoB* *E.coli* strain led to the production of butanol as the main fermentation product from glucose at a yield of 0.3 g/g glucose consumed corresponding to 73 % of the theoretical yield. Moreover, the expression of the both synthetic *CA_C2229-CA_C0764-CA_C0303* and *CA_P0035-CA_C2711-CA_C2710-CA_C2709* operons, favoured the growth which was strongly hampered in the control strain (without plasmid).

4.6 : Ferredoxin NADP⁺ reductase activity determination in the the *E. coli* *MG1655* *ΔldhA::FRTΔpflAB::FRTΔackA-pta::FRTΔiscR::FRTΔfrdABCD::FRTΔaceE::FRTΔmgsA::FRTΔadhE::FRTΔmelB::TT02-ptrc01/RBS01*2-crt-hbd-TT07::FRT ptrc30/RBS01*2-atoB::FRT* pSC101_PGI-cac2229-cac0764-cac0303-udhA_PThl-cap0035-cac2711-cac2710-cac2709 modified strain.

The *E. coli* *MG1655* *ΔldhA::FRTΔpflAB::FRTΔackA-pta::FRTΔiscR::FRTΔfrdABCD::FRTΔaceE::FRTΔmgsA::FRTΔadhE::FRTΔmelB::TT02-Ptrc01/RBS01*2-crt-hbd-TT07::Km ptrc30/RBS01*2-atoB::cm* pSC101_pGI-cac2229-cac0764-cac0303-udhA_pthl-cap0035-cac2711-cac2710-cac2709 strain was grown anaerobically on a 20 g/L glucose mineral medium containing minerals salts as previously described (Meynial-salles *et al.* 2005) supplemented with 4 g/l of yeast extract, 5 mM nitrate and spectinomycin (100 µg/ml) and inoculated with 100 µl of a LB overnight culture. Cells were grown up at 37°C up to an approximatively OD_{550nm} of 2, and the pH was maintained by buffering the culture medium with MOPS. As a control, the *E.coli* *MG1655* *ΔldhA::FRTΔpflAB::FRTΔackApta::FRTΔiscR::FRTΔfrdABCD::FRTΔaceE::FRTΔmgsA::FRTΔadhE::FRTΔmelB::TT02-ptrc01/RBS01*2-crt-hbd-TT07::FRT ptrc30/RBS01*2-atoB::FRT* strain was grown in the same conditions.

Cells were transferred into anaerobic chamber, harvested, washed and 20 times concentrated in 100 mM Tris-HCl 2 mM DTT 10% glycerol (pH 7.6) buffer, frozen at -80°C or immediately used. Cells were further broken by sonication using an ultrasonic disintegrator (vibracell 72434, Bioblock) at 4°C in four cycles of 30 sec with 2-min intervals. Debris were removed by centrifugation at 8600 g for 10 min, 4°C (Sigma centrifuge 2-16K) and the acellular crude extract was loaded onto a SephadexG25 column to eliminate salts and metabolites, before the ferredoxin

NADP⁺ reductase activity determination using ferredoxin as a substrate and following the procedure already described in 1.1 (table D).

Table D: Ferredoxin NADP⁺ reductase specific activities determined on crude extracts using ferredoxin as a substrate:

Strains	Ferredoxin NADP ⁺ reductase specific activity U/mg protein
MG1655 Δ ldhA:: <frt<math>\DeltapflAB::<frt<math>\DeltaackApta::<frt<math>\DeltaiscR::<frt<math>\DeltafrdABCD::<frt<math>\DeltaaceE::<frt<math>\DeltamgsA::<frt<math>\DeltaadhE::<frt<math>\DeltamelB::TT02-Ptrc01/RBS01*2-<i>crt-hbd</i>-TT07ptrc30/RBS01*2-<i>atoB</i></frt<math></frt<math></frt<math></frt<math></frt<math></frt<math></frt<math></frt<math>	0
MG1655 Δ ldhA:: <frt<math>\DeltapflAB::<frt<math>\DeltaackApta::<frt<math>\DeltaiscR::<frt<math>\DeltafrdABCD::<frt<math>\DeltaaceE::<frt<math>\DeltamgsA::<frt<math>\DeltaadhE::<frt<math>\DeltamelB::TT02-Ptrc01/RBS01*2-<i>crt-hbd</i>-TT07 ptrc30/RBS01*2-<i>atoB</i> pSC101_PGI-<i>cac2229-cac0764-cac0303-udhA_pthl-cap0035-cac2711-cac2710-cac2709</i></frt<math></frt<math></frt<math></frt<math></frt<math></frt<math></frt<math></frt<math>	0.11

As shown in table D, a ferredoxin NADP⁺ reductase activity is only detected into the *E. coli* modified strain expressing the CA_C0764 encoding gene.

EXAMPLE 5: Heterologous production of 1, 3 propanediol from glycerol by a new metabolic pathway in an *E. coli* strain with an enhanced Ferredoxin NADP⁺ reductase activity.

5.1: construction of a *E. coli* MG1655 Δ ldhA::\DeltapflAB::\DeltaiscR::\DeltafrdABCD::\DeltaaceE::\DeltamgsA::\DeltaadhE::\DeltaaldA::\DeltaaldB::\DeltaglpD::\DeltaglpA::

5.1.1: Construction of a *E. coli* *MG1655ΔldhA::FRT, ΔpflAB::FRT, ΔiscR::FRT* modified strain:

The *iscR* gene was then replaced with a kanamycin resistance cassette into the *MG1655 ΔldhA::FRTΔpflAB::FRT* strain and the deletion was achieved by generalized P1 phage transduction from the single mutant lysate (Miller 1972) using the *MG1655ΔiscR::km* strain (see 3.1.7).

The Km^R transductants were selected on plate and the replacement of the *iscR* gene by the kanamycin cassette into the *MG1655ΔldhA::FRTΔpflAB::FRT* was checked by PCR analysis using both *iscR*s and *iscR1rv* oligonucleotides. Finally, i) *hslJC* and *ldhAC2* and ii) *pFLAB1* and *pflAB2* couple of primers were also used in PCR analysis to confirm the deletion of *ldhA* and *pflAB* genes respectively in the strain *ΔiscR::km*. The resulting strain was named *MG1655 ΔldhA::FRTΔpflAB::FRT ΔiscR::km*.

The kanamycin-resistance cassette was then eliminated by using the Flp helper pCP20 plasmid (Cheperanov and Wagnagel 1995). Km^R mutants were transformed with pCP20, and ampicillin-resistant transformants were selected at 30°C, after which a few were colony purified at 42°C and then tested for loss of the antibiotic resistance by PCR analysis using oligonucleotides previously described (SEQ ID N°24 and 25). The new strain was named *MG1655 ΔldhA::FRTΔpflAB::FRTΔiscR::FRT*.

5.1.2: Construction of a *E. coli* *MG1655ΔldhA::FRT, ΔpflAB::FRT, ΔiscR::FRT, ΔfrdABCD::FRT* modified strain :

The *frdABCD* genes were then replaced with a chloramphenicol resistance cassette into the *MG1655ΔldhA::FRTΔpflAB::FRTΔiscR::FRT* strain and the deletion was achieved by generalized P1 phage transduction from the single mutant lysate (Miller 1972) using the *MG1655ΔfrdABCD::cm E. coli* strain (see 3.1.9).

The CmR transductants were selected on plate and the replacement of the *frdABCD* genes by the chloramphenicol cassette into the *MG1655ΔldhA::FRTΔpflAB::FRTΔiscR::FRT* was checked by PCR analysis using both *frdABCD1s* and *frdABCD1rv* oligonucleotides. Finally, i) *hslJC* and *ldhAC2*, ii) *pFLAB1* and *pflAB2* and iii) *iscR1s* and *iscR1rv* couple of primers were also used in PCR analysis to confirm the deletion of *ldhA*, *pflAB* and *iscR* genes respectively, in the strain *ΔfrdABCD::cm*. The resulting strain was named *MG1655ΔldhA::FRT ΔpflAB::FRΔiscR::FRTΔfrdABCD::cm*.

The chloramphenicol-resistance cassette was then eliminated by using the Flp helper pCP20 plasmid (Cheperanov and Wagnagel 1995). Cm^R mutants were transformed with pCP20, and ampicillin-resistant transformants were selected at 30°C, after which a few were colony purified at 42°C and then tested for loss of the antibiotic resistance by PCR analysis using oligonucleotides previously described (SEQ ID N°28 and 29). The new strain was named MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT.

5.1.3: Construction of a *E. coli* MG1655 Δ ldhA::FRT, Δ pflAB::FRT, Δ iscR::FRT, Δ frdABCD::FRT Δ aceE::FRT modified strain:

The *aceE* gene was then replaced with a kanamycin resistance cassette into the MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT strain and the deletion was achieved by generalized P1 phage transduction from the single mutant lysate (Miller 1972) using the MG1655 $\cdot$ *aceE::km* *E. coli* strain (see 3.1.11).

The km^R transductants were selected on plate and the replacement of the *aceE* gene by the kanamycin cassette into the MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT was checked by PCR analysis using both *aceE*s and *aceE*rv oligonucleotides. Finally, i) hslJC and ldhAC2, ii) pFLAB1 and pflAB2 iii) iscr1s and iscr1rv and iv) frdABCD1s and frdABCD1rv primers were also used in PCR analysis to confirm the deletion of *ldhA*, *pflAB*, *iscR* and *frdABCD* genes respectively in the strain Δ aceE::km. The resulting strain was named MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ aceE::km.

The kanamycin-resistance cassette was then eliminated by using the Flp helper pCP20 plasmid (Cheperanov and Wagnagel 1995). Km^R mutants were transformed with pCP20, and ampicillin-resistant transformants were selected at 30°C, after which a few were colony purified at 42°C and then tested for loss of the antibiotic resistance by PCR analysis using oligonucleotides previously described (SEQ ID N°32 and 33). The new strain was named MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT.

5.1.4.: Construction of a *E. coli* MG1655 Δ ldhA::FRT, Δ pflAB::FRT, Δ iscR::FRT, Δ frdABCD::FRT Δ aceE::FRT, Δ mgsA::FRT modified strain:

The *mgsA* gene was then replaced with a chloramphenicol resistance cassette into the MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT strain and the deletion was achieved by generalized P1 phage transduction from the single mutant lysate (Miller 1972) using the MG1655 Δ mgsA::cm *E. coli* strain (see 3.1.13).

The Cm^R transductants were selected on plate and the replacement of the *mgsA* gene by the chloramphenicol cassette into the *MG1655ΔldhA::FRTΔpflAB::FRTΔiscR::FRTΔfrdABCD::FRTΔaceE::FRT* was checked by PCR analysis using both *mgsAs* and *mgsArv* oligonucleotides. Finally, i) *hslJC* and *ldhAC2*, ii) *pFLAB1* and *pflAB2*, iii) *iscr1s* and *iscr1rv* iv) *frdABCD1s* and *frdABCD1rv* and v) *aceEs* and *aceErv* couple of primers were also used in PCR analysis to confirm the deletion of *ldhA*, *pflAB*, *iscR*, *frdABCD* and *aceE* genes respectively in the strain *ΔmgsA::cm*. The resulting strain was named *MG1655ΔldhA::FRTΔpflAB::FRTΔiscR::FRTΔfrdABCD::FRTΔaceE::FRTΔmgsA::cm*.

The chloramphenicol-resistance cassette was then eliminated by using the Flp helper pCP20 plasmid (Cheperanov and Wagnernagel 1995). Cm^R mutants were transformed with pCP20, and ampicillin-resistant transformants were selected at 30°C, after which a few were colony purified at 42°C and then tested for loss of the antibiotic resistance by PCR analysis using oligonucleotides previously described (SEQ ID N°36 and 37). The new strain was named *MG1655ΔldhA::FRTΔpflAB::FRTΔiscR::FRTΔfrdABCD::FRTΔaceE::FRTΔmgsA::FRT*.

5.1.5.: Construction of a *E. coli* *MG1655ΔldhA::FRT, ΔpflAB::FRT, ΔiscR::FRT, ΔfrdABCD::FRT ΔaceE::FRT, ΔmgsA::FRT ΔadhE::FRT* modified strain:

The *adhE* gene was then replaced with a chloramphenicol resistance cassette into the *MG1655ΔldhA::FRT ΔpflAB::FRTΔiscR::FRT ΔfrdABCD::FRTΔaceE::FRT ΔmgsA::FRT* strain and the deletion was achieved by generalized P1 phage transduction from the single mutant lysate (Miller 1972) using the *MG1655ΔadhE::cm E. coli* strain (see 4.1.1).

The Cm^R transductants were selected on plate and the replacement of the *adhE* gene by the chloramphenicol cassette into the *MG1655ΔldhA::FRT ΔpflAB::FRTΔiscR::FRT ΔfrdABCD::FRTΔaceE::FRTΔmgsA::FRT* was checked by PCR analysis using both *ychGf* and *adhECr* oligonucleotides. Finally, i) *hslJC* and *ldhAC2*, ii) *pFLAB1* and *pflAB2*, iii) *iscr1s* and *iscr1rv*, iv) *frdABCD1s* and *frdABCD1rv*, v) *aceEs* and *aceErv* and vi) *mgsAs* and *mgsArv* couple of primers were also used in PCR analysis to confirm the deletion of *ldhA*, *pflAB*, *iscR*, *frdABCD*, *aceE* and *mgsA* genes respectively in the strain *ΔadhE::cm*. The resulting strain was named *MG1655ΔldhA::FRT ΔpflAB::FRTΔiscR::FRTΔfrdABCD::FRT ΔaceE::FRTΔmgsA::FRTΔadhE::cm*.

The chloramphenicol-resistance cassette was then eliminated by using the Flp helper pCP20 plasmid (Cheperanov and Wagnernagel 1995). Cm^R mutants were transformed with pCP20, and ampicillin-resistant transformants were selected at 30°C, after which a few were colony

purified at 42°C and then tested for loss of the antibiotic resistance by PCR analysis using oligonucleotides previously described (SEQ ID N°36 and 37). The new strain was named MG1655 *ΔldhA::FRTΔpflAB::FRTΔiscR::FRTΔfrdABCD::FRT ΔaceE::FRTΔmgsA::FRTΔadhE::FRT*.

5.1.6.: Construction of a *E. coli* MG1655 *ΔaldA::FRT-cm-FRT* modified strain:

The *aldA* gene was replaced with a chloramphenicol antibiotic resistance cassette flanked by Flp recognition target (FRT) that is generated by PCR by using primers with 80-nt homology extensions deleting most of the concerned gene. The used technique was described by Datsenko and Wanner in 2000.

Two oligonucleotides were designed and used to replace the *aldA* gene:

1-AldA D f consisting of 100 bases (SEQ ID N°54)

atgtcagtacccgttcaacatcctatgtatatcgatggacagttgttacctggcgtggagacgcgatggattgatgtgtaGTGTAGGCTG
GAGCTGCTTCG

With a region (lower-case letters) homologous to the sequence (1486256-1486336) including the *aldA* gene initiation codon of the *aldA* gene (sequence 1486256 to 1487695), a reference sequence on the website <http://ecocyc.org/>, and a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette of the pKD3 plasmid

2-aldAD r consisting of 100 bases (SEQ ID N°55):

ttaagactgtaaataaaccacctgggtctgcagatattcatgaagccatgtttaccatctgcgccccaataccggattCATATGAAT
ATCCTCCTTAG

With a region (lower-case letters) homologous to the sequence (1487615-1487695) corresponding to the C terminal part encoding sequence of the including the stop codon *aldA* gene (1486256 to 1487695), a reference sequence on the website <http://ecocyc.org/>, and a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette carried by the pKD3 plasmid.

Both oligonucleotides AldADr and aldADf were used to amplify the chloramphenicol resistance cassette from the pKD3 plasmid. The amplified PCR fragment was then introduced by electroporation into the MG1655 *E. coli* strain carrying the RED plasmid helper (pKD46) expressing the system α , β and *exo* genes encoding Gam, Bet and Exo recombinases to promote homologous recombination. The antibiotic-resistant transformants were then selected and the

replacement of the *aldA* gene by the chloramphenicol cassette was checked by PCR analysis using both Ydc F C f and gapCCr oligonucleotides.

3.Ydc F C f (SEQ ID NO 56) :

tgcagcggcgacgatggcgacgttcgccc (homologous to the sequence 1485722 to 1485752)

4.gapCCr (SEQ ID NO57) :

cacgatgacgaccattcatgctatactggc (homologous to the sequence 1488195 to 1488225)

5.1.7.: Construction of a *E. coli* MG1655 Δ ldhA::FRT, Δ pflAB::FRT, Δ iscR::FRT, Δ frdABCD::FRT Δ aceE::FRT, Δ mgsA::FRT Δ adhE::FRT Δ aldA::FRT modified strain:

The *aldA* gene was then replaced with a chloramphenicol resistance cassette into the MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ mgsA::FRT Δ adhE::FRT strain and the deletion was achieved by generalized P1 phage transduction from the single mutant lysate (Miller 1972).

The Cm^R transductants were selected on plate and the replacement of the *adhE* gene by the chloramphenicol cassette into the MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ mgsA::FRT Δ adhE::FRT was checked by PCR analysis using both Ydc F C f and gapCCr oligonucleotides. Finally, i) hslJC and ldhAC2, ii) pFLAB1 and pflAB2, iii) iscr1s and iscr1rv, iv) frdABCD1s and frdABCD1rv, v) aceEs and aceErv vi) mgsAs and mgsArv and vii) ychGf and adhECr couple of primers were also used in PCR analysis to confirm the deletion of *ldhA*, *pflAB*, *iscR*, *frdABCD*, *aceE*, *mgsA* and *adhE* genes respectively in the strain Δ aldA::cm. The resulting strain was named MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ mgsA::FRT Δ adhE::FRT Δ aldA::cm.

The chloramphenicol-resistance cassette was then eliminated by using the Flp helper pCP20 plasmid (Cheperanov and and Wagnernagel 1995). Cm^R mutants were transformed with pCP20, and ampicillin-resistant transformants were selected at 30°C, after which a few were colony purified at 42°C and then tested for loss of the antibiotic resistance by PCR analysis using oligonucleotides previously described (SEQ ID N°56 and 57). The new strain was named MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ mgsA::FRT Δ adhE::FRT Δ aldA::FRT.

5.1.8.: Construction of a *E. coli* MG1655 $\Delta aldB::FRT\text{-}cm\text{-}FRT$ modified strain:

The *aldB* gene was replaced with a chloramphenicol antibiotic resistance cassette flanked by Flp recognition target (FRT) that is generated by PCR by using primers with 80-nt homology extensions deleting most of the concerned gene. The used technique was described by Datsenko and Wanner in 2000.

Two oligonucleotides were designed and used to replace the *aldB* gene:

1-AldB D f consisting of 100 bases (SEQ ID N°58)

tcagaacagccccaacggtttatccgagtagctcaccagcaggcacttggttgctggttaatgctccagcatcatcttGTGTAGGCTG
GAGCTGCTTCG

With a region (lower-case letters) homologous to the sequence (3752996 to 3753075) of the *aldB* gene (sequence 3752996 à 3754534), a reference sequence on the website <http://ecocyc.org/>, and a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette of the pKD3 plasmid

2-AldBD r consisting of 100 bases (SEQ ID N°59):

atgaccaataatcccccttcagcacagattaagccccggcgagtatggttccccctcaagttaaaagcccgctatgacaaCATATGAAT
ATCCTCCTTAG

With a region (lower-case letters) homologous to the sequence (3754455 to 3754534) of the *aldB* gene (sequence 3752996 à 3754534), a reference sequence on the website <http://ecocyc.org/>, and a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette of the pKD3 plasmid.

Both oligonucleotides AldBDr and aldBDf were used to amplify the chloramphenicol resistance cassette from the pKD3 plasmid. The amplified PCR fragment was then introduced by electroporation into the *MG1655 E. coli* strain carrying the RED plasmid helper (pKD46) expressing the system α , β and *exo* genes encoding Gam, Bet and Exo recombinases to promote homologous recombination. The antibiotic-resistant transformants were then selected and the replacement of the *aldB* gene by the chloramphenicol cassette was checked by PCR analysis using both aldBCf et YiaYCr oligonucleotides.

3-aldB C f (SEQ ID NO 60) :

catatttcctcaaagaatataaaaaagaacaattaacgc (homologous to the sequence 3752449 to 3752488)

4-YiaYCr (SEQ ID NO 61) :

tatgttcacgcatggcgccaccagctggggcg (homologous to the sequence 3755040 to 3755070)

5.1.9.: Construction of a *E. coli* MG1655 Δ ldhA::FRT, Δ pflAB::FRT, Δ iscR::FRT, Δ frdABCD::FRT Δ aceE::FRT Δ mgsA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB ::FRT modified strain:

The *aldB* gene was then replaced with a chloramphenicol resistance cassette into the MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ mgsA::FRT Δ adhE::FRT Δ aldA::FRT strain and the deletion was achieved by generalized P1 phage transduction from the single mutant lysate (Miller 1972).

The Cm^R transductants were selected on plate and the replacement of the *adhE* gene by the chloramphenicol cassette into the MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ mgsA::FRT Δ adhE::FRT Δ aldA::FRT was checked by PCR analysis using both aldBCf and YiaYCr oligonucleotides. Finally, i) hslJC and ldhAC2, ii) pFLAB1 and pflAB2, iii) iscr1s and iscr1rv, iv) frdABCD1s and frdABCD1rv, v) aceEs and aceErv vi) mgsAs and mgsArv vii) ychGf and adhECr and viii) Ydc F C f and gapCCr couple of primers were also used in PCR analysis to confirm the deletion of *ldhA*, *pflAB*, *iscR*, *frdABCD*, *aceE*, *mgsA*, *adhE* and *aldA* genes respectively in the strain Δ aldB::cm. The resulting strain was named MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ mgsA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::cm.

The chloramphenicol-resistance cassette was then eliminated by using the Flp helper pCP20 plasmid (Cheperanov and Wagnernagel 1995). Cm^R mutants were transformed with pCP20, and ampicillin-resistant transformants were selected at 30°C, after which a few were colony purified at 42°C and then tested for loss of the antibiotic resistance by PCR analysis using oligonucleotides previously described (SEQ ID N°60 and 61). The new strain was named MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ mgsA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT.

5.1.6.: Construction of a MG1655 Δ glpD::FRT-cm-FRT *E. coli* modified strain:

The *glpD* gene was replaced with a chloramphenicol antibiotic resistance cassette flanked by Flp recognition target (FRT) that is generated by PCR by using primers with 80-nt homology extensions deleting most of the concerned gene. The used technique was described by Datsenko and Wanner in 2000.

Two oligonucleotides were designed and used to replace the *glpD* gene:

1-DglpD f consisting of 100 bases (SEQ ID N°62):

atggaaccaaagatctgattgtgatagggggcgccatcaatggtgctggtatcgcggcagacgccgctggacgcggtttCATATGAA
TATCCTCCTTAG

With a region (lower-case letters) homologous to the sequence (3560036-3560115) including the *glpD* gene initiation codon of the *glpD* gene (sequence 3560036 to 3561541), a reference sequence on the website <http://ecocyc.org/>, and a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette of the pKD3 plasmid

2-DglpD r consisting of 100 bases (SEQ ID N°63):

ttacgacgccagegataacctctgctgcgtatactccaccagccactgactcacacgagattgttgatccgcathtagccTGTAAGGCTG
GAGCTGCTTCG

With a region (lower-case letters) homologous to the sequence (3561462-3561541) corresponding to the C terminal part encoding sequence of the including the stop codon *glpD* gene (3560036 to 3561541), a reference sequence on the website <http://ecocyc.org/>, and a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette carried by the pKD3 plasmid.

Both oligonucleotides DglpDr and DglpDf were used to amplify the chloramphenicol resistance cassette from the pKD3 plasmid. The amplified PCR fragment was then introduced by electroporation into the *MG1655 E. coli* strain carrying the RED plasmid helper (pKD46) expressing the system α , β and *exo* genes encoding Gam, Bet and Exo recombinases to promote homologous recombination. The antibiotic-resistant transformants were then selected and the replacement of the *glpD* gene by the chloramphenicol cassette was checked by PCR analysis using both glpDCf and glpDCr oligonucleotides.

3-glpDC f (SEQ ID N° 64) :

cgtaatacatcgaactgatcc (homologous to the sequence 3559822 to 3559844)

4-glpDCr (SEQ ID N° 65) :

gcgtgggctttgcgtaattccc (homologous to the sequence 3561750 to 3561772)

5.1.7: construction of a *E. coli* MG1655 Δ ldhA::FRT, Δ pflAB::FRT, Δ iscR::FRT, Δ frdABCD::FRT Δ aceE::FRT, Δ mgsA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT Δ glpD::FRT *E. coli* modified strain:

The *glpD* gene was then replaced with a chloramphenicol resistance cassette into the MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ mgsA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT strain and the deletion was achieved by generalized P1 phage transduction from the single mutant lysate (Miller 1972).

The Cm^R transductants were selected on plate and the replacement of the *glpD* gene by the chloramphenicol cassette into the MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ mgsA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT was checked by PCR analysis using both glpDCf and glpDCr oligonucleotides. Finally, i) hslJC and ldhAC2, ii) pFLAB1 and pFLAB2, iii) iscr1s and iscr1rv, iv) frdABCD1s and frdABCD1rv, v) aceEs and aceErvi vi) mgsAs and mgsArvi vii) ychGf and adhECr viii) Ydc F C f and gapCCr and ix) aldBCf and YiaYCr couple of primers were also used in PCR analysis to confirm the deletion of *ldhA*, *pflAB*, *iscR*, *frdABCD*, *aceE*, *mgsA*, *adhE*, *aldA*, *aldB*, genes respectively in the strain Δ glpD::cm. The resulting strain was named MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ mgsA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT Δ glpD::cm.

The chloramphenicol-resistance cassette was then eliminated by using the Flp helper pCP20 plasmid (Cheperanov and Wagnagel 1995). Cm^R mutants were transformed with pCP20, and ampicillin-resistant transformants were selected at 30°C, after which a few were colony purified at 42°C and then tested for loss of the antibiotic resistance by PCR analysis using oligonucleotides previously described (SEQ ID N°64 and 65). The new strain was named MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ mgsA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT Δ glpD::FRT.

5.1.8.: Construction of a *E. coli* MG1655 Δ glpA::FRT-cm-FRT modified strain:

The *glpA* gene was replaced with a chloramphenicol antibiotic resistance cassette flanked by Flp recognition target (FRT) that is generated by PCR by using primers with 80-nt homology extensions deleting most of the concerned gene. The used technique was described by Datsenko and Wanner in 2000.

Two oligonucleotides were designed and used to replace the *glpA* gene:

1-DglpA f consisting of 100 bases (SEQ ID N° 66):

atgaaaactcgcgactcgcaatcaagtgcgtgattatcattggcggcgcgcaacgggagccgggattgcccgcgactg**CATATGA**
ATATCCTCCTTAG

With a region (lower-case letters) homologous to the sequence (2350669-2350748) including the *glpA* gene initiation codon of the *glpA* gene (sequence 2350669 to 2352297), a reference sequence on the website <http://ecocyc.org/>, and a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette of the pKD3 plasmid

2-DglpA r consisting of 100 bases (SEQ ID N°67):

tcaaagcgcacatcttctgctccttccagaccacacaatccctgataaaccagcgggtaaattcgcttcgcgca**GTGTAGGCTGG**
AGCTGCTTCG

With a region (lower-case letters) homologous to the sequence (2352297-2352220) corresponding to the C terminal part encoding sequence of the including the stop codon of *glpA* gene (2350669 to 2352297), a reference sequence on the website <http://ecocyc.org/>, and a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette carried by the pKD3 plasmid.

Both oligonucleotides DglpAr and DglpAf were used to amplify the chloramphenicol resistance cassette from the pKD3 plasmid. The amplified PCR fragment was then introduced by electroporation into the *MG1655 E. coli* strain carrying the RED plasmid helper (pKD46) expressing the system α , β and *exo* genes encoding Gam, Bet and Exo recombinases to promote homologous recombination. The antibiotic-resistant transformants were then selected and the replacement of the *glpA* gene by the chloramphenicol cassette was checked by PCR analysis using both *glpACf* and *glpACr* oligonucleotides.

3-*glpAC f* (SEQ ID N°68) :

ttagcctccgttgctgtcttgc (homologous to the sequence 2349038 to 2349052)

4-glpACr (SEQ ID N° 69) :

gccgcccataatgacagtatcaaagcgc (homologous to the sequence 2352316 to 3352289)

5.1.9: construction of an *E. coli* *MG1655ΔldhA::FRT, ΔpflAB::FRT, ΔiscR::FRT, ΔfrdABCD::FRTΔace::FRT,ΔmgsA::FRTΔadhE::FRTΔaldA::FRTΔaldB::FRTΔglpD::FRT ΔglpA::FRT* modified strain:

The *glpA* gene was then replaced with a chloramphenicol resistance cassette into the *MG1655ΔldhA::FRT ΔpflAB::FRTΔiscR::FRT ΔfrdABCD::FRTΔaceE::FRT ΔmgsA::FRT ΔadhE::FRT ΔaldA::FRTΔaldB::FRTΔglpD::FRT* strain and the deletion was achieved by generalized P1 phage transduction from the single mutant lysate (Miller 1972).

The Cm^R transductants were selected on plate and the replacement of the *glpA* gene by the chloramphenicol cassette into the *MG1655ΔldhA::FRT ΔpflAB::FRTΔiscR::FRT ΔfrdABCD::FRTΔaceE::FRTΔmgsA::FRTΔadhE::FRTΔaldA::FRTΔaldB::FRTΔglpD::FRT* was checked by PCR analysis using both *glpACf* and *glpACr* oligonucleotides. Finally, i) *hslJC* and *ldhAC2*, ii) *pFLAB1* and *pflAB2*, iii) *iscr1s* and *iscr1rv*, iv) *frdABCD1s* and *frdABCD1rv*, v) *aceEs* and *aceErv* vi) *mgsAs* and *mgsArv* vii) *ychGf* and *adhECr* viii) *Ydc F C f* and *gapCCr* ix) *aldBCf* and *YiaYCr* and x) *glpDCf* and *glpDCr* couple of primers were also used in PCR analysis to confirm the deletion of *ldhA*, *pflAB*, *iscR*, *frdABCD*, *aceE*, *mgsA*, *adhE*, *aldA*, *aldB*, *glpD* genes respectively in the strain *ΔglpA::cm*. The resulting strain was named *MG1655 ΔldhA::FRT ΔpflAB::FRTΔiscR::FRTΔfrdABCD::FRTΔaceE::FRTΔmgsA::FRTΔadhE::FRTΔaldA::FRTΔaldB::FRTΔglpD::FRTΔglpA::cm*.

The chloramphenicol-resistance cassette was then eliminated by using the Flp helper pCP20 plasmid (Cheperanov and Wagnernagel 1995). Cm^R mutants were transformed with pCP20, and ampicillin-resistant transformants were selected at 30°C, after which a few were colony purified at 42°C and then tested for loss of the antibiotic resistance by PCR analysis using oligonucleotides previously described (SEQ ID N°68 and 69). The new strain was named *MG1655 ΔldhA::FRTΔpflAB::FRTΔiscR::FRTΔfrdABCD::FRT ΔaceE::FRTΔmgsA::FRTΔadhE::FRTΔaldA::FRTΔaldB::FRTΔglpD::FRTΔglpA::FRT*.

5.2. Construction of the *pSC101-pGI-cac2229-cac0764-cac0303-pntAB* expression vector

The *pntAB* genes were amplified from the genomic DNA of *E. coli* *MG1655* using primers *pntA-NheI*f and *pntB-xma*1r. The primers were designed to introduce a RBS region along with the

pntA gene, as well as placing NheI and XmaI restriction sites upstream *pntA* and downstream *pntB* respectively:

pntAA-NheIf: (SEQ ID N° 70):

AATTGCTAGCATTATATACAAGGAGGAAACAGCTatgcgaattggcataccaagagaacgg

pntB-xma1r: (SEQ ID N° 71)

AATTCCCCTGGGATAATTtacagagctttcaggattgc

The amplified PCR fragment was then subcloned into a Zero Blunt TOPO vector (Invitrogen, Saint Aubin, France) to yield the Zero Blunt TOPO-*pntAB* plasmid and sequenced using universal primers T7P and T3P to assure that no mutations were introduced. The fragment containing the *pntAB* genes was purified on an agarose gel after digestion of the Zero Blunt TOPO-*pntAB* vector with NheI and XmaI. The 9.54 kb *pSC101_pGI-cac2229-cac0764-cac0303* expression vector (3.2) was also digested with NheI and XmaI and ligated to the NheI-XmaI digested *pntAB* fragment, yielding the 12.5 kb *pSC101pGI-cac2229-cac0764-cac0303-pntAB* vector.

5.3. Construction of the *pSC101-pGI-cac2229-cac0764-cac0303-pntAB_pthl-dhaB1-dhaB2-yqhD* expression vector

Both *dhaB1* and *dhaB2* genes were amplified from the *pSPD5* plasmid (Meynial-Salles *et al.* 2005) using primers pdhaB1 and pdhaB2. The primers were designed to introduce a RBS region along with the *dhaB1* gene, as well as placing BamHI and XhoI restriction sites upstream *dhaB1* and downstream *dhaB2* respectively:

pdhaB1f: BamHI-RBS dhaB1 (SEQ ID N°72)

ggatccgtgattggaggagtaaaaatgataagtaaagg

pdhaB2r:XhoI-dhaB2 (SEQ ID N°73)

CTCGAGttactcagctccaattgtgcacggtattcccat

The amplified PCR fragment was then subcloned into a Zero Blunt TOPO vector (Invitrogen, Saint Aubin, France) to yield the Zero Blunt TOPO-*dhaB1-dhaB2* plasmid and sequenced using universal primers T7P and T3P to assure that no mutations were introduced. The

fragment containing the *dhaB1-dhaB2* genes was purified on an agarose gel after digestion of the Zero Blunt TOPO–*dhaB1-dha2* vector with BamHI and XhoI.

The *yqhD* gene was amplified from genomic DNA of *E. coli* MG1655 using primers pyqhD1 and pyqhD2. The primers were designed to introduce the RBS01 region along with the *yqhD* gene, as well as placing XhoI and SfoI restriction sites upstream and downstream respectively:

pyqhD1: XhoI RBS01 yqhD (SEQ ID N°74)

CTCGAGttataacctccttaatgaacaactttaatctgcacacccaacccgcattct

pyqhD2 : SfoI –yqhDrv (SEQ ID N°75)

GGCGCCttagcggggcggttcgtatatacggcggtgacat

The amplified PCR fragment was then subcloned into a Zero Blunt TOPO vector (Invitrogen, Saint Aubin, France) to yield the Zero Blunt TOPO–*yqhD* plasmid and sequenced using universal primers T7P and T3P to assure that no mutations were introduced. The fragment containing the *yqhD* gene was purified on an agarose gel after digestion of the Zero Blunt TOPO–*yqhD* vector with XhoI and SfoI.

The 7 kb pSOS95 plasmid (Genbank accession number AY187686.1) was digested with BamHI (located downstream the *thl* promoter) and SfoI (located upstream the *adc* terminator) and ligated to both the BamHI-XhoI *dhaB1dhaB2* and XhoI-SfoI *yqhD* fragments to yield the *pSOS95 pthl-dhaB1-dhaB2-yqhD* plasmid. The *pSOS95 pthl-dhaB1-dhaB2-yqhD* plasmid was then digested with Sall and the operon containing fragment was purified on agarose gel before to be ligated to the pSC101_pGI-*cac2229-cac0764-cac0303-pntAB* plasmid pre-digested with Sall (5.2.), yielding the *pSC101-pGI-cac2229-cac0764-cac0303-pntAB-ptlh-dhaB1-dhaB2-yqhD* plasmid.

5.4. Introduction of the *pSC101-pGI- cac2229-cac0764-cac0303-pntAB_ptlh-dhaB1-dhaB2-yqhD* expression vector into the MG1655 *ΔldhA::FRTΔpflAB::FRTΔiscR::FRTΔfrdABCD::FRT ΔaceE::FRTΔmgsA::FRTΔaceE::FRTΔmgsA::FRTΔadhE::FRTΔaldA::FRTΔaldB::FRTΔglpD::FRTΔglpA::FRT*.

The *pSC101-pGI-cac2229-cac0764-cac0303-pntAB_pthl-dhaB1-dhaB2-yqhD* expression vector was used to transform the MG1655 $\Delta dhA::FRT\Delta pflAB::FRT\Delta iscR::FRT\Delta frdABCD::FRT\Delta aceE::FRT\Delta mgsA::FRT\Delta aceE::FRT\Delta mgsA::FRT\Delta adhE::FRT\Delta aldA::FRT\Delta aldB::FRT\Delta glpD::FRT\Delta glpA::FRT$ by electroporation (Sambrook and Russel 2001).

The transformants were selected on LB agar plates supplemented with spectinomycin (70 µg/ml) at 37°C. Some transformants were then grown in LB liquid culture supplemented with spectinomycin (100 µg/ml) overnight at 37°C to carry out a DNA plasmid extraction (GenElute HP plasmid miniprep kit, Sigma) and check for the presence of the plasmid. The *pSC101-pGI-cac2229-cac0764-cac0303-pntAB_pthl-dhaB1-dhaB2-yqhD* plasmid was finally controlled by restriction.

The final *E. coli* MG1655 $\Delta dhA::FRT\Delta pflAB::FRT\Delta ackA-pta::FRT\Delta iscR::FRT\Delta frdABCD::FRT$

$\Delta aceE::FRT\Delta mgsA::FRT\Delta adhE::FRT\Delta aldA::FRT\Delta aldB::FRT\Delta glpD::FRT\Delta glpA::FRT$.

pSC101-pGI-cac2229-cac0764-cac0303-pntAB_pthl-dhaB1-dhaB2-yqhD was grown in LB liquid medium supplemented with spectinomycin (100 µg/ml) and kept in 20% glycerol solution at -80°C.

5.5. Physiological characterization of the *E. coli* MG1655 $\Delta dhA::FRT\Delta pflAB::FRT\Delta ackApta::FRT\Delta iscR::FRT\Delta frdABCD::FRT\Delta aceE::FRT\Delta mgsA::FRT\Delta adhE::FRT\Delta aldA::FRT\Delta aldB::FRT\Delta glpD::FRT\Delta glpA::FRTpSC101-pGI-cac2229-cac0764-cac0303-pntAB_pthl-dhaB1-dhaB2-yqhD$ modified strain.

The *E. coli* MG1655 $\Delta dhA::FRT\Delta pflAB::FRT\Delta ackA-pta::FRT\Delta iscR::FRT\Delta frdABCD::FRT\Delta aceE::FRT\Delta mgsA::FRT\Delta adhE::FRT\Delta aldA::FRT\Delta aldB::FRT\Delta glpD::FRT\Delta glpA::FRT$ *pSC101-pGI-cac2229-cac0764-cac0303-pntAB_pthl-dhaB1-dhaB2-yqhD* strain was grown anaerobically on a 20 g/L glycerol mineral medium containing minerals salts as previously described (Meynial-salles *et al.* 2005) supplemented with 4 g/l of yeast extract, 5 mM sodium nitrate and spectinomycin (100 µg/ml) and inoculated with 100 µl of a LB overnight culture. Cells were grown up at 37°C during several days, and the pH was maintained by buffering the culture medium with MOPS. As a control, the *E. coli* MG1655 $\Delta dhA::FRT\Delta pflAB::FRT\Delta ackApta::FRT\Delta iscR::FRT\Delta frdABCD::FRT\Delta aceE::FRT\Delta mgsA::FRT\Delta adhE::FRT\Delta aldA::FRT\Delta aldB::FRT\Delta glpD::FRT\Delta glpA::FRT$ strain was grown in the same conditions.

The comparative phenotypic analysis was performed by measuring both glycerol consumption as well as the concentration of fermentation products for the culture of each strain as is shown in table 5:

Strains	Glycerol consumption	Concentration (g/L)	
		1,3 propanediol	Acetate

	(g/l)		
<i>E. coli</i> <i>MG1655</i> $\Delta dhA::FRT \Delta pflAB::FRT \Delta ackA-ckApta::FRT \Delta iscR::FRT \Delta frdABCD::FRT \Delta aceE::FRT \Delta mgsA::FRT \Delta adhE::FRT \Delta aldA::FRT \Delta aldB::FRT \Delta glpD::FRT \Delta glpA::FRT$	1.2	0	0.1
<i>E. coli</i> <i>MG1655</i> $\Delta dhA::FRT \Delta pflAB::FRT \Delta ackA-pta::FRT \Delta iscR::FRT \Delta frdABCD::FRT \Delta aceE::FRT \Delta mgsA::FRT \Delta adhE::FRT \Delta aldA::FRT \Delta aldB::FRT \Delta glpD::FRT \Delta glpA::FRT$ pSC101-pGI- <i>cac2229-cac0764-cac0303-pntAB</i> <i>pthl-dhaB1-dhaB2-yqhD</i>	23	13.8	3.9

As shown in table 5, the expression of the both *CA_C2229-CA_C0764-CA_C0303-pntAB* and *dhaB1-dhaB2-yqhD* operons into the *MG1655* $\Delta dhA::FRT \Delta pflAB::FRT \Delta ackA-pta::FRT \Delta iscR::FRT \Delta frdABCD::FRT \Delta aceE::FRT \Delta mgsA::FRT \Delta adhE::FRT \Delta aldA::FRT \Delta aldB::FRT \Delta glpD::FRT \Delta glpA::FRT$ *E. coli* led to the production of 1.3 propanediol and acetate as the main fermentation products from glycerol. Moreover, the expression of both *CA_C2229-CA_C0764-CA_C0303-pntAB* and *dhaB1-dhaB2-yqhD* operons favoured the growth which was strongly hampered in the control strain.

EXAMPLE 6: Heterologous production of 1, 2 propanediol by a new metabolic pathway in an *E. coli* strain with an enhanced Ferredoxin NADP⁺ reductase activity.

6.1: construction of a *E. coli* *MG1655* $\Delta dhA::FRT$, $\Delta pflAB::FRT$, $\Delta iscR::FRT$, $\Delta frdABCD::FRT$, $\Delta aceE::FRT$, $\Delta tpiA::FRT$, $\Delta adhE::FRT$, $\Delta aldA::FRT$, $\Delta aldB::FRT$, $\Delta gloA::FRT$, $\Delta hchA::FRT$ modified strain

6.1.1. Construction of an *E. coli* *MG1655* $\Delta tpiA::cm$ modified strain:

The *tpiA* gene was replaced with a chloramphenicol antibiotic resistance cassette flanked by Flp recognition target (FRT) that is generated by PCR by using primers with 80-nt homology extensions deleting most of the concerned gene. The used technique was described by Datsenko and Wanner in 2000.

Two oligonucleotides were designed and used to replace the *tpiA* gene:

DtpiAr consisting 100 bases (SEQ ID N°76):

atgcgacatccttagtgatgggtaactggaaactgaacggcagccgccacatgggtcacgagctgggttttaacctgcgtaCATATGAA
TATCCTCCTTAG

With a region (lower-case letters) homologous to the sequence (4109530-4109449) including the *tpiA* gene initiation codon of the *tpiA* gene (sequence 4108763 to 4109530), a reference sequence on the website <http://ecocyc.org/>, and a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette of the pKD3 plasmid

DtpiAf consisting of 100 bases (SEQ ID N° 77):

CttaagcctgttagccgcttctgcagctttaacgattactgcgaaggcgtcagctttcagagaagcaccaccaaccagcTGTAGGCTG
GAGCTGCTTCG

With a region (lower-case letters) homologous to the sequence (4108762-4108841) corresponding to the C terminal part encoding sequence of the including the stop codon *tpiA* gene (4108763 to 4109530), a reference sequence on the website <http://ecocyc.org/>, and a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette carried by the pKD3 plasmid.

Both DtpiAr and DtpiAf oligonucleotides were used to amplify the chloramphenicol resistance cassette from the pKD3 plasmid. The amplified PCR fragment was then introduced by electroporation into the *MG1655 E. coli* strain carrying the RED plasmid helper (pKD46) expressing the system α , β and *exo* genes encoding Gam, Bet and Exo recombinases to promote homologous recombination. The antibiotic-resistant transformants were then selected and the replacement of the *tpiA* gene by the chloramphenicol cassette was checked by PCR analysis using both *cdh* and YIIQ oligonucleotides.

cdh (SEQ ID N°78) :

ggtgatgatagttatcgccg (homologous to the sequence 4107979 to 4107998)

YIIQ (SEQ ID N°79) :

cgtgccatcgacagcagtc (homologous to the sequence 4110023 to 4110042)

6.1.2. Construction of a *E. coli* MG1655 Δ ldhA::FRT, Δ pflAB::FRT, Δ iscR::FRT, Δ frdABCD::FRT Δ aceE::FRT, Δ tpiA::FRT modified strain:

The *tpiA* gene was then replaced with a chloramphenicol resistance cassette into the MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT strain (see 5.1.3) and the deletion was achieved by generalized P1 phage transduction from the single mutant lysate (Miller 1972) using the MG1655 Δ tpiA::cm *E. coli* strain.

The Cm^R transductants were selected on plate and the replacement of the *tpiA* gene by the chloramphenicol cassette into the MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT was checked by PCR analysis using both *cdh* and YIIQ oligonucleotides. Finally, i) hslJC and ldhAC2, ii) pFLAB1 and pflAB2, iii) iscr1s and iscr1rv iv) frdABCD1s and frdABCD1rv and v) aceEs and aceErv couple of primers were also used in PCR analysis to confirm the deletion of *ldhA*, *pflAB*, *iscR*, *frdABCD* and *aceE* genes respectively in the strain Δ tpiA::cm. The resulting strain was named MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ tpiA::cm.

The chloramphenicol-resistance cassette was then eliminated by using the Flp helper pCP20 plasmid (Cheperanov and Wagnagel 1995). Cm^R mutants were transformed with pCP20, and ampicillin-resistant transformants were selected at 30°C, after which a few were colony purified at 42°C and then tested for loss of the antibiotic resistance by PCR analysis using oligonucleotides previously described (SEQ ID N°78 and 79). The new strain was named MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ tpiA::FRT.

6.1.3. Construction of a *E. coli* MG1655 Δ ldhA::FRT, Δ pflAB::FRT, Δ iscR::FRT, Δ frdABCD::FRT Δ aceE::FRT, Δ tpiA::FRT Δ adhE::FRT modified strain:

The *adhE* gene was then replaced with a chloramphenicol resistance cassette into the MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ tpiA::FRT strain and

the deletion was achieved by generalized P1 phage transduction from the single mutant lysate (Miller 1972) using the MG1655 $\Delta adhE::cm$ *E. coli* strain (see 4.1.1).

The Cm^R transductants were selected on plate and the replacement of the *adhE* gene by the chloramphenicol cassette into the MG1655 $\Delta ldhA::FRT\Delta pflAB::FRT\Delta iscR::FRT\Delta frdABCD::FRT\Delta aceE::FRT\Delta tpiA::FRT$ was checked by PCR analysis using both *ychGf* and *adhECr* oligonucleotides. Finally, i) *hslJC* and *ldhAC2*, ii) *pFLAB1* and *pflAB2*, iii) *iscr1s* and *iscr1rv* iv) *frdABCD1s* and *frdABCD1rv* v) *aceEs* and *aceErv* and vi) *cdh* and *YIIQ* couple of primers were also used in PCR analysis to confirm the deletion of *ldhA*, *pflAB*, *iscR*, *frdABCD*, *aceE* and *tpiA* genes respectively in the strain $\Delta adhE::cm$. The resulting strain was named MG1655 $\Delta ldhA::FRT\Delta pflAB::FRT\Delta iscR::FRT\Delta frdABCD::FRT\Delta aceE::FRT\Delta tpiA::FRT\Delta adhE::cm$.

The chloramphenicol-resistance cassette was then eliminated by using the Fip helper pCP20 plasmid (Cheperanov and Wagnagel 1995). Cm^R mutants were transformed with pCP20, and ampicillin-resistant transformants were selected at 30°C, after which a few were colony purified at 42°C and then tested for loss of the antibiotic resistance by PCR analysis using oligonucleotides previously described (SEQ ID N°40 and N°41). The new strain was named MG1655 $\Delta ldhA::FRT\Delta pflAB::FRT\Delta iscR::FRT\Delta frdABCD::FRT\Delta aceE::FRT\Delta tpiA::FRT\Delta adhE::FRT$.

6.1.4. Construction of an *E. coli* MG1655 $\Delta ldhA::FRT$, $\Delta pflAB::FRT$, $\Delta iscR::FRT$, $\Delta frdABCD::FRT$ $\Delta aceE::FRT$, $\Delta tpiA::FRT\Delta adhE::FRT$ $\Delta aldA::FRT$ modified strain:

The *aldA* gene was then replaced with a chloramphenicol resistance cassette into the MG1655 $\Delta ldhA::FRT\Delta pflAB::FRT\Delta iscR::FRT\Delta frdABCD::FRT\Delta aceE::FRT\Delta tpiA::FRT\Delta adhE::FRT$ strain and the deletion was achieved by generalized P1 phage transduction from the single mutant lysate (Miller 1972) using the MG1655 $\Delta aldA::cm$ *E. coli* strain (see 5.1.6).

The Cm^R transductants were selected on plate and the replacement of the *aldA* gene by the chloramphenicol cassette into the MG1655 $\Delta ldhA::FRT\Delta pflAB::FRT\Delta iscR::FRT\Delta frdABCD::FRT\Delta aceE::FRT\Delta tpiA::FRT\Delta adhE::FRT$ was checked by PCR analysis using both *YdcFCF* and *gapCCr* oligonucleotides. Finally, i) *hslJC* and *ldhAC2*, ii) *pFLAB1* and *pflAB2*, iii) *iscr1s* and *iscr1rv* iv) *frdABCD1s* and *frdABCD1rv* v) *aceEs* and *aceErv* vi) *cdh* and *YIIQ* and vii) *ychGf* and *adhECr* couple of primers were also used in PCR analysis to confirm the deletion of *ldhA*, *pflAB*, *iscR*, *frdABCD*, *ace*, *tpiA* and *adhE* genes respectively in the strain $\Delta aldA::cm$. The resulting strain was named MG1655 $\Delta ldhA::FRT\Delta pflAB::FRT\Delta iscR::FRT\Delta frdABCD::FRT\Delta aceE::FRT\Delta tpiA::FRT\Delta adhE::FRT\Delta aldA::cm$.

The chloramphenicol-resistance cassette was then eliminated by using the Flp helper pCP20 plasmid (Cheperanov and Wagnagel 1995). Cm^R mutants were transformed with pCP20, and ampicillin-resistant transformants were selected at 30°C, after which a few were colony purified at 42°C and then tested for loss of the antibiotic resistance by PCR analysis using oligonucleotides previously described (SEQ ID N° 56 and N°57). The new strain was named MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ tpiA::FRT Δ adhE::FRT Δ aldA::FRT.

6.1.5. Construction of an *E. coli* MG1655 Δ ldhA::FRT, Δ pflAB::FRT, Δ iscR::FRT, Δ frdABCD::FRT Δ aceE::FRT, Δ tpiA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT modified strain:

The *aldB* gene was then replaced with a chloramphenicol resistance cassette into the MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ tpiA::FRT Δ adhE::FRT Δ aldA::FRT strain and the deletion was achieved by generalized P1 phage transduction from the single mutant lysate (Miller 1972) using the MG1655 Δ aldB::cm *E. coli* strain (see 5.1.8).

The Cm^R transductants were selected on plate and the replacement of the *aldB* gene by the chloramphenicol cassette into the MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ tpiA::FRT Δ adhE::FRT Δ aldA::FRT was checked by PCR analysis using both aldBCf and YiaYCr oligonucleotides. Finally, i) hslJC and ldhAC2, ii) pFLAB1 and pflAB2, iii) iscr1s and iscr1rv iv) frdABCD1s and frdABCD1rv v) aceEs and aceErv vi) cdh and YIIQ, ychGf and adhECr and vii) YdcFCF and gapCCr couple of primers were also used in PCR analysis to confirm the deletion of *ldhA*, *pflAB*, *iscR*, *frdABCD*, *aceE*, *tpiA* *adhE* and *aldA* genes respectively in the strain Δ aldB::cm. The resulting strain was named MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ tpiA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::cm.

The chloramphenicol-resistance cassette was then eliminated by using the Flp helper pCP20 plasmid (Cheperanov and Wagnagel 1995). Cm^R mutants were transformed with pCP20, and ampicillin-resistant transformants were selected at 30°C, after which a few were colony purified at 42°C and then tested for loss of the antibiotic resistance by PCR analysis using oligonucleotides previously described (SEQ ID N°60 and N°61). The new strain was named MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ tpiA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT.

6.1.6 Construction of an *E. coli* MG1655Δ*gloA*::FRT-cm-FRT modified strain :

The *gloA* gene was replaced with a chloramphenicol antibiotic resistance cassette flanked by Flp recognition target (FRT) that is generated by PCR by using primers with 80-nt homology extensions deleting most of the concerned gene. The used technique was described by Datsenko and Wanner in 2000.

Two oligonucleotides were designed and used to replace the *gloA* gene:

GLOAD f consisting of 100 bases (SEQ ID N°80):

atgcgtctcttcataccatgctgcgcgttggcgattgcaacgctccatcgattttataccaaagtgcgtgggcatgaaGTGTAGGCTGG
AGCTGCTTCG

With a region (lower-case letters) homologous to the sequence (1725861-1725940) including the *gloA* gene initiation codon of the *gloA* gene (sequence 1725861 to 1726268), a reference sequence on the website <http://ecocyc.org/>, and a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette of the pKD3 plasmid

GLOA D R consisting of 100 bases (SEQ ID N°81)

ttagttgccagaccgcgaccggcgctcttctctcgattaactcaattttgtaaccgtccggatcttcacaaacgcgaCATATGAATA
TCCTCCTTAG

With a region (lower-case letters) homologous to the sequence (1726189–1726268) corresponding to the C terminal part encoding sequence of the including the stop codon *gloA* gene (1725861 to 1726268), a reference sequence on the website <http://ecocyc.org/>, and a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette carried by the pKD3 plasmid.

Both GLOADr and GLOADf oligonucleotides were used to amplify the chloramphenicol resistance cassette from the pKD3 plasmid. The amplified PCR fragment was then introduced by electroporation into the MG1655 *E. coli* strain carrying the RED plasmid helper (pKD46) expressing the system α , β and *exo* genes encoding Gam, Bet and Exo recombinases to promote homologous recombination. The antibiotic-resistant transformants were then selected and the replacement of the *gloA* gene by the chloramphenicol cassette was checked by PCR analysis using both NemACd and RntCr oligonucleotides.

NemACd (SEQ ID N° 82) :

gaagtggctgatgccgggattgaagaatggg (homologous to the sequence 1725331 to 1725361)

Rnt Cr (SEQ ID N°83) :

gggttacgttcagtgaggcgcttctgcgg (homologous to the sequence 1726765 to 1726795)

6.1.7 Construction of an *E. coli* MG1655 Δ ldhA::FRT, Δ pflAB::FRT, Δ iscR::FRT, Δ frdABCD::FRT Δ aceE::FRT, Δ tpiA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT Δ gloA::FRT modified strain:

The *gloA* gene was then replaced with a chloramphenicol resistance cassette into the MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ tpiA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT strain and the deletion was achieved by generalized P1 phage transduction from the single mutant lysate (Miller 1972) using the MG1655 Δ gloA::cm *E. coli* strain.

The Cm^R transductants were selected on plate and the replacement of the *gloA* gene by the chloramphenicol cassette into the MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ tpiA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT was checked by PCR analysis using both NemACd and RntCr oligonucleotides. Finally, i) hslJC and ldhAC2, ii) pFLAB1 and pflAB2, iii) iscr1s and iscr1rv iv) frdABCD1s and frdABCD1rv v) aceEs and aceErv vi) cdh and YIIQ, ychGf and adhECr vii) YdcFCF and gapCCr cand viii) aldBCf and YiaYCr couple of primers were also used in PCR analysis to confirm the deletion of *ldhA*, *pflAB*, *iscR*, *frdABCD*, *aceE*, *tpiA*, *adhE*, *aldA* and *aldB* genes respectively in the strain Δ gloA::cm. The resulting strain was named MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ tpiA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT Δ gloA::cm.

The chloramphenicol-resistance cassette was then eliminated by using the Flp helper pCP20 plasmid (Cheperanov and Wagemagel 1995). Cm^R mutants were transformed with pCP20, and ampicillin-resistant transformants were selected at 30°C, after which a few were colony purified at 42°C and then tested for loss of the antibiotic resistance by PCR analysis using oligonucleotides previously described (SEQ ID N°82 and N°83). The new strain was named MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ tpiA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT Δ gloA::FRT.

6.1.8 Construction of an *E. coli* MG1655 Δ gloB::FRT-cm-FRT modified strain :

The *gloB* gene was replaced with a chloramphenicol antibiotic resistance cassette flanked by Flp recognition target (FRT) that is generated by PCR by using primers with 80-nt homology extensions deleting most of the concerned gene. The used technique was described by Datsenko and Wanner in 2000.

Two oligonucleotides were designed and used to replace the *gloB* gene:

GLOB D f consisting of 100 bases (SEQ ID N°84):

acaatcaggcagcgacctgcttcattcattcaaaaccagatgtaattgtcatcaaggcgggaatactgttaagattcatGTGTAGGCTG
GAGCTGCTTCG

With a region (lower-case letters) homologous to the sequence (234703-234782) including the *gloB* gene initiation codon of the *gloB* gene (sequence 234027 to 234782), a reference sequence on the website <http://ecocyc.org/>, and a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette of the pKD3 plasmid

GLOB D R consisting of 100 bases (SEQ ID N°85)

taatgtaattaatgaagaaacattattgcaacaacctgaagagcggtttgcatggtaaggtaaagaaagataggttctgaCATATGAAT
ATCCTCCTTAG

With a region (lower-case letters) homologous to the sequence (234027-234108) corresponding to the C terminal part encoding sequence of the including the stop codon *gloB* gene (234027 to 234782), a reference sequence on the website <http://ecocyc.org/>, and a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette carried by the pKD3 plasmid.

Both GLOBDr and GLOBDf oligonucleotides were used to amplify the chloramphenicol resistance cassette from the pKD3 plasmid. The amplified PCR fragment was then introduced by electroporation into the *MG1655 E. coli* strain carrying the RED plasmid helper (pKD46) expressing the system α , β and *exo* genes encoding Gam, Bet and Exo recombinases to promote homologous recombination. The antibiotic-resistant transformants were then selected and the replacement of the *gloB* gene by the chloramphenicol cassette was checked by PCR analysis using both MltDCfd and YafS Cr oligonucleotides.

MltDCfd (SEQ ID N° 86) :

ggcgagtaatatcgcttttgcc (homologous to the sequence 233929 to 233950)

YafS Cr (SEQ ID N°87):

gacagtttgagggactcttgccgg (homologous to the sequence 234822 to 234845)

6.1.9. Construction of an *E. coli* MG1655 Δ ldhA::FRT, Δ pflAB::FRT, Δ iscR::FRT, Δ frdABCD::FRT Δ aceE::FRT, Δ tpiA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT Δ gloA::FRT Δ gloB::FRT modified strain:

The *gloB* gene was then replaced with a chloramphenicol resistance cassette into the MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ tpiA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT Δ gloA::FRT strain and the deletion was achieved by generalized P1 phage transduction from the single mutant lysate (Miller 1972) using the MG1655 Δ gloB::cm *E. coli* strain.

The Cm^R transductants were selected on plate and the replacement of the *gloB* gene by the chloramphenicol cassette into the MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ tpiA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT Δ gloA::FRT was checked by PCR analysis using both MltDCfd and YafS Cr oligonucleotides. Finally, i) hslJC and ldhAC2, ii) pFLAB1 and pflAB2, iii) iscr1s and iscr1rv iv) frdABCD1s and frdABCD1rv v) aceEs and aceErv vi) cdh and YIIQ, ychGf and adhECr vii) YdcFCF and gapCCr cand viii) aldBCf and YiaYCr and NemACd and RntCr couple of primers were also used in PCR analysis to confirm the deletion of *ldhA*, *pflAB*, *iscR*, *frdABCD*, *aceE*, *tpiA*, *adhE*, *aldA*, *aldB* and *gloA* genes respectively in the strain Δ gloB::cm. The resulting strain was named MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ tpiA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT Δ gloA::FRT Δ gloB::cm.

The chloramphenicol-resistance cassette was then eliminated by using the Flp helper pCP20 plasmid (Cheperanov and Wagnernagel 1995). Cm^R mutants were transformed with pCP20, and ampicillin-resistant transformants were selected at 30°C, after which a few were colony purified at 42°C and then tested for loss of the antibiotic resistance by PCR analysis using oligonucleotides previously described (SEQ ID N°86 and N°87). The new strain was named MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ tpiA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT Δ gloA::FRT Δ gloB::FRT.

6.1.10. Construction of an *E. coli* MG1655 Δ *hchA*::FRT-cm-FRT modified strain :

The *hchA* gene was replaced with a chloramphenicol antibiotic resistance cassette flanked by Flp recognition target (FRT) that is generated by PCR by using primers with 80-nt homology extensions deleting most of the concerned gene. The used technique was described by Datsenko and Wanner in 2000.

Two oligonucleotides were designed and used to replace the *hchA* gene:

HchAD f consisting of 100 bases (SEQ ID N°88):

atgactgttcaacaagtaaaaaatccgcaggtcgatattgctgaagataatgcattcttccttcagaatattcgcttagCCTTCAGAATA
TTCGCTTAG GTGTAGGCTGGAGCTGCTTCG

With a region (lower-case letters) homologous to the sequence (2033859-2033938) including the *hchA* gene initiation codon of the *hchA* gene (sequence 2033859 to 2034710), a reference sequence on the website <http://ecocyc.org/>, and a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette of the pKD3 plasmid

HchA D R consisting of 100 bases (SEQ ID N°89)

ttaacccgcgtaagctgccagcatttctcgccgccagtttaccacgcattcgctgcaaaaggactgtcgccggtgaCATATGAAT
ATCCTCCTTAG

With a region (lower-case letters) homologous to the sequence (2034631-2034710) corresponding to the C terminal part encoding sequence of the including the stop codon *hchA* gene (2033859 to 2034710), a reference sequence on the website <http://ecocyc.org/>, and a region (upper-case letters) for the amplification of the chloramphenicol resistance cassette carried by the pKD3 plasmid.

Both HchADr and HchADf oligonucleotides were used to amplify the chloramphenicol resistance cassette from the pKD3 plasmid. The amplified PCR fragment was then introduced by electroporation into the MG1655 *E. coli* strain carrying the RED plasmid helper (pKD46) expressing the system α , β and *exo* genes encoding Gam, Bet and Exo recombinases to promote homologous recombination. The antibiotic-resistant transformants were then selected and the

replacement of the *hchA* gene by the chloramphenicol cassette was checked by PCR analysis using both YedS_3Cf and YedvCr oligonucleotides.

YedS_3Cf (SEQ ID N°90) :

tgtgggcttagtctaccagttc (homologous to the sequence 2033243-2033264)

Yedv Cr (SEQ ID N°91):

cgttaccgcaaagaaattaa (homologous to the sequence 2034818 to 2034837)

6.1.11. Construction of an *E. coli* MG1655 Δ ldhA::FRT, Δ pflAB::FRT, Δ iscR::FRT, Δ frdABCD::FRT Δ aceE::FRT, Δ tpiA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT Δ gloA::FRT Δ gloB::FRT Δ hchA::FRT modified strain:

The *hchA* gene was then replaced with a chloramphenicol resistance cassette into the MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ tpiA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT Δ gloA::FRT Δ gloB::FRT strain and the deletion was achieved by generalized P1 phage transduction from the single mutant lysate (Miller 1972) using the MG1655 Δ hchA::cm *E. coli* strain.

The Cm^R transductants were selected on plate and the replacement of the *hchA* gene by the chloramphenicol cassette into the MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ tpiA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT Δ gloA::FRT Δ gloB::FRT was checked by PCR analysis using both YedS_3Cf and YedvCr oligonucleotides. Finally, i) hslJC and ldhAC2, ii) pFLAB1 and pflAB2, iii) iscr1s and iscr1rv iv) frdABCD1s and frdABCD1rv v) aceEs and aceErv vi) cdh and YIIQ, ychGf and adhECr vii) YdcFCF and gapCCr cand viii) aldBCf and YiaYCr, NemACd and RntCr and MltDCfd and YafS Cr couple of primers were also used in PCR analysis to confirm the deletion of *ldhA*, *pflAB*, *iscR*, *frdABCD*, *aceE*, *tpiA*, *adhE*, *aldA*, *aldB*, *gloA* and *gloB* genes respectively in the strain Δ hchA::cm. The resulting strain was named MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FRT Δ frdABCD::FRT Δ aceE::FRT Δ tpiA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT Δ gloA::FRT Δ gloB::FRT Δ hchA::cm.

The chloramphenicol-resistance cassette was then eliminated by using the Flp helper pCP20 plasmid (Cheperanov and Wagnernagel 1995). Cm^R mutants were transformed with pCP20, and ampicillin-resistant transformants were selected at 30°C, after which a few were colony purified at 42°C and then tested for loss of the antibiotic resistance by PCR analysis using

oligonucleotides previously described (SEQ ID N°90 and N°919). The new strain was named MG1655 Δ *ldhA::FRT* Δ *pflAB::FRT* Δ *iscR::FRT* Δ *frdABCD::FRT* Δ *aceE::FRT* Δ *tpiA::FRT* Δ *adhE::FRT* Δ *aldA::FRT* Δ *aldB::FRT* Δ *gloA::FRT* Δ *gloB::FRT* Δ *hchA::FRT*.

6.2 Construction of the *pSC101-pGI-cac2229-cac0764-cac0303_pthl gldA-yqhD* expression vector

The *gldA* gene was amplified from genomic DNA of *E. coli* MG1655 using primers *pgldA1f* and *pgldA2r*. The primers were designed to introduce the RBS01 region along with the *gldA* gene, as well as placing BamHI and XhoI restriction sites upstream and downstream respectively:

pgldA1f: BamHI-RBS *gldA* (SEQ ID N°92)

GGATCCtataacctccttaatggaccgcattattcaatcaccggg

pgldA2r: XhoI-*gldA* (SEQ ID N°93)

CTCGAGttattccactcttgaggaaacgc

The amplified PCR fragment was then subcloned into a Zero Blunt TOPO vector (Invitrogen, Saint Aubin, France) to yield the Zero Blunt TOPO-*gldA* plasmid and sequenced using universal primers T7P and T3P to assure that no mutations were introduced. The fragment containing the *dhaB1-dhaB2* genes was purified on an agarose gel after digestion of the Zero Blunt TOPO-*gldA* vector with BamHI and XhoI.

The *pSOS95 pthl-dhaB1-dhaB2-yqhD* plasmid (see 5.3) was digested with BamHI and XhoI and ligated to the BamHI-XhoI *gldA* fragment to yield the *pSOS95 pthl-gldA-yqhD* plasmid. The *pSOS95 pthl-gldA-yqhD* plasmid was then digested with SalI and the operon containing fragment was purified on agarose gel before to be ligated to the *pSC101-pGI-cac2229-cac0764-cac0303* plasmid pre-digested with SalI (3.2.), yielding the *pSC101-pGI-cac2229-cac0764-cac0303_pthl-gldA-yqhD* plasmid.

6.3 Construction of the *pSC101-pGI-cac2229-cac0764-cac0303-pntAB_pthl sadh_{beij}-yqhD* expression vector

The *sadh* gene from *C. beijerinckii* was amplified from the *pSOS952* plasmid (Dusséaux *et al.* 2013) using primers *psadh11* and *psadh2r*. The primers were designed to amplify the RBS

region along with the *sadh* gene, as well as placing BamHI and XhoI restriction sites upstream and downstream *sadh* respectively:

psadh1f : BamHI-RBSsadh (SEQ ID N°94)

GGATCCtatctttaaggaggacatattttatgaaaggttttgcattgc

psadh2r:XhoI-sadh (SEQ ID N°95)

CTCGAGttataatataactactgctttaattaagtcttttggc

The amplified PCR fragment was then subcloned into a Zero Blunt TOPO vector (Invitrogen, Saint Aubin, France) to yield the Zero Blunt TOPO–*sadh* plasmid and sequenced using universal primers T7P and T3P to assure that no mutations were introduced. The fragment containing the *sadh* gene was purified on an agarose gel after digestion of the Zero Blunt TOPO–*sadh* vector with BamHI and XhoI.

The *pSOS95 pthl-dhaB1-dhaB2-yqhD* plasmid (see 5.3) was digested with BamHI and XhoI and ligated to the BamHI-XhoI *sadh* fragment to yield the *pSOS95 pthl-sadh-yqhD* plasmid. The *pSOS95 pthl-sadh-yqhD* plasmid was then digested with SalI and the operon containing fragment was purified on agarose gel before to be ligated to the pSC101_pGI-cac2229-cac0764-cac0303-pntAB plasmid pre-digested with SalI (5.2.), yielding the *pSC101-pGI-cac2229-cac0764-cac0303-pntAB_pthl-sadh-yqhD* plasmid.

6.4. Introduction of the *pSC101- pGI-cac2229-cac0764-cac0303-pntAB_pthl gldA-yqhD* expression vector into the MG1655Δ*ldhA::FRT*Δ*pflAB::FRT*Δ*iscR::FR*Δ*frdABCD::FRT*Δ*aceE::FRT*Δ*tpiA::FRT*Δ*adhE::FRT*Δ*aldA::FRT*Δ*aldB::FRT*Δ*gloA::FRT*Δ*gloB::FRT*Δ*hchA::FRT* *E. coli* strain.

The *pSC101- pGI-cac2229-cac0764-cac0303-pntAB_pthl gldA-yqhD* expression vector was used to transform MG1655Δ*ldhA::FRT*Δ*pflAB::FRT*Δ*iscR::FR*Δ*frdABCD::FRT*Δ*aceE::FRT*Δ*tpiA::FRT*Δ*adhE::FRT*Δ*aldA::FRT*Δ*aldB::FRT*Δ*gloA::FRT*Δ*gloB::FRT*Δ*hchA::FRT* *E. coli* strain by electroporation (Sambrook and Russel 2001).

The transformants were selected on LB agar plates supplemented with spectinomycin (70 μg/ml) at 37°C. Some transformants were then grown in LB liquid culture supplemented with spectinomycin (100 μg/ml) overnight at 37°C to carry out a DNA plasmid extraction (GenElute HP

plasmid miniprep kit, Sigma) and check for the presence of the plasmid. The *pSC101-pGI-cac2229-cac0764-cac0303-pthlgldA-yqhD* plasmid was finally controlled by restriction profile.

The final MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FR Δ frdABCD::FRT Δ aceE::FRT Δ tpiA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT Δ gloA::FRT Δ gloB::FRT Δ hchA::FRT *pSC101-pGI-cac2229-cac0764-cac0303-pthlgldA-yqhD* *E. coli* strain was grown in LB liquid medium supplemented with spectinomycin (100 µg/ml) and kept in 20% glycerol solution at -80°C.

6.5. Introduction of the *pSC101-pGI-cac2229-cac0764-cac0303-pntAB_pthl sadh_{beij}-yqhD* expression vector into the MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FR Δ frdABCD::FRT Δ aceE::FRT Δ tpiA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT Δ gloA::FRT Δ gloB::FRT Δ hchA::FRT *E. coli* strain.

The *pSC101-pGI-cac2229-cac0764-cac0303-pntAB_pthl sadh_{beij}-yqhD* expression vector was used to transform MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FR Δ frdABCD::FRT Δ aceE::FRT Δ tpiA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT Δ gloA::FRT Δ gloB::FRT Δ hchA::FRT *E. coli* strain by electroporation (Sambrook and Russel 2001).

The transformants were selected on LB agar plates supplemented with spectinomycin (70 µg/ml) at 37°C. Some transformants were then grown in LB liquid culture supplemented with spectinomycin (100 µg/ml) overnight at 37°C to carry out a DNA plasmid extraction (GenElute HP plasmid miniprep kit, Sigma) and check for the presence of the plasmid. The *pSC101-pGI-cac2229-cac0764-cac0303-pntAB_pthl-sadh_{beij}-yqhD* plasmid was finally controlled by restriction profile.

The final MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FR Δ frdABCD::FRT Δ aceE::FRT Δ tpiA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT Δ gloA::FRT Δ gloB::FRT Δ hchA::FRT *pSC101-pGI-cac2229-cac0764-cac0303-pntAB_pthl-sadh_{beij}-yqhD* *E. coli* strain was grown in LB liquid medium supplemented with spectinomycin (100 µg/ml) and kept in 20% glycerol solution at -80°C.

6.6 Physiological characterization of the *E. coli* MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FR Δ frdABCD::FRT Δ aceE::FRT Δ tpiA::FRT Δ adhE::FRT Δ aldA::FRT Δ aldB::FRT Δ gloA::FRT Δ gloB::FRT Δ hchA::FRT *pSC101-pGI-cac2229-cac0764-cac0303-pntAB_pthl-sadh_{beij}-yqhD* and MG1655 Δ ldhA::FRT Δ pflAB::FRT Δ iscR::FR Δ frdABCD::FRT Δ aceE::FRT Δ tpiA::FRT Δ adhE::

***FRTΔaldA::FRTΔaldB::FRTΔgloA::FRTΔgloB::FRTΔhchA::FRT pSC101-pGI- cac2229-cac0764-cac0303-ptlglA-yqhD* modified strains.**

Both *E. coli* MG1655Δ*ldhA::FRTΔpflAB::FRTΔiscR::FRΔfrdABCD::FRTΔaceE::FRTΔtpiA::FRTΔadhE::FRTΔaldA::FRTΔaldB::FRTΔgloA::FRTΔgloB::FRTΔhchA::FRT* (*pSC101-pGI-cac2229-cac0764-cac0303-pntAB_ptl-sadh_{beij}-yqhD*) and MG1655Δ*ldhA::FRTΔpflAB::FRTΔiscR::FRΔfrdABCD::FRTΔaceE::FRTΔtpiA::FRTΔadhE::FRTΔaldA::FRTΔaldB::FRTΔgloA::FRTΔgloB::FRTΔhchA::FRT* (*pSC101-pGI- cac2229-cac0764-cac0303_ptlglA-yqhD*) strains were grown anaerobically on a 20 g/L glucose mineral medium containing minerals salts as previously described (Meynial-salles *et al.* 2005) supplemented with 4 g/l of yeast extract, 5 mM sodium nitrate and spectinomycin (100 μg/ml) and inoculated with 100 μl of a LB overnight culture. Cells were grown up at 37°C during several days, and the pH was maintained by buffering the culture medium with MOPS. As a control, the *E. coli* MG1655Δ*ldhA::FRTΔpflAB::FRTΔiscR::FRΔfrdABCD::FRTΔaceE::FRTΔtpiA::FRTΔadhE::FRTΔaldA::FRTΔaldB::FRTΔgloA::FRTΔgloB::FRTΔhchA::FRT* strain was grown in the same conditions.

The comparative phenotypic analysis was performed by measuring both glucose consumption as well as the concentration of fermentation products for the culture of each strain as is shown in table 6:

Strains	Glucose consumption g/l	Concentration (g/L)	
		1,2 propanediol	Acetate
MG1655Δ <i>ldhA::FRTΔpflAB::FRTΔiscR::FRΔfrdABCD::FRTΔaceE::FRTΔtpiA::FRTΔadhE::FRTΔaldA::FRTΔaldB::FRTΔgloA::FRTΔgloB::FRTΔhchA::FRT</i>	2.3	0.2	0.05
MG1655Δ <i>ldhA::FRTΔpflAB::FRTΔiscR::FRΔfrdABCD::FRTΔaceE::FRTΔtpiA::FRTΔadhE::FRTΔaldA::FRTΔaldB::FRTΔgloA::FRTΔgloB::FRTΔhchA::FRT pSC101-pGI-cac2229-cac0764-cac0303-ptl-sadh_{beij}-yqhD</i>	16.4	6.3	5.2
MG1655Δ <i>ldhA::FRTΔpflAB::FRTΔiscR::FRΔfrdABCD::FRTΔaceE::FRTΔtpiA::FRTΔadhE::FRTΔaldA::FRTΔaldB::FRTΔgloA::FRTΔgloB::</i>	19.8	7.2	6.3

<i>FRTΔhchA::FRT</i> <i>pSC101-pGI-cac2229-cac0764-cac0303_pthl-gldA-yqhD</i>			
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As shown in table 6, the expression of the both *CA_C2229-CA_C0764-CA_C0303* and *gldA-yqhD* operons or both *CA_C2229-CA_C0764-CA_C0303* and *sadh_{beij}-yqhD* operons into the MG1655 *ΔldhA::FRTΔpflAB::FRTΔiscR::FRΔfrdABCD::FRTΔaceE::FRTΔtpiA::FRTΔadhE::FRTΔaldA::FRTΔaldB::FRTΔgloA::FRTΔgloB::FRTΔhchA::FRT* *E.coli* strain led to the production of 1.2 propanediol and acetate as the main fermentation products from glucose. Moreover, the expression of operons, whatever the combination is, favoured the growth which was strongly hampered in the control strain.

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CLAIMS

1. A modified microorganism having Ferredoxin NADP⁺ reductase enzymatic activity, wherein the activity of the polypeptide having at least 70 % identity with the sequence of SEQ ID N°1 is enhanced in comparison with the non-modified microorganism.
2. The microorganism according to claim 1 with enhanced Ferredoxin NADP⁺ reductase enzymatic activity, wherein a polynucleotide coding for the polypeptide having at least 70 % identity with the sequence of SEQ ID N°1 is overexpressed.
3. The microorganism according to claim 2 wherein it contains at least one modification to produce ethanol.
4. The microorganism of claim 3, wherein the at least one modification to produce ethanol comprises:
 - Enhancement of the expression of at least one of the following genes: *pfor*, *fdx*,
udhA
 - and/or
 - Attenuation of the expression of at least one of the following genes: *aceE*, *aceF*,
lpd, *pflA*, *pflB*, *frdABCD*, *ldhA*, *mgsA*, *ackA*, *pta*, *poxB* and *iscR*.
5. The microorganism according to claim 2 wherein it contains at least one modification to produce n-butanol.
6. The microorganism according to claim 5, wherein the at least one modification to produce n-butanol comprises:
 - Enhancement of the expression of at least one of the following genes: *pfor*, *fdx*,
atoB, *hbd*, *crt*, *bcd*, *etfA*, *etfB*, *adhE2*, *udhA*,
 - and/or
 - Attenuation of the expression of at least one of the following genes: *aceE*, *aceF*,
lpd, *pflA*, *pflB*, *frdABCD*, *adhE*, *ldhA*, *mgsA*, *ackA*, *pta*, *poxB* and *iscR*.
7. The microorganism according to claim 2 wherein it comprises at least one modification to produce 1, 3 propanediol.

8. The microorganism of claim 7, wherein the at least one modification to produce 1, 3 propanediol comprises:

- Enhancement of the expression of at least one of the following genes : *pfor*, *fdx*, *pntAB*, *dhaB1*, *dhaB2*, *yqhD*
- and/or
- Attenuation of the expression of at least one of the following genes: *aceE*, *aceF*, *lpd*, *aldA*, *aldB*, *ldhA*, *pflA*, *pflB*, *adhE*, *iscR*, *glpA*, *poxB* and *glpD*.

9. The microorganism according to claim 2 wherein it comprises at least one modification to produce 1, 2 propanediol.

10. The microorganism according to claim 9, wherein the at least one modification to produce 1, 2 propanediol comprises:

- Attenuation of the expression of at least one of the following genes : *ptsG*, *ptsH*, *ptsI*, *crp*, *edd*, *eda*, *gloA*, *aceE*, *aceF*, *lpd*, *aldA*, *aldB*, *ldhA*, *pflA*, *pflB*, *adhE*, *tpiA*, *gapA*, *pykA*, *pykF*, *ackA*, *pta*, *poxB*, *arcA* and *ndh*,
- And/or
- Enhancement of the expression of at least one of the following genes : *pfor*, *fd*, *pntAB*, *gapN*, *galP*, *glk*, *ppsA*, *mgsA*, *yqhD*, *yafB*, *ydhF*, *ycdW*, *yqhE*, *yeaE*, *yghZ*, *yajO*, *tas*, *ydjG*, *ydbC*, *gldA*, *fucO*.

11. A method for modulating the Ferredoxin NADP+ reductase enzymatic activity in a microorganism, wherein the activity of the polypeptide having at least 70 % identity with the sequence of SEQ ID N°1 is enhanced in the said microorganism.

12. The method of claim 11, wherein the Ferredoxin NADP+ reductase enzymatic activity is enhanced by overexpressing a polynucleotide coding for the polypeptide having at least 70 % identity with the sequence of SEQ ID N°1.

13. A method for preparing ethanol wherein the microorganism according to claim 3 or 4 is grown in an appropriate culture medium comprising a source of carbon and the ethanol is recovered.

14. A method for preparing n-butanol wherein the microorganism according to claim 5 or 6 is grown in an appropriate culture medium comprising a source of carbon and the n-butanol is recovered.
15. A method for preparing 1, 3 propanediol wherein the microorganism according to claim 7 or 8 is grown in an appropriate culture medium comprising a source of carbon and the 1, 3-propanediol is recovered.
16. A method for preparing 1, 2 propanediol wherein the microorganism according to claim 9 or 10 is grown in an appropriate culture medium comprising a source of carbon and the 1, 2-propanediol is recovered.
17. Use of a microorganism according to claim 3 or 4 to produce ethanol.
18. Use of a microorganism according to claim 5 or 6 to produce n-butanol.
19. Use of microorganism according to claim 7 or 8 to produce 1,3 propanediol.
20. Use of microorganism according to claim 9 or 10 to produce 1,2 propanediol.
21. A polypeptide having at least 70 % identity with the sequence of SEQ ID N°1 having Ferredoxin NADP+ reductase enzymatic activity.
22. Use of a polypeptide having at least 70 % identity with the sequence of SEQ ID N°1 for the reduction of NADP+ using electrons from reduced ferredoxin in a microorganism or in the culture medium of a microorganism.

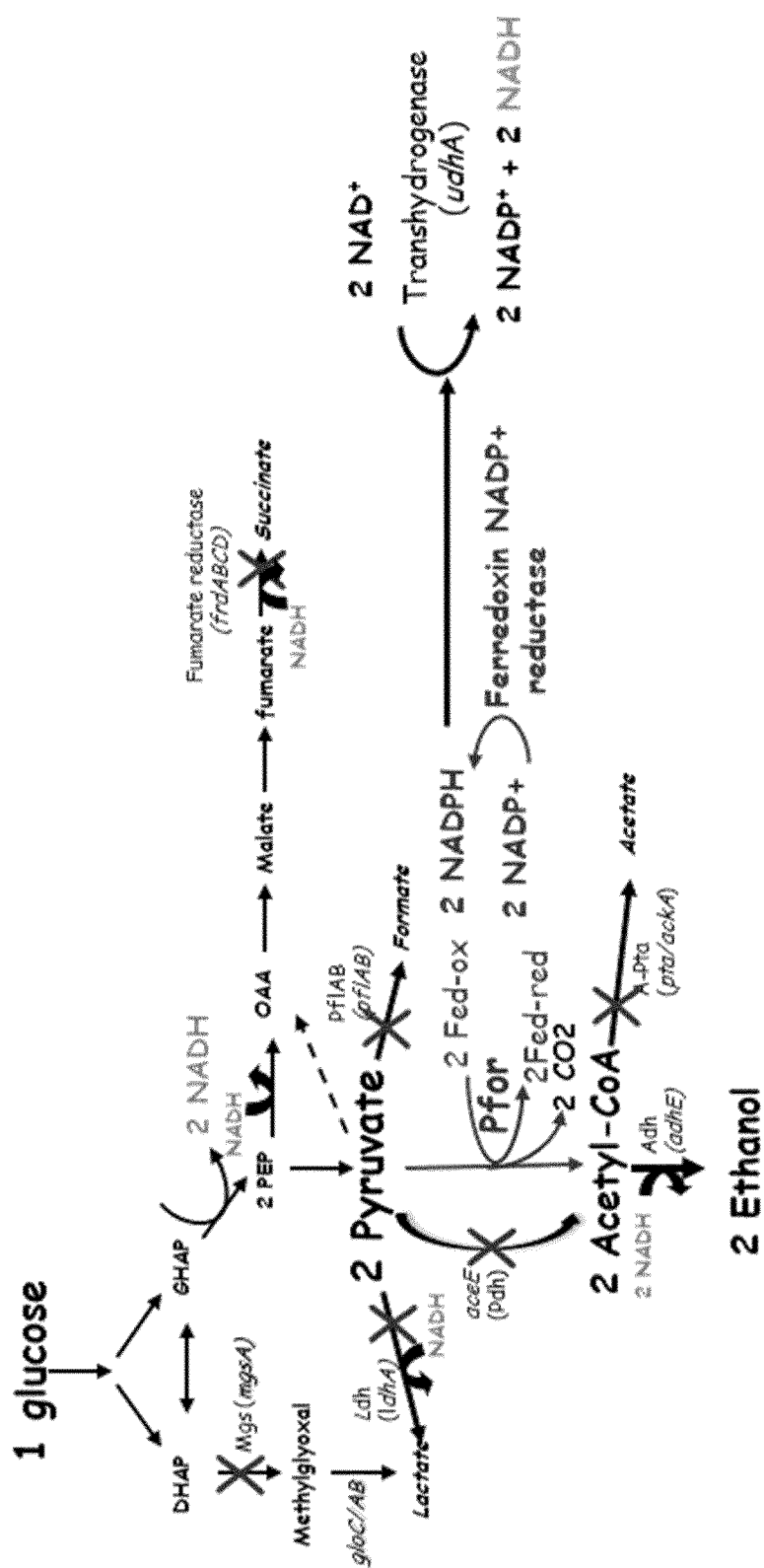


Figure 1



Figure 2

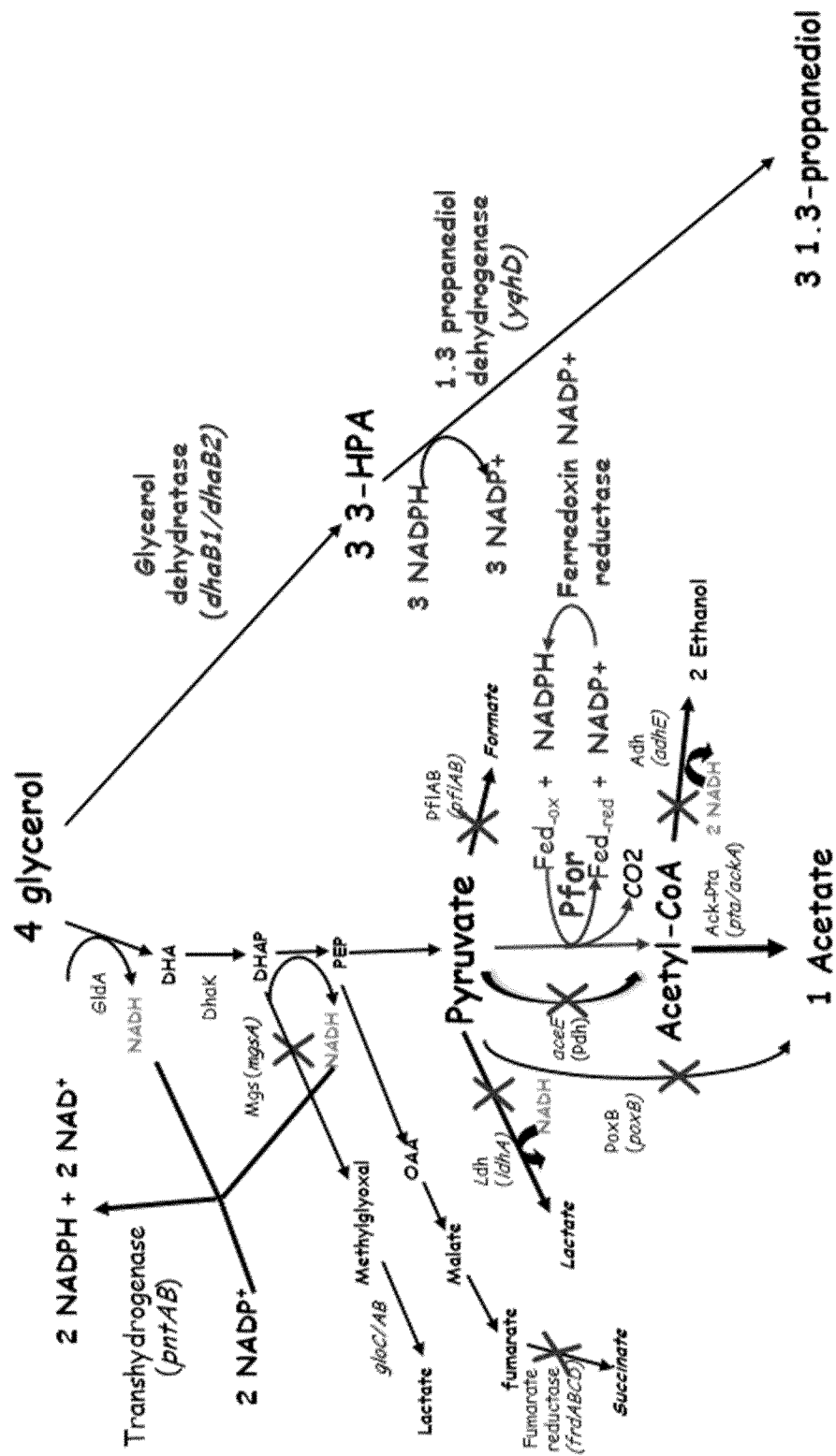


Figure 3

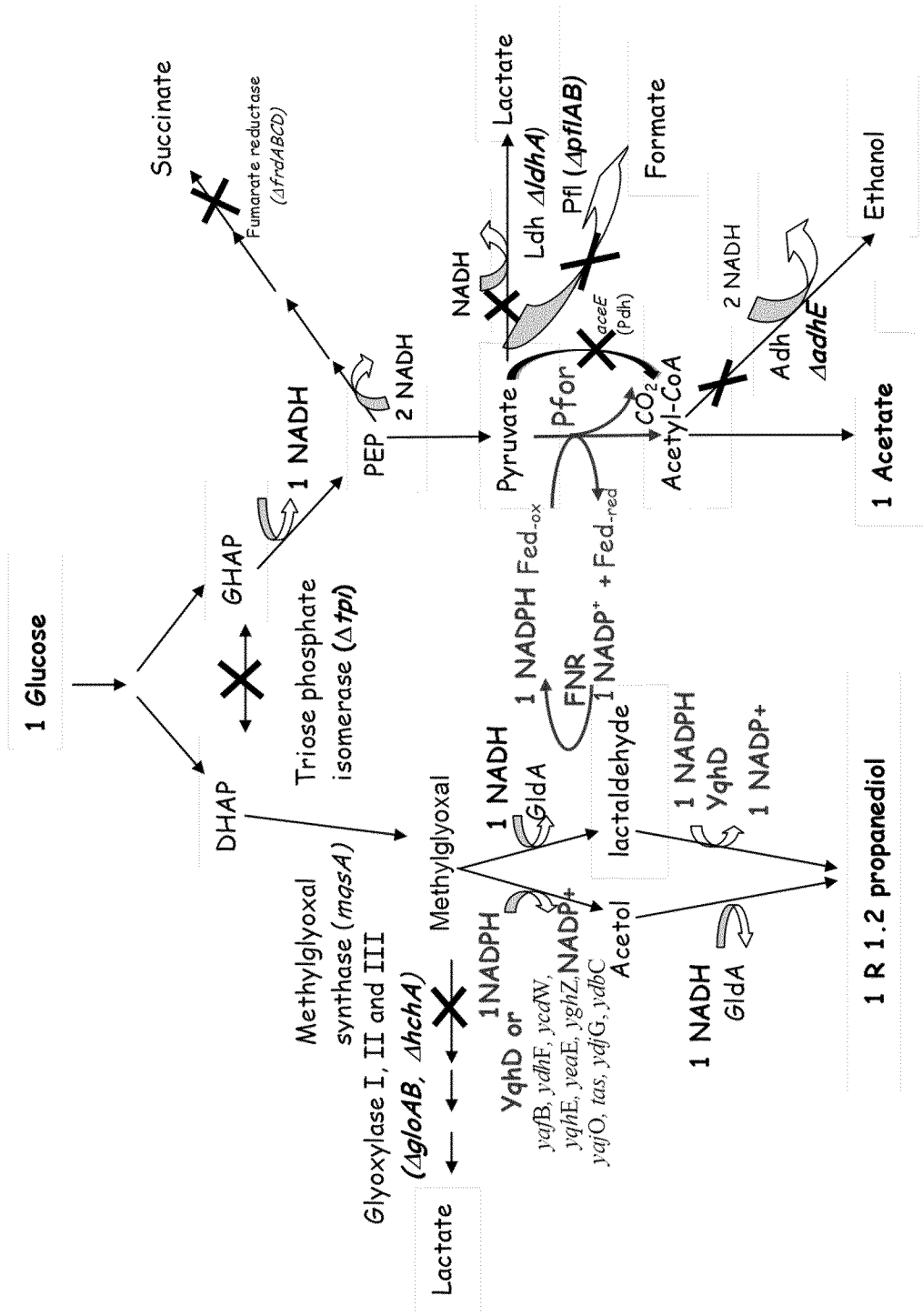


Figure 4

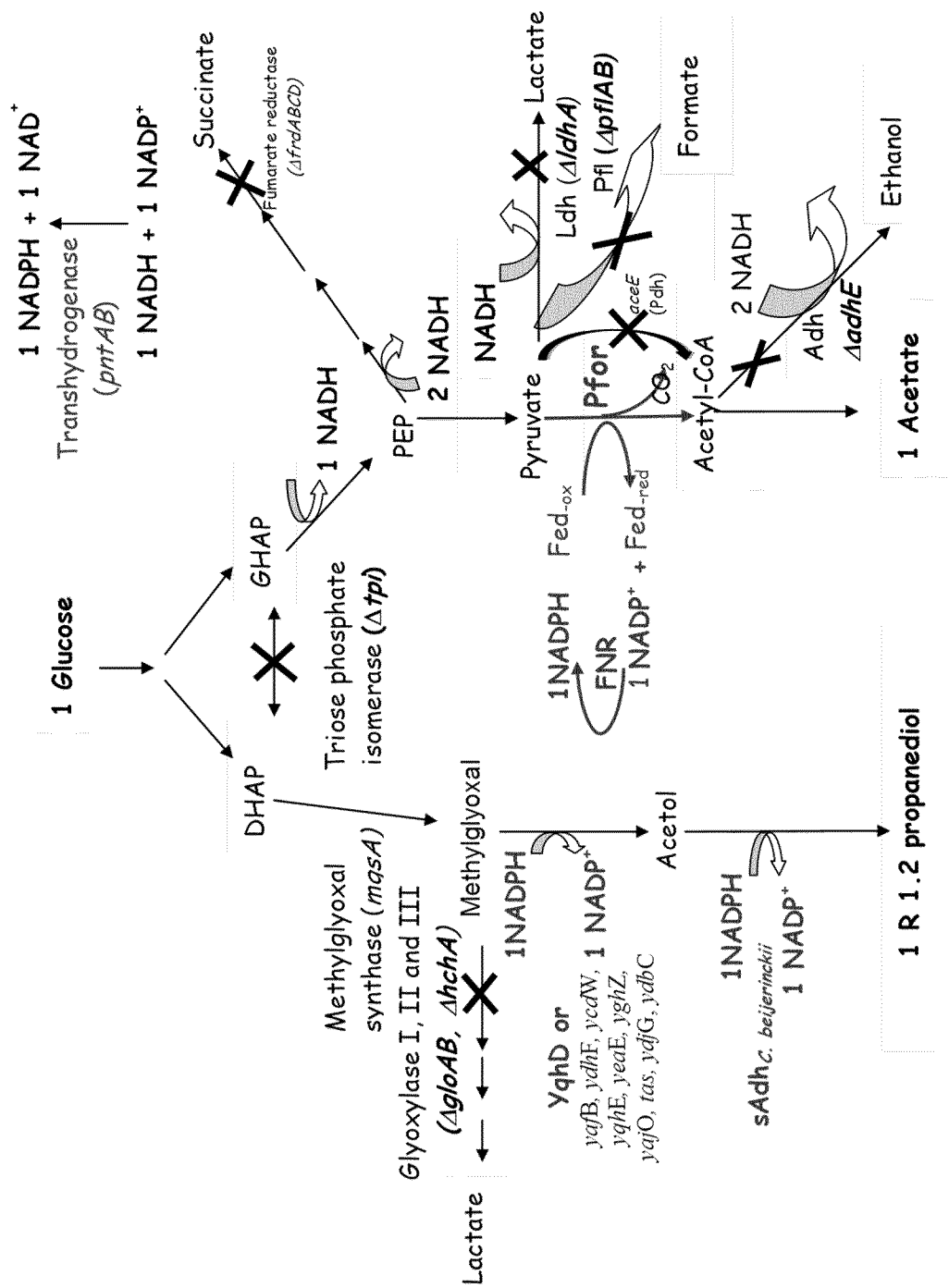


Figure 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/067568

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N9/02 C12P7/06 C12P7/16 C12P7/18
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, FSTA, IBM-TDB, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE UniProt [Online]</p> <p>1 October 2001 (2001-10-01), "SubName: Full=NADPH-dependent glutamate synthase beta chain {ECO:0000313 EMBL:AAK78740.1}";", XP002752983, retrieved from EBI accession no. UNIPROT:Q97L02 Database accession no. Q97L02 sequence</p> <p>-/--</p>	<p>1,2,11, 12,21,22</p>



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

10 October 2016

Date of mailing of the international search report

21/10/2016

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2016/067568

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>-& NOELLING J ET AL: "GENOME SEQUENCE AND COMPARATIVE ANALYSIS OF THE SOLVENT-PRODUCING BACTERIUM CLOSTRIDIUM ACETOBUTYLICUM", JOURNAL OF BACTERIOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 183, no. 16, 1 August 2001 (2001-08-01), pages 4823-4838, XP002950775, ISSN: 0021-9193, DOI: 10.1128/JB.183.16.4823-4838.2001</p> <p>-----</p> <p>ERIN GARZA ET AL: "Engineering a homobutanol fermentation pathway inEG03", JOURNAL OF INDUSTRIAL MICROBIOLOGY & BIOTECHNOLOGY ; OFFICIAL JOURNAL OF THE SOCIETY FOR INDUSTRIAL MICROBIOLOGY, SPRINGER, BERLIN, DE, vol. 39, no. 8, 10 July 2012 (2012-07-10), pages 1101-1107, XP035091406, ISSN: 1476-5535, DOI: 10.1007/S10295-012-1151-8 cited in the application the whole document</p>	1-22
A	<p>-----</p> <p>F. LI ET AL: "Coupled Ferredoxin and Crotonyl Coenzyme A (CoA) Reduction with NADH Catalyzed by the Butyryl-CoA Dehydrogenase/Etf Complex from Clostridium kluyveri", JOURNAL OF BACTERIOLOGY, vol. 190, no. 3, 9 November 2007 (2007-11-09), pages 843-850, XP055090927, ISSN: 0021-9193, DOI: 10.1128/JB.01417-07 cited in the application the whole document</p>	1-22
A	<p>-----</p> <p>G. HERRMANN ET AL: "Energy Conservation via Electron-Transferring Flavoprotein in Anaerobic Bacteria", JOURNAL OF BACTERIOLOGY, vol. 190, no. 3, 1 February 2008 (2008-02-01), pages 784-791, XP055241094, US ISSN: 0021-9193, DOI: 10.1128/JB.01422-07 the whole document</p> <p>-----</p> <p style="text-align: center;">-/--</p>	1-22

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/067568

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>BROOKS B BOND-WATTS ET AL: "Enzyme mechanism as a kinetic control element for designing synthetic biofuel pathways", NATURE CHEMICAL BIOLOGY, vol. 7, no. 4, 27 February 2011 (2011-02-27), pages 222-227, XP055031763, ISSN: 1552-4450, DOI: 10.1038/nchembio.537 the whole document</p> <p>-----</p>	1-22
A	<p>SIMON DUSSÉAUX ET AL: "Metabolic engineering of Clostridium acetobutylicum ATCC 824 for the high-yield production of a biofuel composed of an isopropanol/butanol/ethanol mixture", METABOLIC ENGINEERING, vol. 18, 1 July 2013 (2013-07-01), pages 1-8, XP055241002, US ISSN: 1096-7176, DOI: 10.1016/j.ymben.2013.03.003 the whole document</p> <p>-----</p>	1-22