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(54) Title: BACTERIAL CELLS WITH IMPROVED TOLERANCE TO POLYOLS

(57) Abstract: The present invention relates to bacterial cells genetically modified to improve their tolerance to certain commodity chemicals, such as diols and other polyols, and to methods of preparing and using such bacterial cells for production of polyols and other compounds.

BACTERIAL CELLS WITH IMPROVED TOLERANCE TO POLYOLS

FIELD OF THE INVENTION

The present invention relates to bacterial cells genetically modified to improve their tolerance to certain commodity chemicals, such as diols and other polyols, and to methods of preparing and using such bacterial cells for production of polyols and other compounds.

BACKGROUND OF THE INVENTION

Polyols such as diols are versatile water-miscible compounds used in diverse applications including use as polyester and polyurethane resin precursors, antifreezes, synthetic lubricants, plasticizers and polymer additives, intermediates in the production of pharmaceuticals and fragrances, and as food, cosmetic, and pharmaceutical ingredients (Werle *et al.*, 2012; Köpnick *et al.*, 2012). The predominant industrial use of diols is in the production of polyesters, as nearly all commercially produced polyesters are the product of esterification of dicarboxylic acids with diols (Werle *et al.*, 2012). For example, the dominant use of 1,2-propanediol (45%) is in unsaturated polyester resins (Sullivan *et al.*, 2012). Because of its safety to humans, it is also used in numerous food and cosmetic products, and as a lubricant, antifreeze, and aircraft deicer (Sullivan *et al.*, 2012). Another diol, 2,3-butanediol, can be reduced to butadiene, a component of synthetic rubber, and the largest current usage of 2,3-butanediol is as a cross-linking agent for hard rubbers, as a precursor for insecticides, and as pharmaceutical intermediates (Gräfje *et al.*, 2012).

In the past, butadiene was primarily synthesized from butene obtained from cracked naptha. The recent increase in natural gas production via fracking, coupled with previously high oil prices, however, resulted in an increased price for C_4 and higher hydrocarbons which in turn resulted in a renewed interest in biological production of C_4 compounds such as 1,4-butanediol and 2,3-butanediol. Further, diols that contain stereocenters exist in different stereoisomers, and the use of stereoisomers can impart different physical properties to polymers. Utilization of a specific stereoisomer can also be useful for the purpose of introducing stereocenters into more complex compounds when they are used as intermediates. Biological production of diols can be particularly advantageous when compared to chemical synthesis, in that it can readily allow the production of pure stereoisomers or racemic mixtures of stereoisomers, depending on the enzymes employed. The production of diols in metabolically engineered microbial cells have been reviewed and described in several publications such as, *e.g.*, Sabra *et al.* (2016), Clomburg *et al.* (2011), Jain *et al.* (2015), Li *et al.* (2015) and Xu *et al.* (2014).

For production of bulk chemicals from renewable plant-based carbon feedstocks, high product titers are essential in order to minimize capital equipment and downstream separations costs for product purification. At the high titers required for economical fermentation processes, however, most chemicals exhibit significant toxicity that reduce yields and productivities by negatively affecting microbial growth (Van Dien, 2013; Zingaro *et al.*, 2013).

Escherichia coli being a suitable host for industrial applications, there has been some interest in developing *E. coli* strains with improved tolerance to chemicals of interest for production, such as, *e.g.*, n-butanol, ethanol and isobutanol, or to stress conditions present during fermentation (see, *e.g.*, Haft et al, 2014; Sandberg *et al.*, 2014; Lennen and Herrgård, 2014; Tenaillon *et al.*, 2012; Minty *et al.*, 2011; Dragosits *et al.*, 2013; Winkler *et al.*, 2014; Wu *et al.*, 2014; LaCroix *et al.*, 2015; Jensen *et al.*, 2015 and 2016 ; Doukyu *et al.*, 2012; Shenhar *et al.*, 2012; and Rath and Jawali, 2006).

Despite these and other advances in the art, there is still a need for bacterial cells with improved tolerance to chemicals of interest for bio-based production, such as diols and other polyols. It is an object of the invention to provide such bacterial cells.

SUMMARY OF THE INVENTION

It has been found by the present inventors that certain genetic modifications unexpectedly improve the tolerance of bacterial cells, such as those of, *e.g.*, the *Escherichia* genera, to certain chemical compounds, particularly aliphatic diols and other aliphatic polyols.

Accordingly, the invention provides bacterial cells with improved tolerance to at least one aliphatic polyol, as well as bacterial cells which are capable of producing an aliphatic polyol and have improved tolerance to the aliphatic polyol. Particularly contemplated are aliphatic diols, such as *e.g.*, 2,3-butanediol; 1,2-propanediol; 1,4-butanediol; 1,3-propanediol; 1,2-butanediol; 1,5 pentanediol and/or 1,2-pentanediol.

The invention also relates to compositions comprising such bacterial cells and one or more aliphatic polyols, methods of preparing or screening for such bacterial cells, and methods of producing aliphatic polyols using such bacterial cells.

The invention also relates to methods of producing a diol or other polyol using bacterial cells, comprising supplementing the medium with methionine, wherein the concentration of methionine is from about 0.004 to about 0.2 g L^{-1} gDCW^{-1} (gDCW = grams dry cell weight).

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These and other aspects and embodiments are described further below.

DETAILED DISCLOSURE OF THE INVENTION

In this work, 2,3-butanediol and 1,2-propanediol were selected for performing adaptive laboratory evolutions. Based on the findings reported herein, various aspects of the invention

5   provide for genetically modified bacterial host cells with a higher tolerance to one or more diols or other polyols. When transformed with a recombinant biosynthetic pathway for producing the polyol from a carbon source, the genetically modified bacterial host cells of the invention result in improved production of the polyol from carbon feedstock, since they maintain robust metabolic activity in the presence of higher concentrations of the polyol than

10   the unmodified parent cells.

So, in one aspect, the bacterial cell comprises a biosynthetic, optionally recombinant, pathway for producing an aliphatic polyol and at least one genetic modification which reduces expression of an endogenous gene selected from the group consisting of *metJ, iscR, yhjA, gtrS, ycdU, rzpD, sspA* and *rph*, or a combination of any thereof, optionally wherein the cell

15   further comprises a genetic modification which increases the expression of PyrE and/or a mutation in one or more of NanK, RpsA, RpoB, RpoC, SpoT, NusG, Flu, Lon, and YgaH.

In one aspect, the bacterial cell comprises a biosynthetic, optionally recombinant, pathway for producing an aliphatic polyol and at least one genetic modification which increases one or more of (a) the biosynthesis of methionine in the bacterial cell; (b) the growth of the

20   bacterial cell during polyol-induced methionine starvation; (c) intracellular iron levels during polyol-induced growth inhibition; (d) biosynthesis of iron siderophores during polyol-induced growth inhibition; and (e) the biosynthesis of iron-sulfur clusters during polyol-induced growth inhibition.

In one embodiment of any aspect, the bacterial cell comprises at least one genetic

25   modification which reduces expression of *metJ* and/or *iscR*. The bacterial cell may further comprise genetic modifications which reduce expression of *relA* and *purT*; or genetic modifications which reduce the expression of *acrB, acrA*, or both, optionally in combination with a genetic modification which increases the expression of PyrE, or a mutation in one or more of NanK, RpsA, RpoB, RpoC, SpoT, NusG, Flu, Lon and YgaH.

30   In one aspect, the bacterial cell comprises genetic modifications which reduce expression of *metJ, relA* and *purT*; *metJ* and *acrB* and/or *acrA*; *iscR* and *relA*; or *fabR* and *ygfF*, optionally in combination with a genetic modification which increases the expression of PyrE, and/or a

mutation in one or more of NanK, RpsA, RpoB, RpoC, SpoT, NusG, Flu, Lon, and YgaH. Preferred examples of mutations are disclosed herein.

In one embodiment of any aspect, the genetic modification comprises a knock-down or knock-out of the endogenous gene. In a particular embodiment, the genetic modification is a knock-out.

Preferred, non-limiting polyols include diols, the genetic modification providing for an increased growth rate, a reduced lag time, or both, of the cell in at least one of, *e.g.*, 2,3-butanediol and 1,2-propanediol, as compared to a control. The control may be, for example, the parent bacterial cell.

In one embodiment, the pathway is a recombinant pathway. For example, the bacterial cell may comprise a recombinant biosynthetic pathway for producing at least one of a propanediol, butanediol, pentanediol and a hexanediol.

The bacterial cell may be of any suitable genus or origin. Preferred, non-limiting genera include *Escherichia, Enterobacter, Klebsiella, Lactobacillus, Lactococcus, Bacillus, Pseudomonas, Corynebacterium, Ralstonia, Paenibacillus, Clostridia* and *Citrobacter sp*. *Escherichia coli* is particularly preferred.

In one aspect, there is provided a process for preparing a recombinant *E. coli* cell for producing an aliphatic polyol, comprising genetically modifying an *E. coli* cell to

> (a) introduce a recombinant biosynthetic pathway for producing an aliphatic polyol; and knock-down or knock-out at least one endogenous gene selected from the group consisting of *metJ, rzpD, yhjA, gtrS, ycdU, iscR, sspA* and *rph;* or

> (b) knock-down or knock-out a combination of endogenous genes selected from *metJ, relA* and *purT; metJ* and *acrB* and/or *acrA; iscR* and *relA;* and *fabR* and *ygfF.*

In one aspect, there is provided a process for improving the tolerance of a bacterial cell to an aliphatic diol, comprising genetically modifying the bacterial cell to knock-down or knock-out

> (a) at least one endogenous gene selected from the group consisting of *metJ, rzpD, yhjA, gtrS, ycdU, iscR, sspA* and *rph;* or

(b) a combination of endogenous genes selected from *metJ, relA* and *purT; metJ* and *acrB* and/or *acrA; iscR* and *relA*; and *fabR* and *ygfF*,

optionally also introducing a genetic modification which increases the expression of PyrE, or a mutation in one or more of NanK, RpsA, RpoB, RpoC, SpoT, NusG, Flu, Lon, and YgaH.

In one aspect, there is provided a process for preparing a recombinant *E. coli* cell for producing an aliphatic polyol, comprising genetically modifying an *E. coli* cell to introduce a recombinant biosynthetic pathway for producing an aliphatic polyol, and

(a) knock-down or knock-out at least one endogenous gene selected from the group consisting of *metJ, iscR, yhjA, gtrS, ycdU, rzpD, sspA* and *rph*; or a combination of endogenous genes selected from *metJ, relA* and *purT; metJ* and *acrB, acrA* or both; *iscR* and *relA*; and *fabR* and *ygfF*; and

(b) optionally, upregulating and/or introducing one or more mutations in at least one protein selected from NanK (SEQ ID NO:19), RpsA (SEQ ID NO:37), RpoA (SEQ ID NO:21); RpoB (SEQ ID NO:23), RpoC (SEQ ID NO:25), SpoT (SEQ ID NO:27), NusG (SEQ ID NO:29, Flu (SEQ ID NO:31), Lon (SEQ ID NO:33), and YgaH (SEQ ID NO:35), optionally wherein the one or more mutations are selected from RpoC-L268K, RpoC-L268N, RpoC-L268Q, RpoC-L268R, RpoC-N309F, RpoC-N309S, RpoC-N309T, RpoC-N309W, RpoC-N309Y, RpoC-Y75A, RpoC-Y75C, RpoC-Y75S, RpoC-ΔTPVIE(822-827), RpoB-D549A, RpoB-D549G, RpoB-H447F, RpoB-H447S, RpoB-H447T, RpoB-H447W, RpoB-H447Y, RpoB-I1112S, RpoB-I1112T, RpoB-V931A, RpoB-V931I, RpoB-V931L, NanK-T128S,Flu-L642E, Flu-L642N, Flu-L642Q, Lon-I716S, Lon-I716T, YgaH-V39A, YgaH-V39I,YgaH-V39L,NusG-F144A, NusG-F144I, NusG-F144L, NusG-F144M, NusG-F144V, RpoA-D305A, RpoA-D305G, RpoA-G279A, RpoA-G279F, RpoA-G279I, RpoA-G279L, RpoA-G279M, RpoA-G279V, RpsA-D310A, RpsA-D310F, RpsA-D310I, RpsA-D310L, RpsA-D310M, RpsA-D310V, RpsA-G21A, RpsA-G21F, RpsA-G21I, RpsA-G21L, RpsA-G21M, RpsA-G21V, SpoT-I213A, SpoT-I213F, SpoT-I213L, SpoT-I213M, and SpoT-I213V.

In one aspect, there is provided a process for improving the tolerance of a bacterial cell to an aliphatic polyol, comprising genetically modifying the bacterial cell to

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(a) knock-down or knock-out at least one endogenous gene selected from the group consisting of *metJ, iscR, yhjA, gtrS, ycdU, rzpD, sspA* and *rph*; or a combination of endogenous genes selected from *metJ, relA* and *purT; metJ* and *acrB, acrA* or both; *iscR* and *relA*; and *fabR* and *ygfF*;

(b) optionally introducing one or more mutations in one or more endogenous genes selected from NanK (SEQ ID NO:19), RpsA (SEQ ID NO:37), RpoA (SEQ ID NO:21); RpoB (SEQ ID NO:23), RpoC (SEQ ID NO:25), SpoT (SEQ ID NO:27), NusG (SEQ ID NO:29, Flu (SEQ ID NO:31), Lon (SEQ ID NO:33), and YgaH (SEQ ID NO:35) or the *pyrE/rph* intergenic region;

(c) preparing a population of the genetically modified bacterial cell,; and

(d) selecting from the population in (c) any bacterial cell which has an improved tolerance to the aliphatic polyol.

In one aspect, there is provided a method for producing an aliphatic polyol, comprising culturing the bacterial cell of any aspect or embodiment herein in the presence of a carbon source, and, optionally, isolating the aliphatic polyol.

In one aspect, there is provided a composition comprising a propanediol or a butanediol at a concentration of at least 6% and a plurality of bacterial cells according to any aspect or embodiment herein. The bacterial cells may be, *e.g.*, of the *Escherichia* genus, genetically modified to knock-down or knock-out at least one endogenous gene selected from the group consisting of *metJ, rzpD, yhjA, gtrS, ycdU, iscR, sspA* and *rph*; or a combination of endogenous genes selected from *metJ, relA* and *purT; metJ* and *acrB* and/or *acrA; iscR* and *relA*; and *fabR* and *ygfF.*

In one aspect, there is provided a method for producing an aliphatic diol, comprising (a) culturing a plurality of bacterial cells capable of producing the aliphatic diol in a medium comprising a carbon source, and (b) adding methionine to the medium, wherein the concentration of the added methionine is from about 0.004 g L^{-1} gDCW^{-1} to about 0.2 g L^{-1} gDCW^{-1}, optionally wherein the bacterial cell is the bacterial cell of any preceding aspect or embodiment.

Definitions

Unless otherwise indicated or contradicted by context, a "diol" as used herein is an aliphatic diol, and a "polyol" is an aliphatic polyol. An "aliphatic polyol" herein refers to an organic compound comprising an aliphatic carbon chain to which two or more hydroxyl (-OH) groups are attached, and includes linear aliphatic diols and other linear aliphatic polyols, as well as derivatives thereof. Aliphatic polyols suitable for production in bacteria typically comprise from 3 to 12 carbon atoms, preferably 3 to 10 carbon atoms, more preferably 3 to 8 carbon atoms, and, most preferably, 3 to 6 carbon atoms, and, optionally comprises one or more heteroatoms. Linear aliphatic polyols comprising 2, 3 or 4 hydroxyl groups are preferred and include, but are not limited to, 2,3-butanediol; 1,2-propanediol; 1,5 pentanediol; 1,2-pentanediol; 1,4-butanediol; 1,3-propanediol; 1,2-butanediol; 1,6-hexanediol; 1,8-octanediol; 1,10-decanediol and 1,12-dodecanediol. Particularly contemplated are propanediols, butanediols, pentanediols and hexanediols. Linear aliphatic diols such as, *e.g.*, 2,3-butanediol; 1,2-propanediol; 1,5-pentanediol;1,2-pentanediol; 1,6-hexanediol; 1,4-butanediol and 1,3-propanediol are most preferred.

As used herein, a "recombinant biosynthetic pathway" for a compound of interest refers to an enzymatic pathway resulting in the production of a compound of interest in a host cell, wherein at least one of the enzymes is expressed from a transgene, *i.e.*, a gene added to the host cell genome by transformation. In some cases, the recombinant biosynthetic pathway also comprises a deletion of one or more native genes in the host cell. The compound of interest is typically a diol or other polyol, and may be the actual end product or a precursor or intermediate in the production of another end product.

The terms "tolerant" or "improved tolerance", when used to describe a genetically modified bacterial cell of the invention or a strain derived therefrom, refers to a genetically modified bacterial cell or strain that shows a reduced lag time, an improved growth rate, or both, in the presence of a diol or other polyol than the parent bacterial cell or strain from which it is derived, typically at concentrations of at least 1% v/v, such as at least 1.5% v/v, such as at least 3% v/v, such as at least 5% v/v, such as at least 6% v/v, such as at least 7% v/v, such as at least 7.5% v/v, such as at least 8% v/v, such as at least 10% v/v. An improved growth rate is at least 5%, such as at least 10%, such as at least 20%, such as at least 50%, such as at least 75% higher than that of a control, typically the parent cell or strain. A reduced lag time is at least 10%, such as at least 20%, such as at least 50%, such as at least 75%, such as at least 90% shorter than that of a control, typically the parent cell or strain.

The term "gene" refers to a nucleic acid sequence that encodes a cellular function, such as a protein, optionally including regulatory sequences preceding (5' non-coding sequences) and

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following (3' non-coding sequences) the coding sequence. An "endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "transgene" is a gene, native or heterologous, that has been introduced into the genome by a transformation procedure. Genes names are herein set forth in italicised text with a lower-case first letter (*e.g.*, *metJ*) whereas protein names are set forth in normal text with a capital first letter (*e.g.*, MetJ).

As used herein the term "coding sequence" refers to a DNA sequence that encodes a specific amino acid sequence.

The term "native", when used to characterize a gene or a protein herein with respect to a host cell, refers to a gene or protein having the nucleic acid or amino acid sequence as found in the host cell.

The term "heterologous", when used to characterize a gene or protein with respect to a host cell, refers to a gene or protein which has a nucleic acid or amino acid sequence not normally found in the host cell.

As used herein the term "transformation" refers to the transfer of a nucleic acid fragment, such as a gene, into a host cell. Host cells containing a gene introduced by transformation or a "transgene" are referred to as "transgenic" or "recombinant" or "transformed" cells.

As used herein, a "genetic modification" refers to the introduction a genetically inherited change in the host cell genome. Examples of changes include mutations in genes and regulatory sequences, coding and non-coding DNA sequences. "Mutations" include deletions, substitutions and insertions of one or more nucleotides or nucleic acid sequences in the genome. Other genetic modifications include the introduction of heterologous genes or coding DNA sequences by recombinant techniques.

The term "expression", as used herein, refers to the process in which a gene is transcribed into mRNA, and may optionally include the subsequent translation of the mRNA into an amino acid sequence, *i.e.*, a protein or polypeptide.

As used herein, "reduced expression" or "downregulation" of an endogenous gene in a host cell means that the levels of the mRNA, protein and/or protein activity encoded by the gene are significantly reduced in the host cell, typically by at least 25%, such as at least 50%, such as at least 75%, such as at least 90%, such as at least 95%, as compared to a control. Typically, when the reduced expression is obtained by a genetic modification in the host cell, the control is the unmodified host cell. Sometimes, *e.g.*, in the case of gene knock-out, the

reduction of native mRNA and functional protein encoded by the gene is higher, such as 99% or greater.

"Increased expression", "upregulation", "overexpressing" or the like, when used in the context of a protein or activity described herein, means increasing the protein level or activity within a bacterial cell. An up-regulation of an activity can occur through, *e.g.*, increased activity of a protein, increased potency of a protein or increased expression of a protein. The protein with increased activity, potency or expression can be encoded by genes disclosed herein.

Genetic modifications resulting in a reduced expression of a target gene/protein can include, *e.g.*, knock-down of the gene (*e.g.*, a mutation in a promoter or other expression control sequence that results in decreased gene expression), a knock-out or disruption of the gene (*e.g.*, a mutation or deletion of the gene that results in 99 percent or greater decrease in gene expression), a mutation or deletion in the coding sequence which results in the expression of non-functional protein, and/or the introduction of a nucleic acid sequence that reduces the expression of the target gene, *e.g.* a repressor that inhibits expression of the target or inhibitory nucleic acids (*e.g.* CRISPR etc.) that reduces the expression of the target gene.

Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T. Molecular Cloning: A Laboratory Manual, 4th ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, New York, 2012; and by Silhavy, T. J., Bennan, M. L. and Enquist, L. W. Experiments with Gene Fusions; Cold Spring Harbor Laboratory: Cold Spring Harbor, New York, 1984; and by Ausubel, F. M. *et al.*, In Current Protocols in Molecular Biology, published by John Wiley & Sons (1995); and by Datsenko and Wanner, 2000; and by Baba *et al.*, 2006; and by Thomason *et al.*, 2007.

A "conservative" amino acid substitution in a protein is one that does not negatively influence protein activity. Typically, a conservative substitution can be made within groups of amino acids sharing physicochemical properties, such as, *e.g.*, basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagines), hydrophobic amino acids (leucine, isoleucine, valine and methionine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, and threonine). Most commonly, substitutions can be made between Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly. Other preferred substitutions are set out in Table 1 below.

Table 1 – Examples of amino acid substitutions

Original amino acid	Examples of substitutions	Preferred substitution
Ala (A)	val; leu; ile	Val
Arg (R)	lys; gln; asn	Lys
Asn (N)	gln; his; asp, lys; arg	Gln
Asp (D)	glu; asn	Glu
Cys (C)	ser; ala	Ser
Gln (Q)	asn; glu	Asn
Glu (E)	asp; gln	Asp
Gly (G)	Ala	Ala
His (H)	asn; gln; lys; arg	Arg
Ile (I)	leu; val; met; ala; phe; norleucine	Leu
Leu (L)	norleucine; ile ; val; met; ala; phe	Ile
Lys (K)	arg; gln; asn	Arg
Met (M)	leu; phe; ile	Leu
Phe (F)	leu; val; ile; ala; tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	thr	Thr
Thr (T)	Ser	Ser
Trp (W)	tyr; phe	Tyr
Tyr (Y)	trp; phe ; thr; ser	Phe
Val (V)	ile; leu; met; phe; ala; norleucine	Leu

Specific embodiments of the invention

As described in the Examples, the growth rate of native K-12 MG1655 cells steadily
decreased as a function of diol concentration, starting already at 0.5 % or 1 % v/v, with

toxicity apparently depending on carbon chain length. Maximum concentrations for robust growth in 2,3-butanediol and 1,2-propanediol were 5% and 7.5%, respectively.

So, the invention provides bacterial cells with improved tolerance to diols and other polyols, as well as related processes and materials for producing and using such bacterial cells.

5   1)   Genetic modifications

The genetic modifications according to the invention include those resulting in reduced expression of genes, *e.g.*, by gene knock-down or knock-out, herein referred to as "Group 1 modifications"; as well as silent mutations in coding or non-coding regions and non-silent (*i.e.*, coding) mutations in coding regions, herein referred to as "Group 2 modifications"; and
10 combinations thereof.

In a preferred embodiment, the one or more genetic modifications provide for an increased growth rate, a reduced lag time, or both, of the bacterial cell in at least one of 2,3-butanediol and 1,2-propanediol, *e.g.*, at a concentration of at least 6% or at least 7% as compared to the wild-type bacterial cell.

15   a)   Group 1 modifications

In one aspect, the bacterial cell has a genetic modification which reduces expression of one or more endogenous genes selected from the group consisting of *metJ, rzpD, yhjA, gtrS, ycdU, iscR, sspA* and *rph*. For example, in one particular embodiment, the endogenous gene is *metJ*.

20 In another aspect, there is provided a bacterial cell which comprises genetic modifications reducing the expression of at least two endogenous genes.

In one embodiment, the genetic modifications reduce the expression of *metJ* and one or more other endogenous genes. In one particular embodiment, the other endogenous genes are *relA* and *purT*. In another particular embodiment, the other endogenous gene or genes is
25 *acrB, acrA* or both.

In another embodiment, the bacterial cell comprises genetic modifications which reduce expression of *iscR* and *relA*.

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In another embodiment, the bacterial cell comprises genetic modifications which reduce expression of *fabR* and *ygfF.*

In another embodiment, the at least two endogenous genes bacterial cell comprises genetic modifications which reduce the expression of two or more of *metJ, rzpD, yhjA, gtrS, ycdU,*
iscR, sspA and *rph.* In separate and specific embodiments, the bacterial cell comprises:

- a first genetic modification which reduces the expression of *metJ,* and a second genetic modification which reduces the expression of a gene selected from *rzpD, yhjA, gtrS, ycdU, iscR, sspA* and *rph*;

- a first genetic modification which reduces the expression of *rzpD* and a second genetic modification which reduces the expression of a gene selected from of *metJ, yhjA, gtrS, ycdU, iscR, sspA* and *rph*;

- a first genetic modification which reduces the expression of *yjhA* and a second genetic modification which reduces the expression of a gene selected from of *metJ, rzpD, gtrS, ycdU, iscR, sspA* and *rph*;

- a first genetic modification which reduces the expression of *gtrS* and a second genetic modification which reduces the expression of a gene selected from *metJ, rzpD, yhjA, ycdU, iscR, sspA* and *rph*;

- a first genetic modification which reduces the expression of *ycdU* and a second genetic modification which reduces the expression of a gene selected from *metJ, rzpD, yhjA, gtrS, iscR, sspA* and *rph*;

- a first genetic modification which reduces the expression of *iscR* and a second genetic modification which reduces the expression of a gene selected from *metJ, rzpD, yhjA, gtrS, ycdU, sspA* and *rph,* and, optionally, a third genetic modification which reduces the expression of relA *;*

- a first genetic modification which reduces the expression of *sspA* and a second genetic modification which reduces the expression of a gene selected from *metJ, rzpD, yhjA, gtrS, ycdU, iscR* and *rph*; and

- a first genetic modification which reduces the expression of *rph* and a second genetic modification which reduces the expression of a gene selected from *metJ, rzpD, yhjA, gtrS, ycdU, iscR* and *sspA.*

In one specific embodiment, either one or both of the first and second genetic modifications is a knock-out of the gene, optionally a deletion. In an alternative embodiment at least one of the first and second genetic modifications is a knock-down of the gene.

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In one aspect, there is provided a bacterial cell according to any one of the preceding aspects and embodiments, wherein the genetic modification is a knock-down of the one or more endogenous genes, resulting in at least 25%, such as at least 50%, such as at least 75%, such as at least 90%, such as at least 95%, reduction in the level of mRNA encoded by the gene.

In one aspect, there is provided a bacterial cell according to any one of the preceding aspects and embodiments, wherein the genetic modification is a knock-down of the one or more endogenous genes, resulting in at least 25%, such as at least 50%, such as at least 75%, such as at least 90%, such as at least 95%, reduction in the level of protein encoded by the gene.

In one aspect, there is provided a bacterial cell according to any one of the preceding aspects and embodiments, wherein the genetic modification is a knock-out of the one or more endogenous genes.

Knock-down or knock-out of a gene can be accomplished by any method known in the art for bacterial cells, and include, *e.g.*, lambda Red mediated recombination, P1 phage transduction, and single-stranded oligonucleotide recombineering/MAGE technologies (see, *e.g.*, Datsenko and Wanner, 2000; Thomason *et al.*, 2007; Wang *et al.*, 2009). Typically, a knock-down of a gene can be accomplished by, for example, a mutation in the promoter region resulting in decreased transcription, a deletion or mutation in the coding region of the gene resulting in a reduced or fully or substantially eliminated activity of the protein, or by the presence of antisense sequences that interfere with transcription or translation of the gene, resulting in reduced expression of the protein. Preferably, the knocking-down of a gene results in at least 20% reduction in the expression level of the gene product in the bacterial cell, such as at least 30%, such as at least 40%, such as at least 50%, such as at least 60%, such as at least 70%, such as at least 80%, such as at least 90%, such as at least 95% or higher.

A knock-out of a gene includes elimination of a gene's expression, such as by introducing a mutation in the coding sequence and/or promoter so that at least a portion (up to and including all) of the coding sequence and/or promoter is disrupted, shifted or deleted, resulting in loss of expression of the protein, or expression only of a non-functional mutant or non-functional fragment of the endogenous protein. As used herein, the symbol "DELTA" denotes a deletion of an endogenous gene. Preferably, a knock-out of a gene results in 1% or less of the native gene product being detectable, such as no detectable gene product.

b) Group 2 modifications

In certain embodiments, a mutant protein is expressed in the bacterial cell, *e.g.*, from a mutated version of an endogenous gene, or from a transgene encoding the mutant protein. For example, the bacterial cell may comprise one or more mutations in at least one protein selected from NanK (SEQ ID NO:19), RpsA (SEQ ID NO:37), RpoA (SEQ ID NO:21); RpoB (SEQ ID NO:23), RpoC (SEQ ID NO:25), SpoT (SEQ ID NO:27), NusG (SEQ ID NO:29, Flu (SEQ ID NO:31), Lon (SEQ ID NO:33), and YgaH (SEQ ID NO:35), *e.g.*, wherein the one or more mutations are selected from RpoC-L268K, RpoC-L268N, RpoC-L268Q, RpoC-L268R, RpoC-N309F, RpoC-N309S, RpoC-N309T, RpoC-N309W, RpoC-N309Y, RpoC-Y75A, RpoC-Y75C, RpoC-Y75S, RpoC-ΔTPVIE(822-827), RpoB-D549A, RpoB-D549G, RpoB-H447F, RpoB-H447S, RpoB-H447T, RpoB-H447W, RpoB-H447Y, RpoB-I1112S, RpoB-I1112T, RpoB-V931A, RpoB-V931I, RpoB-V931L, NanK-T128S,Flu-L642E, Flu-L642N, Flu-L642Q, Lon-I716S, Lon-I716T, YgaH-V39A, YgaH-V39I,YgaH-V39L,NusG-F144A, NusG-F144I, NusG-F144L, NusG-F144M, NusG-F144V, RpoA-D305A, RpoA-D305G, RpoA-G279A, RpoA-G279F, RpoA-G279I, RpoA-G279L, RpoA-G279M, RpoA-G279V, RpsA-D310A, RpsA-D310F, RpsA-D310I, RpsA-D310L, RpsA-D310M, RpsA-D310V, RpsA-G21A, RpsA-G21F, RpsA-G21I, RpsA-G21L, RpsA-G21M, RpsA-G21V, SpoT-I213A, SpoT-I213F, SpoT-I213L, SpoT-I213M, and SpoT-I213V. The bacterial cell may further comprise a Group 1 modification as set out herein.

In one embodiment, the bacterial cell comprises a Group 1 modification according to any aspect or embodiment herein as well as a mutation in one or more of NanK (*e.g.*, NanK-T128S), RpsA (*e.g.*, RpsA-G21V, RpsA-G21I, RpsA-G21L, RpsA-G21M, RpsA-G21F, RpsA-G21A), RpoB (*e.g.*, RpoB-H447Y, RpoB-H447F, RpoB-H447W, RpoB-H447T, RpoB-H447S, RpoB-D549G, RpoB-D549A, RpoB-V931A, RpoB-V931L, RpoB-V931I, RpoB-I1112S, and/or RpoB-I1112T), RpoC (*e.g.*, RpoC-L268R, RpoC-L268K, RpoC-L268Q, RpoC-L268N, RpoC-ΔTPVIE(822-827), RpoC-N309Y, RpoC-N309F, RpoC-N309W, RpoC-N309T, RpoC-N309S, RpoC-Y75C, RpoC-Y75S, and/or RpoC-Y75A), SpoT (*e.g.*, SpoT-I213L, SpoT-I213V, SpoT-I213M, SpoT-I213A, or SpoT-I213F), NusG (*e.g.*, NusG-F144V, NusG-F144I, NusG-F144L, NusG-F144M, or NusG-F144A), Flu (*e.g.*, Flu-L642Q, Flu-L642N, or Flu-L642E), Lon (*e.g.*, Lon-I716S or Lon-I716T), and YgaH (*e.g.*, YgaH-V39A, YgaH-V39L, or YgaH-V39I) and/or a mutation in *rph* or the *pyrE/rph* intergenic region which increases the expression of PyrE, wherein the one or more mutations improve tolerance to an aliphatic polyol such as, *e.g.* 2,3-butanediol.

In one embodiment, the bacterial cell comprises a Group 1 modification according to any aspect or embodiment herein as well as a mutation in one or more of RpoA (*e.g.*, RpoA-D305G, RpoA-D305A, RpoA-G279V, RpoA-G279I, RpoA-G279L, RpoA-G279M, RpoA-G279F, and/or RpoA-G279A) and RpsA (*e.g.*, RpsA-D310V, RpsA-D310I, RpsA-D310L, RpsA-D310M,

RpsA-D310F, or RpsA-D310A), and/or a mutation in *rph* or the *pyrE/rph* intergenic region which increases the expression of PyrE, wherein the one or more mutations improve tolerance to an aliphatic polyol such as, *e.g.*, 1,2-propanediol.

5   In an alternative embodiment, the bacterial cell comprises a Group 1 modification according to any preceding aspect or embodiment as well as an upregulation of at least one of the endogenous genes NanK, RpsA, SpoT, NusG, PyrE, Flu, Lon, and YgaH, *e.g.*, by transforming the bacterial cell with a transgene expressing the endogenous protein. To cause an up-regulation through increased expression of a protein, the copy number of a gene or genes encoding the protein may be increased. Alternatively, a strong and/or inducible promoter can

10   be used to direct the expression of the gene, the gene being expressed either as a transient expression vehicle or homologously or heterologously incorporated into the bacterial genome. In another embodiment, the promoter, regulatory region and/or the ribosome binding site upstream of the gene can be altered to achieve the over-expression. The expression can also be enhanced by increasing the relative half-life of the messenger or other forms of RNA. Any

15   one or a combination of these approaches can be used to effect upregulation of a desired target protein as needed.

In one embodiment, the bacterial cell comprises one or more mutations which increase(s) the expression level or activity of PyrE, optionally in combination with a Group 1 modification. *E. coli* K-12 MG1655 and W3110, plus their common ancestor strain W1485, are known to

20   exhibit pyrimidine starvation in minimal media due to the presence a frameshift mutation occurring in *rph* relative to other E. coli strains (Jensen *et al.*, 1993). This mutation disrupts the transcriptional/translational coupling required for efficient translation of *pyrE*, encoding orotate phosphoribosyltransferase in the pyrimidine biosynthesis pathway. Compensatory mutations that correct this deficiency are well-known in the art. One of these mutations is an

25   82 bp deletion near the 3' terminus of *rph*, due to presence of two homologous GCAGAAGGC sequences flanking this 82 bp region (Conrad *et al.*, 2009). In addition to the 82 bp deletion, a 1 bp deletion at coordinate 3815809 in the pyrE/rph intergenic region has previously been encountered in strains evolved for growth on a minimal glucose medium (LaCroix *et al.*, 2015), and a wide array of other frameshift mutations, substitutions, and coding mutations

30   near the 3' terminus of *rph* were encountered in a short-term selection/evolution of combinatorial mutant libraries in minimal medium at an elevated temperature of 42°C (Sandberg *et al.*, 2014). Without being limited to theory, all of these mutations can serve the same function of increasing expression of PyrE, with the selective pressure for these mutations being even stronger in minimal media with particular imposed stresses (certain

35   chemicals or heat) than in minimal media alone. In one embodiment, the bacterial cell comprises mutations in *rph* or the *pyrE/rph* intergenic region, such as, *e.g.*, the 82 bp

deletion near the 3' terminus of *rph*, the 1 bp deletion in the intergenic region between *pyrE* and *rph*, or both.

In separate and specific embodiments, the bacterial cell comprises
-   a mutation which increases the expression of PyrE and a knock-out or knockdown of at least one of *metJ, rzpD, yhjA, gtrS, ycdU, iscR,* and *sspA,* such as *metJ;*
-   a mutation selected from NanK-T128S, RpsA-G21V, RpoB-H447Y, RpoC-L268R, RpoB-D549G, RpoB-V931A, RpoC-ΔTPVIE(822-827), RpoC-N309Y, SpoT-I213L, NusG-F144V, RpoC-Y75C, Flu-L642Q, RpoC-L268R, RpoB-I1112S, Lon-I716S, and YgaH-V39A, or a conservative substitution of any thereof, and a knock-out or knockdown of at least one of *metJ, rzpD, yhjA, gtrS, ycdU, iscR, sspA,* such as *metJ;*
-   a mutation selected from RpoA-D305G, RpsA-D310V, and RpoA-G279V, or a conservative substitution of any thereof, and a knock-out or knockdown of at least one of *metJ, rzpD, yhjA, gtrS, ycdU, iscR, sspA,* such as *metJ;*
-   a mutation which increases the expression of PyrE and a knock-out or knockdown of *metJ, relA,* and *purT* in combination;
-   a mutation selected from NanK-T128S, RpsA-G21V, RpoB-H447Y, RpoC-L268R, RpoB-D549G, RpoB-V931A, RpoC-ΔTPVIE(822-827), RpoC-N309Y, SpoT-I213L, NusG-F144V, RpoC-Y75C, Flu-L642Q, RpoC-L268R, RpoB-I1112S, Lon-I716S, and YgaH-V39A, or a conservative substitution of any thereof, and a knock-out or knockdown of *metJ, relA,* and *purT* in combination;
-   a mutation selected from RpoA-D305G, RpsA-D310V, and RpoA-G279V, or a conservative substitution of any thereof, and a knock-out or knockdown of *metJ, relA,* and *purT* in combination;
-   a mutation increasing the expression of PyrE and a knock-out or knockdown of a combination of *metJ* and *acrB* and/or *acrA;*
-   a mutation selected from NanK-T128S, RpsA-G21V, RpoB-H447Y, RpoC-L268R, RpoB-D549G, RpoB-V931A, RpoC-ΔTPVIE(822-827), RpoC-N309Y, SpoT-I213L, NusG-F144V, RpoC-Y75C, Flu-L642Q, RpoC-L268R, RpoB-I1112S, Lon-I716S, and YgaH-V39A, or a conservative substitution of any thereof, and a knock-out or knockdown of a combination of *metJ* and *acrB* and/or *acrA;*
-   a mutation selected from RpoA-D305G, RpsA-D310V, and RpoA-G279V, or a conservative substitution of any thereof, and a knock-out or knockdown of a combination of *metJ* and *acrB* and/or *acrA;*

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- a mutation increasing the expression of PyrE and a knock-out or knockdown of *iscR* and *relA* in combination;

- a mutation selected from NanK-T128S, RpsA-G21V, RpoB-H447Y, RpoC-L268R, RpoB-D549G, RpoB-V931A, RpoC-ΔTPVIE(822-827), RpoC-N309Y, SpoT-I213L, NusG-F144V, RpoC-Y75C, Flu-L642Q, RpoC-L268R, RpoB-I1112S, Lon-I716S, and YgaH-V39A, or a conservative substitution of any thereof, and a knock-out or knockdown of *iscR* and *relA* in combination; or

- a mutation selected from RpoA-D305G, RpsA-D310V, and RpoA-G279V, or a conservative substitution of any thereof, and a knock-out or knockdown of *iscR* and *relA* in combination.

In other separate and specific embodiments, the bacterial cell comprises

- a mutant RpoC comprising a RpoC-L268R, RpoC-L268K, RpoC-L268Q or RpoC-L268N mutation and at least one genetic modification which reduces the expression of *metJ, relA* and *purT*;

- a mutant RpoC comprising a RpoC-L268R, RpoC-L268K, RpoC-L268Q, or RpoC-L268N mutation and at least one genetic modification which reduces the expression of *metJ* and *acrB, acrA* or both;

- a mutant RpoC comprising a RpoC-L268R, RpoC-L268K, RpoC-L268Q or RpoC-L268N mutation and at least one genetic modification which reduces the expression of *metJ, relA, purT,* and *acrB, acrA* or both;

- a mutant RpoC comprising a RpoC-L268R, RpoC-L268K, RpoC-L268Q, or RpoC-L268N mutation and a mutant NanK comprising a NanK-T128S mutation, and at least one genetic modification which reduces the expression of *metJ, relA,* and *purT,* and *acrB, acrA* or both;

- a mutant RpoC comprising a RpoC-L268R, RpoC-L268K, RpoC-L268Q, or RpoC-L268N mutation and a mutant NanK comprising a NanK-T128S mutation, and at least one genetic modification which reduces the expression of *metJ* and *acrB, acrA* or both;

- a mutant RpoC comprising a RpoC-L268R, RpoC-L268K, RpoC-L268Q, or RpoC-L268N mutation and a mutant NanK comprising a NanK-T128S mutation, and at least one genetic modification which reduces the expression of *metJ, relA, purT,* and *acrB, acrA* or both;

- a mutant RpoC comprising a RpoC-L268R, RpoC-L268K, RpoC-L268Q or RpoC-L268N mutation, a mutant NanK comprising a NanK-T128S mutation, and a mutant Flu comprising a

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Flu-L642Q, Flu-L642N, or Flu-L642E mutation, and at least one genetic modification which reduces the expression of *metJ, relA, purT, elfD and acrB, acrA* or both;

- a mutant RpoB comprising a RpoB-I1112S or RpoB-I1112T mutation and at least one genetic modification which reduces the expression of *iscR, relA,* and *acrB, acrA* or both;

- a mutant RpoB comprising a RpoB-I1112S or RpoB-I1112T mutation and at least one genetic modification which reduces the expression of *iscR, relA,* and *acrB, acrA* or both;

- a mutant RpoB comprising a RpoB-I1112S or RpoB-I1112T mutation, and a mutant Lon comprising a Lon-I716S or Lon-I716T mutation, and at least one genetic modification which reduces the expression of *iscR, relA,* and *acrB, acrA* or both;

- a mutant RpoB comprising a RpoB-I1112S or RpoB-I1112T mutation, a mutant Lon comprising a Lon-I716S or Lon-I716T mutation, and a mutant YgaH comprising a YgaH-V39A, YgaH-V39L, or YgaH-V39I mutation, and at least one genetic modification which reduces the expression of *iscR, relA,* and *acrB, acrA* or both; or

- a mutant RpoB comprising a RpoB-I1112S or RpoB-I1112T mutation, a mutant Lon comprising a Lon-I716S or Lon-I716T mutation, a mutant YgaH comprising a YgaH-V39A, YgaH-V39L, or YgaH-V39I mutation, a genetic modification that increases the expression of PyrE, and at least one genetic modification which reduces the expression of *iscR, relA,* and *acrB, acrA* or both.

AcrB is part of a protein complex which includes AcrA (AcrAB-TolC), with TolC also serving as the outer membrane component of a number of other protein complexes. Accordingly, a knock-down or knock-out AcrA can result in the same phenotype as a knockdown or knock-out of AcrB. So, in any aspect or embodiment herein relating to a knock-down or knock-out of *acrB*, a knock-down or knock-out of *acrA*, or of *acrA* and *acrB*, can be used as an alternative.

In a specific embodiment, the bacterial cell comprises a knockdown or knockout of *metJ, acrB, relA,* and *purT*; and one or more Group 2 modifications selected from the group consisting of: a mutation in NanK such as NanK-T128S or a conservative substitution thereof; a mutation in RpoC such as RpoC-L268R or a conservative substitution thereof; and/or a mutation in Flu such as Flu-L642Q or a conservative substitution thereof. Optionally, the bacterial cell also comprises a knockdown or knockout of *elfD*.

In another specific embodiment, the bacterial cell comprises a knockdown or knockout of *iscR* and *relA*; and one or more Group 2 modifications selected from the group consisting of: a mutation in RpoB such as RpoB-I1112S or a conservative substitution thereof; a mutation in

Lon such as I716S or a conservative substitution thereof; a mutation in YgaH such as YgaH-V39A or a conservative substitution thereof; and/or a mutation increasing the expression of PyrE.

2) Production pathways

5   Bacterial strains capable of producing diols and other polyols can be found, *e.g.*, in the genera *Enterobacter, Klebsiella, Serratia, Lactobacillus, Bacillus, Paenibacillus, Clostridia, Thermoanaerobacterium, Bacteroides, Pantoea,* and *Citrobacter* sp. (Sabra *et al.*, 2016; Jiang *et al.*, 2014). For example, production of up to 150 g/L and 87.7 g/L 2,3-butanediol and 1,3-propanediol from glucose or glycerol, respectively, have been reported in *Klebsiella*
10 *pneumoniae* and *Clostridium* IK124, respectively (Ma *et al.*, 2009; Hirschmann *et al.*, 2005).

In some aspects, however, the bacterial cell comprises a recombinant pathway for producing the diol or other polyol of interest. A recombinant pathway can, for example, be added to introduce the capability to produce the diol or other polyol in a bacterial cell which does not have a native pathway to do so, typically by transforming the cell with one or more
15 heterologous enzymes catalyzing the desired reaction(s). Alternatively, in cases where the bacterial cell has native pathway for production of the diol or other polyol of interest, a recombinant pathway can nonetheless be introduced in order to increase the production yield, *e.g.*, by overexpressing one or more native enzymes or transforming the cell with heterologous enzymes.

20 So, in one aspect, there is provided a bacterial cell with improved tolerance to at least one aliphatic polyol according to any aspect or embodiment described herein, wherein the bacterial cell further comprises a recombinant biosynthetic pathway for producing an aliphatic polyol of interest, such as, *e.g.*, 2,3-butanediol, 1,2-propanediol, 1,4-butanediol, 1,3-propanediol, 1,2-butanediol, 1,5-pentanediol and/or 1,2-pentanediol. In a particular
25 embodiment, the bacterial cell further comprises a recombinant biosynthetic pathway for producing 2,3-butanediol. In another particular embodiment, the bacterial cell further comprises a recombinant biosynthetic pathway for producing 1,2-propanediol. In another particular embodiment, the bacterial cell further comprises a recombinant biosynthetic pathway for producing 1,4-butanediol. In another particular embodiment, the bacterial cell
30 further comprises a recombinant biosynthetic pathway for producing 1,3-propanediol. In another particular embodiment, the bacterial cell further comprises a recombinant biosynthetic pathway for producing 1,2-butanediol. In another particular embodiment, the bacterial cell further comprises a recombinant biosynthetic pathway for producing 1,5-pentanediol. In another particular embodiment, the bacterial cell further comprises a
35 recombinant biosynthetic pathway for producing 1,2-pentanediol.

20

In principle, any such recombinant biosynthetic pathway which is known in the art can be introduced into the cell by standard recombinant technologies. Biosynthetic pathways suitable for production of diols in bacteria are well-known in the art and have been described by, *e.g.*, Xu *et al.* (2014), Jiang *et al.* (2014), Sabra *et al.* (2016), Saxena *et al.* (2010), Altaras and

5   Cameron (2000), Clomburg and Gonzalez (2011), Zhu *et al.* (2016), Jain *et al.* (2015), Yim *et al.* (2011), Nakamura and Whited (2003), and Kataoka *et al.* (2013). Some specific, preferred pathways are, however, exemplified below and in Example 1, the section entitled "Biological production of 1,2-propanediol and 2,3-butanediol". It is to be understood that, when a specific enzyme of these biosynthetic pathways is mentioned by name such as, *e.g.*,

10   "acetolate synthase", the enzyme may be any characterized and sequenced enzyme, from any species, that have been reported in the literature so long as it provides the desired activity. In some embodiments, the enzyme is an overexpressed gene which is native to the host cell used. In some embodiments, the enzyme is a functionally active fragment or variant of an enzyme which is heterologous or native to the host cell. Also, in some embodiments,

15   the recombinant biosynthetic pathway comprises a knock-down or a knock-out of one or more genes, typically for the purpose of avoiding competing reactions reducing the yield of the desired aliphatic polyol.

So, in one embodiment, the biosynthetic pathway is for producing 2,3-butanediol from the cellular glycolytic intermediate pyruvate, and comprises genes, optionally overexpressed

20   and/or heterologous, encoding:

- an acetolactate synthase, *e.g.*, BudB from *Enterobacter cloacae*, catalyzing the conversion of two pyruvate molecules to acetolactate;
- an acetolactate decarboxylase, *e.g.*, BudA from *Enterobacter cloacae,* catalyzing the conversion of acetolactate to acetoin; and

25   - a 2,3-butanediol dehydrogenase (or acetoin reductase), *e.g.*, BudC from *Enterobacter cloacae*, catalyzing the conversion of acetoin to 2,3-butanediol

Typically, the native genes *adhE*, *gloA*, *ldhA*, *tpiA*, and/or *zwf are* knocked-down or -out to reduce lactate production, ethanol production, and carbon flux into the pentose phosphate

30   pathway.

In another embodiment, the biosynthetic pathway is for producing 2,3-butanediol from the cellular glycolytic intermediate pyruvate, and comprises genes, optionally overexpressed and/or heterologous, encoding:

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- an acetolactate synthase, *e.g.*, BudB from *Enterobacter cloacae*, catalyzing the conversion of two pyruvate molecules to acetolactate, which spontaneously decarboxylates to diacetyl;

- a diacetyl reductase, *e.g.*, BudC from *Klebsiella pneumoniae,* catalyzing the conversion of diacetyl to acetoin; and

- a 2,3-butanediol dehydrogenase (or acetoin reductase), *e.g.*, BudC from *Enterobacter cloacae*, catalyzing the conversion of acetoin to 2,3-butanediol.

In another embodiment, the biosynthetic pathway is for producing 2,3-butanediol from the cellular glycolytic intermediate pyruvate, and comprises genes, optionally overexpressed and/or heterologous, encoding:

- an acetolactate synthase, *e.g.*, BudB from *Enterobacter cloacae*, catalyzing the conversion of two pyruvate molecules to acetolactate;

- an acetolactate decarboxylase, *e.g.*, BudA from *Enterobacter cloacae,* catalyzing the conversion of acetolactate to acetoin;

- an acetoin dehydrogenase, *e.g.*, BudC from *Klebiella pneumoniae*, catalyzing the conversion of acetoin to diacetyl;

- an acetylacetoin synthase, *e.g.*, from *Bacillus licheniformis*, catalyzing the conversion of two diacetyl molecules to acetylacetoin;

- an acetylacetoin reductase, *e.g.*, from *Bacillus licheniformis*, catalyzing the conversion of acetylacetoin to acetylbutanediol; and

- an acetylbutanediol reductase, *e.g.*, from *Bacillus licheniformis*, catalyzing the conversion of acetylbutanediol to 2,3-butanediol.

Optionally, the biosynthetic pathway does not constitute an acetolactate decarboxylase nor an acetoin dehydrogenase, and acetolactate is instead spontaneously converted to acetoin.

In one embodiment, the biosynthetic pathway is for producing 1,2-propanediol from the cellular glycolytic intermediate dihydroxyacetone phosphate, and comprises genes, optionally overexpressed and/or heterologous, encoding:

- a methylglyoxal synthase, *e.g.*, MgsA from *E. coli*, catalyzing the conversion of dihydroxyacetone phosphate to methylglyoxal;

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- a methylglyoxal reductase or glycerol dehydrogenase, *e.g.*, GlyD and GlyH from *E. coli*, catalyzing the conversion of methylglyoxal to lactaldehyde; and
- a lactaldehyde reductase or 1,2-propanediol reductase, *e.g.*, FucO from *E. coli*, catalyzing the conversion of lactaldehyde to 1,2-propanediol.

Optionally, native lactate dehydrogenases which convert pyruvate to lactate, such as (in *E. coli*), LdhA, can be deleted (Altaras and Cameron, 2000).

In another embodiment, the biosynthetic pathway is for producing 1,2-propanediol from the cellular glycolytic intermediate dihydroxyacetone phosphate, and comprises genes, optionally overexpressed and/or heterologous, encoding:

- a methylglyoxal synthase, *e.g.*, MgsA from *E. coli*, catalyzing the conversion of dihydroxyacetone phosphate to methylglyoxal;
- an aldehyde oxidoreductase, *e.g.* YqhD from *E. coli*, catalyzing the conversion of methylglyoxal to acetol; and
- a glycerol reductase, *e.g.*, GlyD and GlyH from *E. coli*, catalyzing the conversion of acetol to 1,2-propanediol.

Optionally, native lactate dehydrogenases which convert pyruvate to lactate, such as (in *E. coli*), LdhA, can be deleted (Altaras and Cameron, 2000).

In another embodiment, the biosynthetic pathway is for producing 1,2-propanediol from the cellular glycolytic intermediate pyruvate, and comprises genes, optionally overexpressed and/or heterologous, encoding:

- a lactate dehydrogenase, *e.g.*, LdhA from *E. coli*, catalyzing the conversion of pyruvate to lactate;
- a lactaldehyde dehydrogenase, *e.g.* AldA from *E. coli*, catalyzing the conversion of lactate to lactaldehyde; and
- a lactaldehyde reductase or 1,2-propanediol reductase, *e.g.*,FucO from *E. coli*, catalyzing the conversion of lactaldehyde to 1,2-propanediol.

In another embodiment, the biosynthetic pathway is for producing 1,2-propanediol from the cellular glycolytic intermediate pyruvate, and comprises genes, optionally overexpressed and/or heterologous, encoding:

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- a methylglyoxal synthase, *e.g.*, MgsA from *E. coli*, catalyzing the conversion of dihydroxyacetone phosphate to methylglyoxal;

- a Type I glyoxylase, *e.g.* GloA from *E. coli*, catalyzing the conversion of methylglyoxal and glutathione to (*S*)-lactoylglutathione;

- a Type II glyoxylase, *e.g.* GloB from *E. coli*, catalyzing the conversion of (*S*)-lactoylglutathione to lactate and glutathione;

10

- a lactaldehyde dehydrogenase, *e.g.* AldA from *E. coli*, catalyzing the conversion of lactate to lactaldehyde; and

- a lactaldehyde reductase or 1,2-propanediol reductase, *e.g.*,FucO from *E. coli*, catalyzing the conversion of lactaldehyde to 1,2-propanediol.

15   Optionally, native lactate dehydrogenases which convert pyruvate to lactate, such as (in *E. coli*), LdhA, can be deleted (Altaras and Cameron, 2000).

In another embodiment, the biosynthetic pathway is for producing 1,2-propanediol from the cellular glycolytic intermediate pyruvate, and comprises genes, optionally overexpressed
20   and/or heterologous, encoding:

- a lactate dehydrogenase, *e.g.*, LdhA from *E. coli*, catalyzing the conversion of pyruvate to lactate;

- a CoA transferase, *e.g.*, Pct from *Clostridium propionicum* DSM 1682, catalyzing the conversion of lactate and CoA to lactoyl-CoA;

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- an aldehyde dehydrogenase, *e.g.*, a CoA-dependent succinate semialdehyde dehydrogenase (PdcD) from *Yersinia enterocolitica* subsp. *enterocolitica* 8081, catalyzing the conversion of lactoyl-CoA to lactaldehyde; and

- an alcohol dehydrogenase, *e.g.* a 3-hydroxypropionate dehydrogenase (MmsB) from *Bacillus cereus* ATCC 14579, catalyzing the conversion of lactaldehyde to 1,2-propanediol.

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In one embodiment, the biosynthetic pathway is for producing 1,4-butanediol from the cellular tricarboxylic acid intermediate succinate, and comprises genes, optionally overexpressed and/or heterologous, encoding:

- a succinyl-CoA synthetase, *e.g.*, SucCD from *E. coli*, catalyzing the conversion of succinate to
5   succinyl-CoA;
- a CoA-dependent succinate semialdehyde dehydrogenase, *e.g.*, SucD from *E. coli*, catalyzing the conversion of succinyl-CoA to succinyl semialdehyde;
- a 4-hydroxybutyrate dehydrogenase, *e.g.*, 4HBd from *Porphyromonas gingivalis*, catalyzing the conversion of succinyl semialdehyde to 4-hydroxybutryrate;
10 - a 4-hydroxybutyryl-CoA transferase, *e.g.*, Cat2 from *Porphyromonas gingivalis*, catalyzing the conversion of 4-hydroxybutyrate to 4-hydroxybutyryl-CoA;
- a 4-hydroxybutyryl-CoA reductase, *e.g.*, the bifunctional enzyme 025B from *Clostridium beijerinckii*, catalyzing the conversion of 4-hydroxybutyryl-CoA to 4-hydroxybutyrylaldehyde; and
15 - an alcohol dehydrogenase, *e.g.*, the bifunctional enzyme 025B from *Clostridium beijerinckii*, catalyzing the conversion of 4-hydroxybutryrylaldehyde to 1,4-butanediol.

Optionally, native malate dehydrogenase, such as (in *E. coli*), Mdh, can be deleted. Optionally, one or more subunits of a global regulator of gene expression under microaerobic and/or aerobic
20 conditions, such as (in *E. coli*), ArcAB, can be deleted. Optionally, native lactate and/or alcohol dehydrogenases, and/or pyruvate formate lyase, such as (in *E. coli*) LdhA, AdhE, and PflB, can be deleted. Optionally, pyruvate dehydrogenase can be modified by deleting the native lipoamide dehydrogenase (*e.g.*, LpdA in *E. coli*) and heterologously expressing an anaerobically functional LpdA such as from *Klebsiella pneumoniae*. The heterologously expressed LpdA can optionally harbor a
25 mutation reducing NADH sensitivity, such as D354K. Optionally, tricarboxylic acid cycle flux can increased by introducing a mutation to reduce NADH inhibition of citrate synthase, *e.g.*, by introducing a GltA-R163L mutation to *E. coli* GltA. Optionally, an α-ketoglutarate decarboxylase can be overexpressed, *e.g.*, SucA from *E. coli*, to additionally convert the tricarboxylic acid cycle intermediate α-ketoglutarate to succinyl semialdehyde (Yim *et al.*, 2011).

30 In one embodiment, the biosynthetic pathway is for producing 1,3-propanediol from the cellular glycolytic intermediate dihydroxyacetone phosphate, and comprises genes, optionally overexpressed and/or heterologous, encoding:

-   a glycerol-3-phosphate dehydrogenase and glycerol-3-phosphate phosphatase, *e.g.*, DAR1 and GPP2 from *Saccharomyces cerevisiae*, catalyzing the conversion of dihydroxyacetone phosphate to glycerol;

-   a glycerol dehydratase, *e.g.*, DhaB1, DhaB2, and DhaB3 from *Klebsiella pneumoniae*, catalyzing the conversion of glycerol to 3-hydroxypropionaldehyde; and

-   an aldehyde oxidoreductase, *e.g.*, YqhD from *E. coli*, catalyzing the conversion of 3-hydroxypropionaldehyde to 1,3-propanediol.

Optionally, PEP-dependent glucose transport is eliminated via deletion of one or more genes in glucose-specific PTS enzyme II, *e.g.*, PtsG in *E. coli*, and an ATP-dependent glucose transport system composed of galactose permease (*e.g.*, GalP in *E. coli*) and glucokinase (*e.g.*, Glk in *E. coli*) are overexpressed or heterologously expressed. Optionally, glyceraldehyde-3-phosphate dehydrogenase (*e.g.*, Gap in *E. coli*), is downregulated (Nakamura and Whited, 2003).

In one embodiment, the biosynthetic pathway is for producing 1,3-butanediol from the cellular intermediate acetyl-CoA, and comprises genes, optionally overexpressed and/or heterologous, encoding:

-   a 3-ketothiolase, *e.g.*, PhaA from *Ralstonia eutropha* NBRC 102504, catalyzing the conversion of acetyl-CoA to acetoacetyl-CoA;

-   an acetoacetyl-CoA reductase, *e.g.*, PhaB from *Ralstonia eutropha* NBRC 102504, catalyzing the conversion of acetoacetyl-CoA to 3-hydroxybutyryl-CoA;

-   a butyraldehyde dehydrogenase, *e.g.*, Bld from *Clostridium saccharoperbutylacetonicum* ATCC 27012, catalyzing the conversion of 3-hydroxybutyryrl-CoA to 3-hydroxybutyraldehyde; and

-   an aldehyde-alcohol dehydrogenase, *e.g.*, AdhE from *E. coli*, catalyzing the conversion of 3-hydroxybutyraldehyde to 1,3-butanediol.

In one embodiment, 1,5-pentanediol is produced from glutaric acid, optionally via glutaryl-CoA, via reduction of the 1- and 5-carboxylic acids to alcohols. Pathways describing the production of glutaric acid from the intracellular amino acid L-lysine have been described (Adkins *et al.*, 2013; Park *et al.*, 2013). Biosynthesis of the glutaryl-CoA intermediate has been described by Cheong *et al.*, 2016.

3)   Processes

In one aspect, there is provided a process for preparing a recombinant bacterial cell, *e.g.*, an
E. coli cell. Also provided is a process for improving the tolerance of a bacterial cell, *e.g.*, an
E. coli cell, to a diol or other polyol. Also provided is a method of identifying a bacterial cell
5   which is tolerant to at least one diol or other polyol. Also provided is a process for preparing a
recombinant bacterial cell, *e.g.*, an *E. coli* cell, for producing a diol or other polyol.

These processes may comprise one or more steps of genetically modifying a bacterial cell to
knock-down or knock-out one or more endogenous genes of any aspect or embodiment of
the Group 1 modifications and/or introducing one or more mutations in the endogenous
10   protein(s) or gene(s) of any Group 2 aspect or embodiment. This can be achieved by, *e.g.*,
transforming the bacterial cell with genetic constructs, *e.g.*, vectors, antisense nucleic acids
or siRNA, which result, *e.g.*, in the knock-out or knock-down of a gene, introduce a mutation
into an endogenous gene, or which encode the mutated protein from a transgene.

The genetic constructs, particularly vectors, can also comprise suitable regulatory sequences,
15   typically nucleotide sequences located upstream (5' non-coding sequences), within, or
downstream (3' non-coding sequences) of a coding sequence, and which influence the
transcription, RNA processing or stability, or translation of the associated coding sequence.
Regulatory sequences may include promoters (*e.g.*, constitutive promoters or inducible
promoters), translation leader sequences, introns, polyadenylation recognition sequences,
20   RNA processing sites, effector binding sites and stem-loop structures.

Alternatively, bacterial cells can be exposed to selection pressure (as described in the
Examples) or to conditions which introduce random mutations in endogenous genes, and
bacterial cells which comprise one or more Group 1 and/or Group 2 modifications according
to any preceding aspects and embodiments can then be identified. Typically, this involves
25   preparing a population of the genetically modified bacterial cell, having different Group 1
and/or Group 2 modifications, and then selecting from this population any bacterial cell which
has an improved tolerance to the diol or other polyol, *e.g.*, an aliphatic diol or other polyol.

In one specific embodiment, the Group 1 modification is a knock-down or knock-out of one or
more endogenous genes selected from *metJ, rzpD, yhjA, gtrS, ycdU, iscR, sspA and rph* or,
30   *e.g.*, a knock-down or knock-out of *metJ* in combination with *relA* and *purT* or with *acrB*
and/or *acrA*, and/or a knock-down or knock-out of *iscR* and *relA*. In one specific embodiment,
the Group 2 modification is a mutation in at least one endogenous protein or gene selected
from NanK, RpsA, RpoB, RpoC, SpoT, NusG, Flu, Lon, or YgaH, such as *e.g.*, NanK-T128S,
RpoA-D305G, RpoA-D305A, RpsA-D310V, RpsA-D310I, RpsA-D310L, RpsA-D310M, RpsA-

D310F, RpsA-D310A, RpoA-G279V, RpoA-G279I, RpoA-G279L, RpoA-G279M, RpoA-G279F, RpoA-G279A, RpsA-G21V, RpsA-G21I, RpsA-G21L, RpsA-G21M, RpsA-G21F, RpsA-G21A, RpoB-H447Y, RpoB-H447F, RpoB-H447W, RpoB-H447T, RpoB-H447S, RpoC-L268R, RpoC-L268K, RpoC-L268Q, RpoC-L268N, RpoB-D549G, RpoB-D549A, RpoB-V931A, RpoB-V931L, RpoB-V931I, RpoC-ΔTPVIE(822-827), RpoC-N309Y, RpoC-N309F, RpoC-N309W, RpoC-N309T, RpoC-N309S, SpoT-I213L, SpoT-I213V, SpoT-I213M, SpoT-I213A, SpoT-I213F, NusG-F144V, NusG-F144I, NusG-F144L, NusG-F144M, NusG-F144A, RpoC-Y75C, RpoC-Y75S, RpoC-Y75A, Flu-L642Q, Flu-L642N, Flu-L642E, RpoB-I1112S, RpoB-I1112T, Lon-I716S, Lon-I716T, YgaH-V39A, YgaH-V39L, or a YgaH-V39I mutation and/or a mutation which increases the expression of PyrE, such as, *e.g.* a mutation in *rph* or the *pyrE/rph* intergenic region.

In one embodiment, the process comprises genetically modifying the bacterial cells, *e.g.*, the E. coli cells, to express a mutant NanK, RpoC, Flu, RpoB, Lon, YgaH, such as, *e.g.*, NanK-T128S, RpoC-L268R, Flu-L642Q, RpoB-I1112S, Lon-I716S, and/or YgaH-V39A mutation, or a conservative substitution of any thereof, and/or a mutation which increases the expression of PyrE.

The processes may further comprise

- a step of selecting any bacterial cell which has an improved tolerance to a diol or other polyol at a predetermined concentration, such as at least 1% v/v or higher, such as at least 1.5% v/v or higher, such as at least 3% v/v or higher, such as at least 5% v/v or higher, such as at least 6% v/v or higher, such as at least 7% v/v or higher, such as at least 8% v/v or higher, such as at least 10% v/v or higher;

- an optional step of introducing a recombinant biosynthetic pathway for producing the diol or other polyol; or

- both of the above steps, in any order.

In one embodiment, the diol is 2,3-butanediol, and the predetermined concentration is at least 1% v/v or higher, such as at least 1.5% v/v or higher, such as at least 3% v/v or higher, such as at least 5% v/v or higher, such as at least 6% v/v or higher, such as at least 7% v/v or higher, such as at least 10% v/v or higher. In one embodiment, the diol is 1,2-propanediol, and the predetermined concentration is at least 1% v/v or higher, such as at least 1.5% v/v or higher, such as at least 3% v/v or higher, such as at least 5% v/v or higher, such as at least 6% v/v or higher, such as at least 7% v/v or higher, such as at least 7.5% v/v or higher, such as at least 8% v/v or higher, such as at least 10% v/v or higher. In

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one embodiment, the diol is 1,5-pentanediol, and the predetermined concentration is at least 0.5% v/v or higher, such as at least 1% v/v or higher, such as at least 2% v/v or higher, such as at least 3% v/v or higher, such as at least 5% v/v or higher, such as at least 6% v/v or higher, such as at least 7% v/v or higher, such as at least 7.5% v/v or higher, such as at least 8% v/v or higher, such as at least 10% v/v or higher. In one embodiment, the diol is 1,2-pentanediol, and the predetermined concentration is at least 0.5% v/v or higher, such as at least 1% v/v or higher, such as at least 2% v/v or higher, such as at least 3% v/v or higher, such as at least 5% v/v or higher, such as at least 6% v/v or higher, such as at least 7% v/v or higher, such as at least 7.5% v/v or higher, such as at least 8% v/v or higher, such as at least 10% v/v or higher.

In a particular embodiment, the predetermined concentration is at most 7%, such as at most 8%, such as at most 9%, such as at most 10%, such as at most 15%, such as at most 20%.

Assays for assessing the tolerance of a modified bacterial cell to the diol or other polyol typically evaluate the growth rate, lag time, or both, of the bacterial cell at predetermined concentrations for the diol or other polyol in question, typically as compared to a control. Preferably, the control is the native or unmodified parent cell or strain, and an improved tolerance is identified as an improved growth rate, a reduced lag-time or both. For example, an improved growth rate can be at least 5%, such as at least 10%, such as at least 20%, such as at least 50%, such as at least 75% higher than that of the control, while a reduced lag time can be at least 10%, such as at least 20%, such as at least 50%, such as at least 75%, such as at least 90% shorter than that of the control. Specific assays are described, in detail, in the Examples.

Also provided is a method of producing a diol or other polyol, comprising culturing the bacterial cell obtained by any one of these methods, or the bacterial cell of any preceding aspect or embodiment, under conditions where the diol or other polyol is produced. Typically, these conditions include the presence of a suitable carbon source or mixes of different suitable carbon sources. Non-limiting examples of suitable carbon sources include, *e.g.*, sucrose, D-glucose, D-xylose, L-arabinose, glycerol; raw carbon feedstocks such as crude glycerol and cane syrup; as well as hydrolysates produced from cellulosic or lignocellulosic materials. For further details see, *e.g.*, Sabra *et al.*, 2016; Clomburg *et al.*, 2011; Jain *et al.*, 2015; Li *et al.*, 2015; Jiang *et al.*, 2014; and Xu *et al.*, 2014.

The inventors have further discovered that methionine supplementation can improve endogenous production of diols in diol-overproducing strains during fermentation. In particular, robust growth of K-12 MG1655 in 6% 2,3-butanediol or 8% 1,2-propanediol was significantly restored by the addition of methionine, with a growth rate approaching that of

evolved strains in such media, whereas evolved strains did not have a significantly enhanced growth rate with the addition of methionine.

Accordingly, in one embodiment, there is provided a method for producing an aliphatic diol, comprising culturing a bacterial cell capable of producing the aliphatic diol in the presence of a carbon source and adding methionine to the cultivation medium, wherein the concentration of the added methionine is at least about 0.004 g L^{-1} $gDCW^{-1}$ (gDCW = grams dry cell weight), such as at least about 0.007 g L^{-1} $gDCW^{-1}$, such as at least 0.015 g L^{-1} $gDCW^{-1}$, such as at least about 0.03 g L^{-1} $gDCW^{-1}$, such as at least about 0.07 g L^{-1} $gDCW^{-1}$, such as at least about 0.2 g L^{-1} $gDCW^{-1}$. In a particular embodiment, the added methionine concentration is at most 0.03 g L^{-1} $gDCW^{-1}$, such as at most 0.07 g L^{-1} $gDCW^{-1}$, such as at most 0.2 g L^{-1} $gDCW^{-1}$. In some embodiments, the added methionine concentration is in the range from about 0.0004 to about 0.2, such as from about 0.007 to about 0.2, such as from about 0.015 to about 0.2, such as from about 0.03 to about 0.2, such as from about 0.07 to about 0.2 g L^{-1} $gDCW^{-1}$. Optionally, the bacterial cell may comprise one or more genetic modifications according to any aspect or embodiment described herein. In one embodiment, the medium comprises no more than 10, such as no more than 8, such as no more than 6, such as no more than 5, such as no more than 4 other natural amino acids, *e.g.*, selected from alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine at a biologically relevant level, *e.g.*, at a concentration of at least 0.0002 g L^{-1} $gDCW^{-1}$. Optionally, the method further comprises isolating the aliphatic diol.

4) Compositions

A bacterial cell which has an increased tolerance to a diol or other polyol can be useful for preparing producer cells for the production of the diol or other polyol. Bacterial cells according to the invention may have an increased growth rate, an decreased lag time, or both. For example, the bacterial cell may have Group 1 and/or Group 2 modifications providing for an increased growth rate, a reduced lag time, or both, of the cell in at least one of a propanediol, butanediol, pentanediol or a hexanediol, *e.g.*, 2,3-butanediol, 1,2-propanediol; 1,4-butanediol, 1,3-propanediol, 1,2-butanediol, 1,5 pentanediol and/or 1,2-pentanediol, such as in 2,3-butanediol, 1,2-propanediol, or both.

In one aspect, there is provided a composition of a plurality of bacterial cells according to any aspect or embodiment described herein, *e.g.*, an *in vitro* culture of such bacterial cells, optionally in a suitable culture medium and/or a chemically-defined medium comprising a carbon source. In one embodiment, the composition is substantially homogenous with respect to the bacterial cells.

In one aspect, there is provided a composition comprising a plurality of bacterial cells according to any preceding aspect or embodiment and a diol or other polyol. In one embodiment, the diol or other polyol is present at a concentration at which the genetic modification(s) and/or mutant(s) comprised in the bacterial cells results in an improved
5   tolerance as compared to the parent bacterial cells, *e.g.*, wild-type or native bacterial cells. The concentrations at which bacterial cells according to the invention have improved tolerance are shown in Example 1, *e.g.*, in "Cross-compound tolerance testing". Typically, the concentration of the a diol or other polyol is at least 1% v/v or higher, such as at least 1.5% v/v or higher, such as at least 3% v/v or higher, such as at least 5% v/v or higher, such as
10   at least 6% v/v or higher, such as at least 7.5% v/v or higher, such as at least 10% v/v or higher, such as at least 20% v/v or higher; such as at least 30% v/v or higher; such as in the range of 1% to 30% v/v, such as in the range of 2% to 20% v/v; such as in the range of 5% to 15% v/v or 5 to 10% v/v.

In one embodiment, the composition comprises 2,3-butanediol. In one embodiment, the
15   composition comprises 1,2-propanediol. In one embodiment, the composition comprises 1,5-pentanediol. In one embodiment, the composition comprises 1,2-pentanediol. In one embodiment, the composition comprises 1,4-butanediol. In one embodiment, the composition comprises 1,3-propanediol. In one embodiment, the composition comprises 1,2-butanediol.

20   As described in Example 1; "Cross-compound tolerance testing", some of the genetic modifications according to the invention also confer tolerance to other chemicals, such as to other polyols or diols, to hexanoate and/or to p-coumarate. Accordingly, in one embodiment, there is provided a composition comprising

    -    hexanoate at a concentration of at least 0.1 g/L, such as at least 1 g/L, such as at least 5 g/L,
25   such as at least 10 g/L, such as at least 20 g/L, or p-coumarate at a concentration of at least
           1 g/L, such as at least 2.5 g/L, such as at least 5 g/L, such as at least 7.5 g/L, such as at least
           15 g/L; and

    -    a plurality of bacterial cells according to any preceding aspect or embodiment.

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Preferably, the bacterial cells are of the *Escherichia, Lactobaccillus, Lactococcus, Corynebacterium, Bacillus, Ralstonia,* or *Pseudomonas* genera, such as, *e.g., E. coli* cells, and comprise

a) at least one genetic modification which reduces expression of an endogenous gene selected from the group consisting of *metJ, rzpD, yhjA, gtrS, ycdU, iscR, sspA* and *rph*, or a combination of any thereof;

b) genetic modifications which reduce expression of *metJ, relA* and *purT*,

c) genetic modifications which reduce expression of *metJ* and *acrB* and/or *acrA*, or

d) genetic modifications which reduce expression of *iscR* and *relA*.

Such bacterial cells may further comprise one or more Group 2 modifications as described in any aspect or embodiment herein.

Assays for assessing the tolerance of a modified bacterial cell to a diol or other polyol typically evaluate the growth rate, lag time, or both, of the bacterial cell at one or more predetermined concentrations of the compound, typically as compared to a control (*e.g.*, no compound). The predetermined concentrations(s) could be, for example, 6% v/v, 7% v/v or 8% v/v. Preferably, the control is the native or unmodified parent cell or strain, and an improved tolerance is identified as an improved growth rate, a reduced lag-time or both. For example, an improved growth rate can be at least 5%, such as at least 10%, such as at least 20%, such as at least 50%, such as at least 75% higher than that of the control, while a reduced lag time can be at least 10%, such as at least 20%, such as at least 50%, such as at least 75%, such as at least 90% shorter than that of the control. Specific assays are described, in detail, in the Examples.

5) Bacterial cells

Also provided are strains, clones and other progeny of the bacterial cells of these and other aspects and embodiments, as well as cell cultures of such bacterial cells or strains. Typically, as used herein, a "strain" typically refers to a group of cells which are descendants of a initial single colony of parent cells whereas a "clone" is a group of cells which are the descendants of an initial genetically modified single parent cell.

Non-limiting examples of bacterial cells suitable for modification according to any one of the aspects and embodiments described herein include bacteria of the *Escherichia, Enterobacter, Klebsiella, Lactobaccillus, Lactococcus, Corynebacterium, Bacillus, Ralstonia, Paenibacillus, Clostridia, Citrobacter sp.* or *Pseudomonas* genera, such as from the *Escherichia, Lactobacillus, Lactococcus, Corynebacterium, Bacillus, Ralstonia,* or *Pseudomonas* genera. In one embodiment, the bacterial cell is an *E. coli* cell, such as a cell of the commercially available and/or fully characterized strains K-12 MG1655, BW25113, BL21, BL21(DE3), K-12 W3110, W, JM109, or Crooks (ATCC 8739). In a specific embodiment, the bacterial cell is derived from an *E. coli* K12 strain. In another embodiment, the bacterial cell is a *Lactobacillus*

cell, such as a cell of the commercially available and/or fully characterized strains *Lactobacillus plantarum* JDM1, *Lactobacillus plantarum* WCFS1, and *Lactobacillus plantarum* NCIMB 8826. In another embodiment, the bacterial cell is a *Lactococcus* cell, such as a cell of the commercially available and/or fully characterized strains *Lactococcus lactis lactis* CV56,

5   *Lactococcus lactis lactis* NIZO B40, and *Lactococcus lactis cremoris* NZ9000. In another embodiment, the bacterial cell is a *Bacillus* cell, such as a cell of the commercially available and/or fully characterized strains *Bacillus subtilis* 168 and *Bacillus subtilis* PY79. In one embodiment, the bacterial cell is a *Pseudomonas* cell, such as a cell of the commercially available and/or fully characterized strain *Pseudomonas putida* KT2440. In another

10   embodiment, the bacterial cell is a *Ralstonia* cell, such as a cell of the commercially available and/or fully characterized strains *Ralstonia eutropha* H16 and *Ralstonia eutropha* JMP134. In another embodiment, the bacterial cell is a *Corynebacterium* cell, such as a cell of the commercially available and/or fully characterized strains 534 (ATCC 13032), K051, MB001, R, SCgG1, and SCgG2.

15   While aspect and embodiments relating to bacterial cells herein typically refer to genes or proteins according to their designation in *E. coli*, for bacterial cells of another family or species, it is within the level of skill in the art to identify the corresponding gene or protein, *i.e.*, the ortholog and/or paralog, in the other family or species, typically by identifying sequences having moderate or high homology to the *E. coli* sequence, optionally taking the

20   function of the protein expressed by the gene and/or the locus of the gene in the genome into account. Table 2A below sets out the function of the protein encoded by each specific gene, the corresponding E.C. number (if applicable), its locus in the *E. coli* K-12 MG1655 genome and the SEQ ID number of the coding or non-coding sequence and, where applicable, the encoded amino acid sequence.

25   Table 2B below sets out some examples of homologs or orthologs in selected organisms, identified in a preliminary and non-limiting analysis. Indeed, homologs or orthologs of these proteins exist also in other bacteria, and other homologs or orthologs not identified in this preliminary search can exist in the species listed in Table 2B. The skilled person is well-familiar with different searching and/or screening methods for identifying homologs or

30   orthologs across different species. To briefly summarize some of the preliminary findings in Table 2B:

-    RelA, PurT, YfgF, and PyrE are widely conserved and were identified in all organisms.
-    AcrB homologs or orthologs were identified in the Gram-negative species and *Bacillus subtilis*.

35   -   Rph was found to be conserved in all organisms with the exception of *Lactococcus lactis*.

- FabR, RzpD, and YhjA had the longest alignments with *Pseudomonas putida* proteins, with other more partial alignments to FabR annotated as transcriptional regulators in all other organisms.
- IscR was found in Gram-negative organisms and *Bacillus subtilis*, with other more partial alignments annotated as transcriptional regulators in other organisms.
- SspA was found to be conserved in Gram-negative organisms.
- GtrS, YcdU, MetJ, Flu (with the exception of partial conservation in *Pseudomonas putida*), and YgaH were not widely conserved.
- RpoA, RpoB, RpoC, SpoT, RpsA and NusG were found to be widely conserved in all organisms.
- Lon was found to be conserved in Gram-negative organisms.

Table 2 – Protein function and Locus IDs

E. coli gene designation	Protein function	E.C. number	Locus ID	SEQ ID NO:
metJ	MetJ transcriptional repressor	N/A	b3938	1
rzpD	DLP12 prophage; predicted murein endopeptidase	N/A	b0556	2
yhjA	predicted cytochrome C peroxidase	N/A	b3518	3
gtrS	CPS-53 (KpLE1) prophage; predicted inner membrane protein	N/A	b2352	4
ycdU	predicted inner membrane protein	N/A	b1029	5
iscR	IscR DNA-binding transcriptional dual regulator	N/A	b2531	6
sspA	stringent starvation protein A	N/A	b3229	7
Rph	ribonuclease PH	2.7.7.56	b3643	8
relA	GDP pyrophosphokinase / GTP pyrophosphokinase	2.7.6.5	b2784	9
purT	phosphoribosylglycinamide formyltransferase 2	2.1.2.-; 2.7.2.1	b1849	10

acrB	AcrAB-TolC multidrug efflux system – permease subunit	N/A	b0462	11
acrA	AcrAB-TolC multidrug efflux system – membrane fusion protein	N/A	b0463	12
fabR	FabR DNA-binding transcriptional repressor	N/A	b3963	13
ygfF	cyclic di-GMP phosphodiesterase	3.1.4.52	b2503	14
pyrE	Orotate phosphoribosyltransferase	2.4.2.10	b3642	15 (DNA) 16 (protein)
pyrE/rph intergenic region	-	-	-	17
nanK	N-acetylmannosamine kinase	2.7.1.60	b3222	18 (DNA) 19 (protein)
rpoA	RNA polymerase subunit α	2.7.7.6	b3295	20 (DNA) 21 (protein)
rpoB	RNA polymerase subunit β	2.7.7.6	b3987	22 (DNA) 23 (protein)
rpoC	RNA polymerase subunit β'	2.7.7.6	b3988	24 (DNA) 25 (protein)
spoT	bifunctional (p)ppGpp synthase/hydrolase SpoT	3.1.7.2	b3650	26 (DNA) 27 (protein)
nusG	transcription termination factor NusG	N/A	b3982	28 (DNA) 29 (protein)
flu	CP4-44 prophage; self recognizing antigen 43 (Ag43) autotransporter	N/A	b2000	30 (DNA) 31 (protein)
lon	DNA-binding, ATP-dependent protease La	3.4.21.53	b0439	32 (DNA) 33 (protein)
ygaH	L-valine exporter – YgaH subunit	N/A	b2683	34 (DNA) 35 (protein)
rpsA	30S ribosomal subunit protein S1	N/A	b0911	36 (DNA) 37 (protein)

Table 2B – Homologs or orthologs identified by protein BLAST (BLASTP) of E. coli K-12 MG1655 proteins against protein databases from selected reference organisms. Hits with the largest e-value are shown, and hits are only shown when the e-value < 1.0.

Protein (# aa)	B. subtilis 168	P. putida KT2440	L. plantarum JDM1	L. lactis KF147	Ralstonia eutropha H16	Coryne-bacterium glutamicum ATCC 13032
MetJ (105 aa)			38% identity (50 aa) "histidine protein kinase; sensor protein" (YP_003063223.1)			26% identity (99 aa) "hypothetical protein NCgl2236" (NP_601518.1)
RelA (744 aa)	39% identity (694 aa) "GTP pyro-phospho-kinase" (NP_390638.2)	32-48% identity (681-751 aa) "(p)ppGpp synthetase I SpoT/RelA" (NP_743813.1, NP_747403.1)	37% identity (732 aa) "GTP pyrophospho-kinase" (YP_003063260.1)	38% identity (697 aa) "GTP pyro-phospho-kinase/guanosine-3,5-bis(diphosphate) 3-pyrophospho-hydrolase" (YP_003352549.1)	32-42% identity (684-717 aa) "GTP pyro-phospho-kinase" (YP_725845.1, YP_725468.1)	37% identity (688 aa) "guanosine poly-phosphate pyrophospho-hydrolase/ synthetase" (NP_600866.1)
PurT (392 aa)	57% identity (378 aa) "phospho-ribosylglycinamide formyl-transferase" (NP_388105.1)	69% identity (392 aa) "phospho-ribosylglycinamide formyltransferase 2" (NP_743615.1)	22-26% identity (329-363 aa) "phospho-ribosylamino-imidazole carboxylase ATPase subunit" (YP_003063771.1, YP_003062522.1)	24% identity (351 aa) "phospho-ribosylamino-imidazole carboxylase NCAIR mutase subunit" (YP_003354057.1)	59% identity (382 aa) "phospho-ribosyl-glycin-amide formyl-transferase 2" (YP_726361.1)	57% identity (396 aa) "phospho-ribosyl-glycinamide formyltransferase 2" (NP_601954.1)
AcrB (1049 aa)	24% identity (806 aa) "surfactin self-resistance transporter" (NP_388553.1)	54-66% identity (1016-1039 aa) "hydrophobe/ amphiphile efflux (HAE1) family transporter" (NP_743544.1, NP_745594.1)	25% identity (88 aa) "prenyl-transferase" (YP_003062880.1)	25% identity (72 aa) "hypo-thetical protein LLKF_0319" (YP_003352791.1), 30% identity (63 aa) "multidrug resistance ABC transporter ATP-binding/permease" (YP_003353188.1)	26-61% identity (1033-1066 aa) "cation/ multidrug efflux pump" (YP_728154.1, YP_728030.1, YP_727793.1, YP_726250.1, YP_726630.1, YP_725100.1, YP_727320.1)	31% identity (64 aa) "short chain dehydrogenase" (NP_601672.1)
FabR (234 aa)	29% identity (80 aa) "HTH-type tran-scriptional regulator YxbF" (NP_391864.1)	38% identity (203 aa) "TetR family transcriptional regulator" (NP_746964.1)	28% identity (96 aa) "transcription regulator" (YP_003063005.1), 25% identity (223 aa) "transcription regulator" (YP_003061828.1)	24-31% identity (98-136 aa) "TetR family transcriptional regulator" (YP_003354897.1, YP_003354066.1)	23-31% identity (97-147 aa) "TetR/AcrR family tran-scriptional regulator" (YP_726724.1, YP_728152.1, YP_727318.1); 29% identity (142 aa) "response regulator" (YP_724722.1)	23-33% identity (48-110 aa) "transcriptional regulator" (NP_600383.1, NP_601305.1, NP_600189.1)

YfgF (747 aa)	26% identity (434 aa) "di-guanylate cyclase" (YP_054577.1)	26-28% identity (467-484 aa) "sensory box protein/GGDEF family protein" (NP_743917.1), "sensory box protein" (NP_742833.1)	23% identity (155-231 aa) "diguanylate cyclase/ phospho-diesterase domain-containing protein" (YP_003062219.1, YP_003063933.1)	23% identity (241 aa) "cyclic di-GMP-specific phospho-diesterase" (YP_003353123.1)	28% identity (439 aa) "sensor protein" (YP_725212.1)	33% identity (258 aa) "diguanylate cyclase" (NP_600263.1)
RzpD (153 aa)	23% identity (98 aa) "ferrous iron permease EfeU" (NP_391707.2)	25% identity (126 aa) "DNA-directed RNA polymerase subunit beta" (NP_742613.1)	29% identity (51 aa) "hypothetical protein JDM1_0327" (YP_003061913.1), 28% identity (90 aa) "SLT domain protein" (YP_003062580.1)	37% identity (35 aa) "metal ABC transporter substrate-binding protein" (YP_003353799.1)		35% identity (37 aa) "3-oxoacyl-ACP synthase" (NP_601696.1)
YhjA (465 aa)	42% identity (36 aa) "mena-quinol-cytochrome c reductase b/c subunit" (NP_390135.1)	43% identity (294 aa) "cytochrome c551 peroxidase" (NP_745087.1)	32% identity (57 aa) "hypothetical protein JDM1_1287" (YP_003062871.1)		26% identity (114 aa) "C-type cytochrome (SoxD)" (YP_727997.1)	37% identity (41 aa) "glycosyl-transferase" (NP_600879.1)
GtrS (443 aa)			30% identity (77 aa) "hypothetical protein JDM1_2362" (YP_003063946.1)			33% identity (36 aa) "hypothetical protein NCgl1728" (NP_601005.1)
YcdU (328 aa)		46% identity (33 aa) "hydrophobe/ amphiphile efflux-1 (HAE1) family transporter" (NP_745564.1)			33% identity (33 aa) "response regulator" (YP_727560.1)	26% identity (46 aa) "hypothetical protein NCgl0613" (NP_599874.1)
IscR (162 aa)	32% identity (131 aa) "Rrf2 family tran-scriptional regulator" (NP_390630.2); 29% identity (136 aa) "HTH-type transcriptional regulator YwgB" (NP_391638.1); 31% identity (137 aa) "Rrf2 family transcriptional regulator" (NP_388819.1)	65% identity (142 aa) "BadM/Rrf2 family transcriptional regulator" (NP_743002.1)	34% identity (70 aa) "transcription regulator" (YP_003062417.1)	24% identity (140 aa) "Rrf2 family transcriptional regulator" (YP_003353004.1)	54% identity (136 aa) "tran-scriptional regulator of iron sulfur cluster assembly (IscR)" (YP_725666.1)	30-35% identity (44-101 aa) "transcriptional regulator" (NP_600099.1, NP_601856.1, NP_600589.1)
SspA (212 aa)		57% identity (200 aa) "stringent starvation protein A" (NP_743480.1)	45% identity (22 aa) "hypothetical protein JDM1_0823" (YP_003062407.1)		46% identity (203 aa) "stringent starvation protein A" (YP_727831.1)	56% identity (16 aa) "hypothetical protein NCgl2333" (NP_601617.1)
Rph (228 aa)	58% identity (222 aa) "ribo-nuclease PH" (NP_390715.1)	69% identity (228 aa) "ribo-nuclease PH" (NP_747395.1)		26% identity (207 aa) "polyribo-nucleotide nucleotidyl-transferase"	62% identity (221 aa) "ribo-nuclease PH" (YP_725462.1)	59% identity (217 aa) "ribonuclease PH" (NP_601703.2)

PyrE (213 aa)	25-34% identity (in stretches) "orotate phospho-ribosyl-transferase" (NP_389439.1)	67% identity (213 aa) "orotate phospho-ribosyl-transferase" (NP_747392.1)	29% identity (138 aa) "orotate phospho-ribosyl-transferase" (YP_003063746.1)	24-30% identity (in stretches) "orotate phospho-ribosyl-transferase" (YP_003354448.1)	56% identity (215 aa) "orotate phospho-ribosyl-transferase" (YP_724744.1)	29% identity (139 aa) "orotate phospho-ribosyl-transferase" (NP_601967.1)
NanK (291 aa)	30% identity (310 aa) "glucokinase" (NP_390365.2)	32% identity (65 aa) "DNA gyrase subunit A" (NP_743923.1), 27% identity (75 aa) "D,D-heptose 1,7-bisphosphate phosphatase" (NP_742229.1)	25-28% identity (256-296 aa) "sugar kinase and transcription regulator" (YP_003063958.1, YP_003061957.1, YP_003064483.1, YP_003063668.1, YP_003062678.1, YP_003064434.1), 27% identity (313 aa) "glucokinase" (YP_003062902.1)	28% identity (313 aa) "glucokinase" (YP_003354608.1), 29% identity (293 aa) "ROK family glucokinase/transcription regulator" (YP_003354028.1)	41% identity (37 aa) "ATP-dependent helicase" (YP_725935.1)	27% identity (319 aa) "glucose kinase" (NP_601389.1)
RpoA (329 aa)	46% identity (312 aa) "DNA-directed RNA polymerase subunit alpha" (NP_388024.1)	74% identity (326 aa) "DNA-directed RNA polymerase subunit alpha" (NP_742645.1)	47% identity (311 aa) "DNA-directed RNA polymerase subunit alpha" (YP_003062460.1)	41% identity (319 aa) "DNA-directed RNA polymerase subunit alpha" (YP_003354712.1)	61% identity (323 aa) "DNA-directed RNA polymerase subunit alpha" (YP_727894.1)	45% identity (315 aa) "DNA-directed RNA polymerase subunit alpha" (NP_599801.1)
RpoB (1342 aa)	47-59% identity (in stretches) "DNA-directed RNA polymerase subunit beta" (NP_387988.2)	72% identity (1360 aa) "DNA-directed RNA polymerase subunit beta" (NP_742613.1)	47-52% identity (in stretches) "DNA-directed RNA polymerase subunit beta" (YP_003062426.1)	46-47% identity (in stretches) "DNA-directed RNA polymerase subunit beta" (YP_003354373.1)	66% identity (1370 aa) "DNA-directed RNA polymerase subunit beta" (YP_727933.1)	42.56% identity (in stretches) "DNA-directed RNA polymerase subunit beta" (NP_599733.1)
RpoC (1407 aa)	50% identity (in stretches) "DNA-directed RNA polymerase subunit beta'" (NP_387989.2)	75% identity (1399 aa) "DNA-directed RNA polymerase subunit beta'" (NP_742614.1)	44-51% identity (in stretches) "DNA-directed RNA polymerase subunit beta'" (YP_003062427.1)	48-52% identity (in stretches) "DNA-directed RNA polymerase subunit beta'" (YP_003354372.1)	67% identity (1397 aa) "DNA-directed RNA polymerase subunit beta'" (YP_727932.1)	46-50% identity (in stretches) "DNA-directed RNA polymerase subunit beta'" (NP_599734.1)
SpoT (702 aa)	40% identity (719 aa) "GTP pyrophosphokinase" (NP_390638.2)	37-55% identity (681-701 aa) "(p)ppGpp synthetase I SpoT/RelA" (NP_747403.1, NP_743813.1)	38% identity (741 aa) "GTP pyrophosphokinase" (YP_003063260.1)	40% identity (725 aa) "GTP pyrophosphokinase/guanosine-3,5-bis(diphosphate) 3-pyrophosphohydrolase (YP_003352549.1)	36-47% identity (674-720 aa) "GTP pyrophosphokinase" (YP_725468.1, YP_725845.1)	38% identity (723 aa) "guanosine polyphosphate pyrophosphohydrolase/synthetase" (NP_600866.1)
NusG (181 aa)	44% identity (177 aa) "transcription termination/antitermination protein NusG" (NP_387982.1)	73% identity (174 aa) "transcription antitermination protein NusG" (NP_742608.1)	42% identity (183 aa) "transcription antitermination protein NusG" (YP_003062140.1)	38% identity (173 aa) "transcription antitermination protein NusG" (YP_003354744.1)	64% identity (179 aa) "transcription antitermination factor NusG" (YP_727938.1)	37% identity (194 aa) "transcription antitermination factor NusG" (NP_599720.1)
Flu (1039 aa)		25-29% identity (414-484 aa) "outer membrane autotransporter" (NP_745213.1, NP_744035.1)		25% identity (83 aa) "gluconate permease" (YP_003354819.1)		31% identity (83 aa) "carboxylesterase type B" (NP_600361.2)

Lon (784 aa)	56% identity (765 aa) "Lon protease 1" (NP_390698.1)	41-70% identity (757-763 aa) "ATP-dependent protease La" (NP_744451.1, NP_743601.1)	29% identity (104 aa) "endopeptidase La" (YP_003063372.1)	31% identity (103 aa) "hypothetical protein LLKF_2407" (YP_003354798.1)	70% identity (773 aa) "ATP-dependent Lon protease" (YP_725987.1)	25% identity (114 aa) "ATPase with chaperone activity" (NP_601235.2)
YgaH (111 aa)			31% identity (101 aa) "hypothetical protein JDM1_1611" (YP_003063195.1), 37% identity (38 aa) "hypothetical protein JDM1_0741" (YP_003062325.1)	25% identity (56 aa) "peptide deformylase" (YP_003353006.1)		
RpsA (557 aa)	29-39% identity (in stretches) "30S ribosomal protein S1 homolog" (NP_390169.1)	74% identity (554 aa) "30S ribosomal protein S1" (NP_743928.2)	32-37% identity (in stretches) "30S ribosomal protein S1" (YP_003063166.1)	29-41% identity (in stretches) "30S ribosomal protein S1" (YP_003353306.1)	67% identity (529 aa) "30S ribosomal protein S1" (YP_725313.1)	31-46% identity (in stretches) "30S ribosomal protein S1" (NP_600575.1)

So, in one aspect, there is provided a bacterial cell according to any one of the preceding aspects and embodiments, wherein each recited gene is instead (i) a gene encoding the corresponding (homolog or ortholog) protein in Table 2A or 2B above, (ii) a gene located at the corresponding locus, or (iii) both.

In particular, without being limited to theory, improved tolerance toward an aliphatic diol or other aliphatic polyol can be achieved by one or more genetic modifications which increase one or more of (a) the biosynthesis of methionine in the bacterial cell; (b) growth of the bacterial cell during polyol-induced methionine starvation, and (c) reduced efflux of precursors or intermediates required for methionine biosynthesis. This can, *e.g.*, be achieved by a reduced expression of metJ, optionally also of *relA* and *purT*, and/or one or more other genetic modifications described herein.

In one embodiment, the bacterial cell has a genetic modification which reduces the expression of one or more endogenous proteins selected from the group consisting of

- A transcriptional repressor of a methionine regulon
- A murein endopeptidase
- A cytochrome C peroxidase
- A DNA-binding transcriptional dual regulator
- A stringent starvation protein, synthesized predominantly when cells are exposed to amino acid starvation
- An ribonuclease PH
- A GDP pyrophosphokinase / GTP pyrophosphokinase
- A phosphoribosylglycinamide formyltransferase
- A permease subunit of a multidrug efflux system

- A DNA-binding transcriptional repressor
- A cyclic di-GMP phosphodiesterase.

EXAMPLE 1

Methods

5   <u>Screening for tolerance in wild-type cells</u>

Escherichia coli K-12 MG1655 was grown overnight in M9 minimal medium + 1% glucose and subcultured the following morning to an initial OD_{600} of 0.05 in M9 + 1% glucose. Cells were grown to mid-exponential phase (OD_{600} 0.7-1.0) and were back-diluted with fresh medium to an OD_{600} of 0.7. The diluted cells were used to inoculate M9 + 1% glucose containing varying
10 concentrations of diols, and growth was measured in FlowerPlates in a Biolector microbioreactor system (m2p-labs) at 37°C with 1000 rpm shaking. The culture volume in each well was 1.4 mL.

<u>Adaptive laboratory evolution of tolerant strains</u>

Based on the screening results, existence of biological production routes, and application
15 potential of different diols, two diols were selected for evolutions: 2,3-butanediol and 1,2-propanediol. *E. coli* K-12 MG1655 was grown overnight in M9 minimal medium and 150 µL was transferred the next day into 8 tubes containing 15 mL of M9 + 1% glucose + 5% (v/v) 2,3-butanediol or 1,2-propanediol on a Tecan Evo robotic platform custom-designed for performing adaptive laboratory evolutions (ALE). Cells were cultured on a 37°C heat block
20 with stirring by magnetic stir bars. Culture OD_{600} was monitored at times determined by a predictive custom script, and when the OD_{600} reached approximately 0.3, 150 µL of culture was inoculated into a new tube with the same media concentration. Instrument downtime would occasionally result in cells overgrowing to saturation or an OD_{600} greater than 0.3, and reinoculations were occasionally performed from cryogenic stocks of the population. When
25 the growth rate was observed to substantially increase, the media concentration was changed. These concentration changes were to 5.5%, 6.5%, 7%, and 8% for 1,2-propanediol and to 6.5%, 7%, and 8% for 2,3-butanediol. Approximately 100 µL of each population (8 per chemical) were plated on LB agar and incubated at 37°C overnight.

Primary screening of ALE isolates

Five colonies from wild-type K-12 MG1655 and 10 individual colonies deriving from each population were inoculated into 300 µL M9 + 1% glucose in 96 well deepwell plates and incubated in a 300 rpm plate shaker at 37°C. The next day, cells were diluted 10X in M9 +

5   1% glucose and 30 µL was transferred into clear-bottomed 96 well half-deepwell plates (with rectangular wells) containing M9 + 1% glucose and M9 + 1% glucose + 8.89% (v/v) 2,3-butanediol or 1,2-propanediol, such that the final concentration of diol was 8% (v/v). In addition, cryogenic glycerol stocks of the overnight culture were saved in a 96 well plate format. Half deepwell plates were incubated at 37°C with 225 rpm shaking in a Growth

10   Profiler (Enzyscreen), with optical scans of the plates taken at 15 minute intervals. Green pixel values integrated over a 1 mm diameter circular area in each well were converted to OD_{600} values using a previously determined calibration between OD_{600} and green pixel values. Resulting growth curves were visually inspected for isolates exhibiting the most robust or unique growth patterns within each population. In general, it was attempted to select three

15   isolates per population for further analysis, and all populations were represented in the resequenced isolates.

Secondary screening of ALE isolates

Selected isolates from the primary screen were restruck onto LB agar from the cryogenic stock made from the overnight culture plate for the primary screen. Five K-12 MG1655

20   colonies and three individual colonies from each isolate were inoculated as biological replicates into a new 96 well deepwell plate containing 300 µL of M9 + 1% glucose, and grown overnight as for the primary screen. The next day, a cryogenic stock and half deepwell plates containing M9 + 1% glucose with or without diols were inoculated using the plate of overnight cultures, and growth was measured as described for the primary screen.

25   Resulting growth curves were visually inspected for isolates exhibiting robust and reproducible growth between replicates in high concentrations of diols.

Re-sequencing of ALE isolates

A total of 20 isolates were selected from the secondary screen for whole-genome resequencing. An individual colony was taken from the LB agar plates prepared following the

30   primary screen, inoculated into 2 mL LB, and grown overnight at 37°C in a 250 rpm shaker. The following morning, 0.5 mL of cells were transferred to microcentrifuge tubes and centrifuged at 16000 x g for 2 minutes. The supernatant was removed and pellets were stored at -20°C until further processing. Genomic DNA was extracted from thawed cell pellets using a PureLink genomic DNA extraction kit, with further concentration and

purification performed by ethanol precipitation. To generate libraries for sequencing, the Illumina TruSeq Nano kit was used according to the manufacturers' directions using an input quantity of 200 ng of genomic DNA from each isolate. Sequencing was performed on an Illumina MiSeq sequencer, with a minimum 20X average genomic coverage ensured for each isolate based on the number of reads. Fastq output files were analyzed for variants compared to the K-12 MG1655 reference genome (accession number NC_000913.3) using breseq.

Construction of gene knockouts

Probable important losses-of-function were determined by identifying genes across all isolates that harbored mutations, especially those occurring in multiple populations, and by the presence of at least one mutation that either generated a premature stop codon, a frameshift mutation, or the presence of an insertion element sequence within the gene. For those genes, the corresponding knockout strain from the Keio collection of single knockout mutants (where each gene is replaced with a cassette consisting of a kanamycin resistance gene flanked by FRT sites) was used as a donor strain for P1*vir* phage transduction (Baba *et al.*, 2006). Briefly, the Keio strain was grown to early exponential phase in LB + 5 mM CaCl$_2$ and 80 μL of a P1*vir* stock raised on K-12 MG1655 was added. After significant lysis was observed after 1.5 to 2 hours, the lysate was filter-sterilized to remove cells and stored at 4°C. Strain K-12 MG1655 was grown overnight in LB + 5 mM CaCl$_2$ and 100 μL of the overnight culture was mixed with 100 μL of the P1*vir* lysate of the Keio collection mutant, and the mixture was incubated at 37°C without shaking for 20 minutes. The entire mixture was then plated on LB agar containing 1.25 mM sodium pyrophosphate as a chelating agent and 25 μg/mL kanamycin. One colony was then restruck on LB + 1.25 mM Na$_2$P$_4$O$_7$ + 25 μg/mL kanamycin plate and analyzed for presence of the Keio cassette in place of the wild-type gene by colony PCR. When further knockouts were constructed in the same strain, the Keio cassette was flipped out to generate a scar sequence such that Kan^R marker could be recycled. This was performed by transforming with pCP20, which constitutively expresses a flippase recombinase, and plating cells on LB agar + 100 μg/mL ampicillin and incubating at 30°C. The next day, one or more colonies was tested by colony PCR for loss of the Keio cassette, and successful mutants were then cured of pCP20 by elevated temperature curing at 40°C. Strains were verified to be cured of plasmid by plating on LB agar + 100 μg/mL ampicillin and incubation at 30°C. P1*vir* transductions were then performed using these mutant strains as recipients.

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Biolector growth screening of evolved isolates and reconstructed mutants

Biological triplicate cultures of each strain were grown to saturation overnight in 96 well
deepwell plates containing 300 µL M9 + 1% glucose. The next day, cells were diluted 1:10 in
deionized water in a clear 96 well plate and the OD_{600} was measured on a BioTek plate
5   reader. 48 well FlowerPlates containing a final volume of 1.4 mL of M9 + 1% glucose + 8%
(v/v) 1,2-propanediol or 7% (v/v) 2,3-butanediol were inoculated to OD_{600} 0.03 (with plate
reader pathlength, 200 µL volume) with the overnight culture and sealed with Breathseal
film. Light backscatter intensity was monitored in a Biolector microbioreactor system at 37°C
with 1000 rpm shaking. The Biolector screening concentration of 2,3-butanediol had to be
10   reduced to 7% from 8% due to lack of growth at the higher concentration. Oxygen transfer
rates are lower in the Biolector than in the Growth Profiler screening setup, resulting in
reduced aeration of cultures.

Keio collection screening for loss-of-function mutations

For primary screening, Keio collection mutants were inoculated directly from a cryogenic
15   stock of the Keio collection into 300 µL LB medium containing 25 µg/mL kanamycin in 96 well
deepwell plates and grown at 37°C with 300 rpm shaking overnight. The Keio background
strain, BW25113, was also inoculated into wells of this plate as a control. A cryogenic stock
was made from each plate, and the cryogenic stock was replica plated into another 96 well
deepwell plate containing 300 µL M9 + 1% glucose and grown overnight. The next day, cells
20   were inoculated 1:100 into clear bottomed 96 well half-deepwell plates containing M9 + 1%
glucose plus 6% and 7% 2,3-butanediol or 6% and 8% 1,2-propanediol, and cultivated in a
Growth Profiler as previously described for screening of ALE isolates.

As a secondary screen, promising Keio collection mutants were struck on LB + 25 µg/mL
kanamycin from the cryogenic stock plate prepared during primary screening above and
25   biological triplicate colonies were inoculated into a 96 well deepwell plate containing 300 µL
M9 + 1% glucose. The next day, cells were inoculated into plates for cultivation on the
Growth Profiler as described above.

Methionine supplementation

Cultures of selected strains/isolates were grown as described above for Biolector growth
30   screening of evolved isolates and reconstructed mutants. L-methionine was supplemented to
the media to a final concentration of 0.3 g/L.Generation of 2,3-butanediol production strains

The Keio collection strain containing *hsdR::kan*, JW4313, was used as the donor strain for P1*vir* phage transduction into recipient strains K-12 MG1655 and all 23BD evolved isolates as described in 'Construction of gene knockouts'. Plasmid pCP20, which encodes a constitutively expressed yeast flippase recombinase (FLP), was transformed into each P1 transduced strain

5   to remove the kanamycin resistance marker, generating the equivalent Δ*hsdR* strains. Plasmid pET-RABC was obtained from Dr. Cuiqing Ma and Dr. Chao Gao (Shandong University; Xu *et al.*, 2014). The *hsdR* deletion was found to be necessary for transformation in K-12 MG1655 due to the presence of EcoKI (HsdM/HsdR/HsdS) restriction sites in the plasmid. The plasmid was transformed into each Δ*hsdR* strain by adding the plasmid to cells

10   resuspended in TSS buffer followed by heat shocking for 30 seconds at 42°C, placing the cells on ice, resuspending in LB, and outgrowing at 37°C for 1-2 hours. The outgrown cells were plated on LB agar plates containing 50 µg/mL kanamycin to select for transformants.

2,3-butanediol production run

Individual colonies of 2,3-butanediol production strains were picked as biological replicates

15   and inoculated into 300 µL of M9 medium containing 5% (w/v) glucose, 1% (w/v) yeast extract, and 50 µg/mL kanamycin in 96-well deepwell plates with metal sandwich covers. Plates were grown overnight in a plate shaker at 37°C with 300 rpm shaking. The next morning, 22 µL of cells were inoculated into 2 mL of M9 medium containing 5% (w/v) glucose, 50 µg/mL kanamycin, and 1% (w/v) yeast extract in 24-well deepwell plates, and

20   grown in a plate shaker at 30°C and 300 rpm shaking. After 48 hours, culture supernatants were collected.

HPLC analysis of 2,3-butanediol

Culture supernatants were injected (30 µL) onto an Aminex HPX-87H ion exclusion column held at 30°C on a Dionex UltiMate HPLC system equipped with a Shodex RI-101 refractive

25   index detector held at 45°C. The mobile phase was 5 mM sulfuric acid and was kept at a constant flow rate of 0.6 mL/min. 2,3-butanediol (from a standard composed of a mixture of racemic and *meso* forms) was found to elute as two overlapping peaks. Concentrations were calculated using a standard calibration curve (linear response with R^2 = 0.9999) and adding up the areas of both peaks.

30   ## Cross-compound tolerance screening

96 well deepwell plates containing 300 µL of M9 + 1% glucose were inoculated directly from cryogenic stocks made from precultures for the secondary screening of ALE isolates and were grown overnight at 37°C with 300 rpm shaking. The next day, cells were diluted 1:100 into

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96 well half-deepwell plates containing the following final concentrations of each chemical in M9 + 1% glucose:

Butanol	1.4% v/v
Glutarate	40 g/L
p-coumarate	7.5 g/L
Putrescine	32 g/L
HMDA	32 g/L
Adipate	45 g/L
Isobutyrate	7.5 g/L
Hexanoate	3 g/L
Octanoate	8 g/L
2,3-butanediol	6% v/v
1,2-propanediol	6% v/v
sodium chloride	0.6 M

5

Plates were cultivated in a Growth Profiler for 48 hours as described for screening of ALE isolates. Green pixel integrated values from each well were converted to OD_{600} values using a calibration curve and the resulting OD_{600} vs. elapsed time data was processed using custom scripts to determine the time required for each culture to reach an OD of 1.0 (t_{OD1}). This value is a combined measure of growth rate and lag time in each culture. The median value was taken for biological triplicates of each isolate and was normalized to the median t_{OD1} for

10

K-12 MG1655 controls (5 replicates). The ratio of $t_{OD1(evolved)}/t_{OD1(wild-type)}$ is presented.

The same cultivation method as described above was also used to determine growth parameters (growth rate and lag time) in different defined concentrations of other diols, as described in the next section.

Analysis of growth parameters (growth rate and lag time)

15

For data obtained with the Biolector microbioreactor system, self-baselined growth series were imported directly into a custom software platform that automatically detects growth phases and exports growth rates and lag times. In this software, a line was fit to a detected linear region in semilog space to determine the growth rate.

20

For data obtained with the Growth Profiler, an algorithm was implemented that automatically detected the pixel integration region in each well in each image by locating the darkest pixels in each well. These values were converted to OD_{600} with a calibration run in the same manner. Growth parameters were automatically determined as described for Biolector data above, but with a newer version of the software that implemented a direct exponential fit of a detected growth phase in linear space. Additionally, the software implemented an adaptive

smoothing algorithm that split the data into variable sized windows that minimize the standard deviation of growth values within a time interval, and generated spline fits between points. Finally, the software discarded regions where growth curves were fit but the signal-to-noise ratio was less than 1, to eliminate automatic detection of false growth phases.

5   While automatic detection succeeded in detecting and fitting the dominant growth phase more than 95% of the time, all data was additionally manually curated to ensure that the main growth phase was always selected and that false growth phases were not detected when growth was essentially absent.

Results

10   Wild-type tolerance to diols

E. coli K-12 MG1655 exhibited a steadily decreasing growth rate as a function of diol concentration in general (Table 3). Toxicity appeared to depend on carbon chain length, with toxicity increasing in order of 1,2-propanediol, 2,3-butanediol, and the pentanediols. Toxicity was much greater for 1,2-pentanediol than for 1,5-pentanediol, with growth observed at

15   maximum concentrations of 1% and 3.5%, respectively. Maximum concentrations for robust growth in 2,3-butanediol and 1,2-propanediol were 5% and 7.5%, respectively.

Table 3. *Growth of K-12 MG1655 in varying concentrations of different diols (neutralized).*

diol % (v/v)	2,3-butanediol Mean		std. error		1,2-propanediol mean		std. error	
	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
0	0.988	2.3	0.079	0.2	0.701	0.9	0.019	0.1
0.5	0.944	2.2	0.144	0.6	0.701	0.9	0.042	0.2
1	0.791	1.8	0.058	0.2	0.662	0.8	0.022	0.2
2	0.754	2.1	0.065	0.4	0.595	0.8	0.021	0.4
3.5	0.498	1.6	0.060	0.8	0.442	0.5	0.024	0.4
5	0.265	-1.2	0.017	0.8	0.406	2.3	0.014	0.3
7.5	0.054	-	0.094	-	0.579	8.3	0.034	0.2
10	0.000	-	0.000	-	0.000	-	0.000	-

diol % (v/v)	1,5-pentanediol mean		std. error		1,2-pentanediol mean		std. error	
	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
0	0.699	0.8	0.009	0.0	0.701	0.9	0.019	0.1

0.5	0.613	0.3	0.003	0.1	0.524	0.4	0.025	0.3
1	0.569	0.5	0.013	0.2	0.346	1.3	0.032	0.8
2	0.437	0.9	0.003	0.1	0.000	-	0.000	-
3.5	0.158	-8.8	0.075	15.1	0.000	-	0.000	-
5	0.000	-	0.000	-	0.000	-	0.000	-
7.5	0.000	-	0.000	-	0.000	-	0.000	-
10	0.000	-	0.000	-	0.000	-	0.000	-

Based on these results and aiming for an initial growth rate of approximately 0.3-0.4 h^{-1}, it was decided to begin evolutions at a concentration of 5% (v/v) for both 2,3-butanediol and 1,2-propanediol.

5   Resequencing of tolerant isolates

Variants detected in 2,3-butanediol and 1,2-propanediol evolved isolates are presented in Tables 4 and 5. Each strain name corresponds to the chemical the strain was isolated from, the population the strain was isolated from, and the original number of the strain assigned during primary screening (*e.g.* 23BD1-6 is an 2,3-butanediol-evolved strain isolated from
10   population 1). In each table, strains are arranged such that all that were isolated from the same population are presented in the same rows. Strains with an asterisk (*) following their name are hypermutator strains, and only the mutation identified that can be associated with generating the hypermutator phenotype (mutations in *mutS*, *mutY*, or *mutL*) and those mutations that are shared with other mutations in the same gene in other strains are shown.
15   For the 1,2-propanediol populations, the majority of isolates were hypermutator strains, with the exception of 12PD4-6, 12PD6-3, and 12PD6-9. A large number of called missing coverage deletions in 12PD6-9 were likely a result of an adapter problem, and these are not considered. For mutator strains, only mutations in genes (or surrounding intergenic regions) that were common between the mutator isolates and the non-mutator isolates are listed.

20   Mutations that occur independently across multiple populations, or that appear fixed in a highly variable population, are likely causative and of highest interest. For 2,3-butanediol, these include mutations in *metJ*, *relA*, *nanK*, *purT*, *rpoB*, and *rpoC*. Mutations also occur in *acrB* in 2 populations. Of these mutations, those of *metJ*, *relA*, *purT*, and *acrB* are likely loss-of-function mutations, due to the presence of frameshift mutations, large deletions, or IS
25   element insertions in at least one population of individual isolate that possesses mutations in that gene. Other mutations are likely gain-of-function or weakening of function, for example coding mutations in genes encoding subunits of RNA polymerase (RpoB and RpoC), which are essential, and the T128S mutation in NanK, which is present in nearly every population.

For 1,2-propanediol, mutations in *metJ* were all coding, however they are also assumed to be loss-of-function mutations due to the co-occurrence of probable loss-of-function mutations for 2,3-butanediol. Because most isolates were hypermutators, SNPs are expected to be more common than other types of mutations. There were additionally mutations in *relA* in

5   most isolates, which are also presumed to be losses-of-function based on loss-of-function mutations found for 2,3-butanediol evolved isolates. Mutations in *fabR* and *yfgF* co-occurred in population 12PD6 and both were presumed to be losses-of-function due to an intergenic IS element insertion upstream of *fabR* in 12PD7-5, and an IS element insertion and large deletion in *yfgG* in 12PD6-3 (a non-mutator strain) and 12PD8-7, respectively. Mutations

10   also occurred in *clsA* across multiple mutator 12PD populations (not shown in Table 3) that appeared to have different lineages based on the mutation in the mutator gene, with 12PD3-10 having a premature stop codon in that gene (W428*).

Table 4. Variants detected in 2,3-butanediol-evolved isolates

23BD1-6

coordinate	gene	change
998193	elfA	IS5 element insertion
1347480	rnb	IS5 element insertion
1931977	purT	V366G (T→G)
2794550	gabP	W100G (T→G)
2911491	[relA][gudD]	7528 bp deletion
3369969	nanK	T128S (T→A)
4128380	metJ	G6C (C→A)
4182583	rpoB	H447Y (C→T)

23BD2-4

coordinate	gene	change
580116	ybcW/ylcI	IS5 element insertion
962056	rpsA	G21V (G→T)
1979639	insA/uspC	noncoding SNP (T→C)
2470411	gtrS	noncoding SNP (A→G)
2911491	[relA][gudD]	7528 bp deletion
3369969	nanK	T128S (T→A)
4128293	metJ	IS5 element insertion
4186152	rpoC	L268R (T→G)

23BD3-3*

coordinate	gene	change
483726	acrB	1 bp deletion
2911491	[relA][gudD]	7528 bp deletion
3369969	nanK	T128S (T→A)
4128316	metJ	V27A (A→G)
4182890	rpoB	D549G (A→G)
4184036	rpoB	V931A (T→C)
4184514	rpoB	noncoding SNP (C→T)
4398051	mutL	7 bp deletion

23BD4-3

coordinate	gene	change
1230727	hlyE/umuD	noncoding SNP (C→A)
1347775	rnb	E380* (C→A)
1879829	yeaR	IS186 element insertion
1931977	purT	V366G (T→G)
2810756	ygaH/mprA	noncoding SNP (G→T)
2913536	relA	W39* (C→T)
4031019	fadB/pepQ	noncoding SNP (G→A)
4128361	metJ	Y12C (T→C)
4187815	rpoC	15 bp deletion

23BD1-9

coordinate	gene	change
998193	elfA	IS5 element insertion
1347480	rnb	IS5 element insertion
1931977	purT	V366G (T→G)
2794550	gabP	W100G (T→G)
2911491	[relA][gudD]	7528 bp deletion
3369969	nanK	T128S (T→A)
3762316	rhsA	2905 bp deletion
4128380	metJ	G6C (C→A)
4182583	rpoB	H447Y (C→T)

23BD2-7

coordinate	gene	change
580116	ybcW/ylcI	IS5 element insertion
962056	rpsA	G21V (G→T)
1096841	ycdU/serX	IS2 element insertion
2911491	[relA][gudD]	7528 bp deletion
3369969	nanK	T128S (T→A)
4128293	metJ	IS5 element insertion
4186152	rpoC	L268R (T→G)

23BD3-4*

coordinate	gene	change
483726	acrB	1 bp deletion
2911491	[relA][gudD]	7528 bp deletion
3369969	nanK	T128S (T→A)
4128316	metJ	V27A (A→G)
4182890	rpoB	D549G (A→G)
4184036	rpoB	V931A (T→C)
4398051	mutL	7 bp deletion

23BD4-4

coordinate	gene	change
1230727	hlyE/umuD	noncoding SNP (C→A)
1879829	yeaR	IS186 element insertion
1931977	purT	V366G (T→G)
2913536	relA	W39* (C→T)
4128361	metJ	Y12C (T→C)
4187815	rpoC	15 bp deletion

23BD2-9

coordinate	gene	change
580116	ybcW/ylcI	IS5 element insertion
962056	rpsA	G21V (G→T)
2911491	[relA][gudD]	7528 bp deletion
3369969	nanK	T128S (T→A)
4128293	metJ	IS5 element insertion
4186152	rpoC	L268R (T→G)

23BD3-9*

coordinate	gene	change
483726	acrB	1 bp deletion
2795142	gabP	V297A (T→C)
2911491	[relA][gudD]	7528 bp deletion
3369969	nanK	T128S (T→A)
4128316	metJ	V27A (A→G)
4182890	rpoB	D549G (A→G)
4184036	rpoB	V931A (T→C)
4398051	mutL	7 bp deletion

23BD4-7

coordinate	gene	change
2911491	[relA][gudD]	7528 bp deletion
3369969	nanK	T128S (T→A)
4128169	metJ	D76A (T→G)
4186274	rpoC	N309Y (A→T)

Column group 1

coordinate	gene	change
23BD5-1		
1230727	hlyE/umuD	noncoding SNP (C→A)
1879829	yeaR	IS186 element insertion
1931977	purT	V366G (T→G)
2913536	relA	W39* (C→T)
3823036	spoT	I213L (A→C)
4128361	metJ	Y12C (T→C)
4187815	rpoC	15 bp deletion
23BD6-1		
575786	[nmpC][borD]	3027 bp deletion
1930993	purT	V38G (T→G)
2912611	relA	10 bp deletion
4128250	metJ	L49R (A→C)
4178172	nusG	F144V (T→G)
4185573	rpoC	Y75C (A→G)
23BD7-4		
998719	elfD	IS2 element insertion
1931668	purT	L263W (T→G)
2073463	flu	L642Q (T→A)
2911491	[relA][gudD]	7528 bp deletion
3178128	tolC	L5R (T→G)
3369969	nanK	T128S (T→A)
3668878	yhjA	IS2 element insertion
4128386	metJ	W4G (A→C)
4186152	rpoC	L268R (T→G)
4466841	treR	IS5 element insertion
23BD8-2		
461034	lon	I716S (T→G)
2661816	iscR	T106P (T→G)
2810459	ygaH	V39A (T→C)
2913641	relA	V4E (A→T)
3815809	pyrE/rph	1 bp deletion
4184579	rpoB	I1112S (T→G)

Column group 2

coordinate	gene	change
23BD5-7		
679090	ybeT	L106P (A→G)
824028	ybhP	L157P (A→G)
1722386	ydhK	T89M (C→T)
2911491	[relA][gudD]	7528 bp deletion
3369969	nanK	T128S (T→A)
3438773	zntR	S120R (T→G)
4128379	metJ	G6D (C→T)
4186274	rpoC	N309Y (A→T)
23BD7-5		
484098	acrB	2 bp insertion (→AT)
998719	elfD	IS2 element insertion
1931499	purT	IS5 element insertion
2073463	flu	L642Q (T→A)
2911491	[relA][gudD]	7528 bp deletion
3369969	nanK	T128S (T→A)
4128386	metJ	W4G (A→C)
4186152	rpoC	L268R (T→G)
23BD8-7		
365741	lacZ	1 bp deletion
2661816	iscR	T106P (T→G)
2810459	ygaH	V39A (T→C)
2913641	relA	V4E (A→T)
3815809	pyrE/rph	1 bp deletion
4128212	metJ	F62L (A→G)
4184579	rpoB	I1112S (T→G)

Column group 3

coordinate	gene	change
23BD5-10		
2911491	[relA][gudD]	7528 bp deletion
3369969	nanK	T128S (T→A)
4128169	metJ	D76A (T→G)
4186274	rpoC	N309Y (A→T)
23BD7-7		
998719	elfD	IS2 element insertion
1413735	rcbA	Y2* (A→T)
1931668	purT	L263W (T→G)
2073463	flu	L642Q (T→A)
2911491	[relA][gudD]	7528 bp deletion
3178128	tolC	L5R (T→G)
3369969	nanK	T128S (T→A)
3668878	yhjA	IS2 element insertion
4128386	metJ	W4G (A→C)
4186152	rpoC	L268R (T→G)
4466841	treR	IS5 element insertion

Table 5. *Variants detected in 1,2-propanediol-evolved isolates.*

12PD1-2*

coordinate	gene	change
2406631	lrhA	A4V (G→A)
2780330	ypjA	noncoding SNP (G→A)
2912885	relA	I256T (A→G)
3377215	sspA	L69P (A→G)
3440116	rpoA	D305G (T→C)
3815801	pyrE/rph	1 bp deletion
4128316	metJ	V27A (A→G)
4399151	mutL	1 bp deletion

12PD2-8*

coordinate	gene	change
2780330	ypjA	noncoding SNP (G→A)
2912885	relA	I256T (A→G)
3377215	sspA	L69P (A→G)
3440116	rpoA	D305G (T→C)
3815801	pyrE/rph	1 bp deletion
4128316	metJ	V27A (A→G)
4399151	mutL	1 bp deletion

12PD3-7*

coordinate	gene	change
2780330	ypjA	noncoding SNP (G→A)
2912885	relA	I256T (A→G)
3377215	sspA	L69P (A→G)
3440116	rpoA	D305G (T→C)
3815801	pyrE/rph	1 bp deletion
4128316	metJ	V27A (A→G)
4399151	mutL	1 bp deletion

12PD4-6

coordinate	gene	change
962923	rpsA	D310V (A→T)
1879829	yeaR	IS186 element insertion
2912634	relA	T340P (T→G)
3377240	sspA	T61P (T→G)
4128078	metJ	*106Y (T→G)

12PD1-4*

coordinate	gene	change
2406631	lrhA	A4V (G→A)
2780330	ypjA	noncoding SNP (G→A)
2912885	relA	I256T (A→G)
3377215	sspA	L69P (A→G)
3440116	rpoA	D305G (T→C)
3815801	pyrE/rph	1 bp deletion
4128316	metJ	V27A (A→G)
4399151	mutL	1 bp deletion

12PD2-9*

coordinate	gene	change
2780330	ypjA	noncoding SNP (G→A)
2912885	relA	I256T (A→G)
3377215	sspA	L69P (A→G)
3440116	rpoA	D305G (T→C)
3815801	pyrE/rph	1 bp deletion
4128316	metJ	V27A (A→G)
4399151	mutL	1 bp deletion

12PD3-8*

coordinate	gene	change
2780330	ypjA	noncoding SNP (G→A)
2912885	relA	I256T (A→G)
3377215	sspA	L69P (A→G)
3440116	rpoA	D305G (T→C)
3815801	pyrE/rph	1 bp deletion
4128316	metJ	V27A (A→G)
4399151	mutL	1 bp deletion

12PD4-8*

coordinate	gene	change
1879829	yeaR	IS186 element insertion
2858929	mutS	T613P (A→C)
2913035	relA	L206P (A→G)
2913196	relA	noncoding SNP (T→C)
3815801	pyrE/rph	1 bp deletion
4128197	metJ	T67A (T→C)

12PD1-10*

coordinate	gene	change
2780330	ypjA	noncoding SNP (G→A)
2912885	relA	I256T (A→G)
3377215	sspA	L69P (A→G)
3440116	rpoA	D305G (T→C)
4128316	metJ	V27A (A→G)
3815801	pyrE/rph	1 bp deletion
4399151	mutL	1 bp deletion

12PD3-10*

coordinate	gene	change
2912357	relA	H432R (T→C)
3377215	sspA	L69P (A→G)
3377215	sspA	L69P (A→G)
4128247	metJ	R50H (C→T)
3815801	pyrE/rph	1 bp deletion
4399151	mutL	1 bp deletion

12PD4-9*

coordinate	gene	change
2405949	lrhA	noncoding SNP (G→A)
2858929	mutS	T613P (A→C)
2913035	relA	L206P (A→G)
2913196	relA	noncoding SNP (T→C)
3815801	pyrE/rph	1 bp deletion
4128197	metJ	T67A (T→C)

Column group 1

coordinate	gene	change
12PD5-1*		
2780291	ypjA	noncoding SNP (G→A)
3377215	sspA	L69P (A→G)
3815801	pyrE/rph	1 bp deletion
4128316	metJ	V27A (A→G)
4161466	fabR	G115S (G→A)
4399151	mutL	1 bp deletion
12PD6-3		
2406831	lrhA/alaA	IS2 element insertion
2628616	yfgF	IS2 element insertion
3440194	rpoA	G279V (C→A)
4035240	rrsA	1768 bp deletion
4161155	fabR	T11N (C→A)
4208083	sE/gltV/rrlE/rrj	5021 bp deletion
4261586	yjbM/dusA	noncoding SNP (A→C)
12PD7-5*		
2913035	relA	L206P (A→G)
2913196	relA	noncoding SNP (T→C)
3815801	pyrE/rph	1 bp deletion
4128316	metJ	V27A (A→G)
4160984	sthA/fabR	IS2 element insertion
4161391	fabR	T90A (A→G)
12PD8-6*		
2912885	relA	I256T (A→G)
3376927	sspA	L165P (A→G)
3815801	pyrE/rph	1 bp deletion
3816407	rph	noncoding SNP (G→A)
4128197	metJ	T67A (T→C)

Column group 2

coordinate	gene	change
12PD5-3*		
2780291	ypjA	noncoding SNP (G→A)
3377215	sspA	L69P (A→G)
3815801	pyrE/rph	1 bp deletion
4128316	metJ	V27A (A→G)
4161466	fabR	G115S (G→A)
4399151	mutL	1 bp deletion
12PD6-9		
2628621	yfgF	62 bp deletion
2780609	ypjA	noncoding SNP (A→C)
2780609	ypjA	noncoding SNP (A→C)
3440194	rpoA	G279V (C→A)
4161155	fabR	T11N (C→A)
12PD7-6*		
1879829	yearR	IS186 element insertion
2913035	relA	L206P (A→G)
2913196	relA	noncoding SNP (T→C)
3815801	pyrE/rph	1 bp deletion
3815521	pyrE	V83A (A→G)
4128247	metJ	R50H (C→T)
12PD8-7*		
2406923	lrhA/alaA	noncoding SNP (T→C)
2628850	yfgF	409 bp deletion
2912885	relA	I256T (A→G)
3376927	sspA	L165P (A→G)
3815801	pyrE/rph	1 bp deletion
3816407	rph	1 bp deletion
4128197	metJ	noncoding SNP (G→A)

Column group 3

coordinate	gene	change
12PD8-10*		
2912885	relA	I256T (A→G)
3376927	sspA	L165P (A→G)
3815801	pyrE/rph	1 bp deletion
3816407	rph	noncoding SNP (G→A)
4128197	metJ	T67A (T→C)

Characterization of selected isolates

Each re-sequenced isolate was characterized using the Biolector system for growth in M9
media containing 7% (v/v) 2,3-butanediol or 8% (v/v) 1,2-propanediol in biological
triplicates. Tables showing the calculated average growth rates and lag times for each isolate
5   of each detected phase (using custom automated growth parameter determination software)
are shown in Table 6 for 2,3-butanediol, and Table 7 for 1,2-propanediol. Standard errors
are standard deviations about the mean of the growth rate and lag time for the three
independent biological replicates. In the presence of diols, many strains exhibited diauxic or
triauxic growth patterns, manifesting in the presence of multiple growth phases. A value is
10   only shown for second and third phases if two or more replicates had a growth phase
detected, and that value is the average of the parameters calculated for those determined
growth phases.

Large differences in growth behavior amongst evolved isolates can be noted. Better growing
strains are defined by both the slope of the curve (higher growth rate) and at what time the
15   cultures begin growing (reduced lag time). Wild-type K-12 MG1655 did not grow in 7% 2,3-
butanediol within 48 hours in 2 out of 3 biological replicates (the remaining biological
replicate had a growth rate of 0.32 h^{-1} with a 28.3 h lag time). All other isolates grew
robustly but with a variety of lag times.

Table 6. *Growth rates and lag times of re-sequenced 2,3-butanediol evolved isolates in M9*
20   *+ 7% (v/v) 2,3-butanediol.*

| | phase 1 | | | | phase 2 | | | |
| | mean | | std. error | | Mean | | std. error | |
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	-	-	-	-	-	-	-	-
23BD1-6	0.258	4.4	0.037	0.7	0.544	13.8	0.109	0.4
23BD1-9	0.297	5.2	0.033	1.9	0.547	13.9	0.063	0.3
23BD2-4	0.464	6.5	0.014	0.5	0.515	15.0	0.139	3.6
23BD2-7	0.543	7.7	0.025	0.4	0.409	14.2	0.023	0.4
23BD2-9	0.476	14.0	0.091	0.7	0.326	19.4	0.036	11.4
23BD3-3	0.430	12.9	0.062	2.7	0.371	21.1	0.119	13.8
23BD3-4	0.471	6.8	0.081	1.4	-	-	-	-
23BD3-9	0.379	12.2	0.069	1.2	-	-	-	-
23BD4-3	0.386	9.4	0.105	0.6	0.495	14.3	0.041	0.9
23BD4-4	0.393	21.4	0.043	0.7	0.554	37.2	0.176	1.3
23BD4-7	0.411	17.3	0.072	1.1	0.592	24.6	0.042	11.7

23BD5-1	0.597	8.9	0.054	1.8	0.944	16.7	0.209	0.4
23BD5-7	0.244	4.2	0.049	0.7	0.667	17.0	0.180	0.6
23BD5-10	0.389	9.0	0.050	0.3	0.473	13.5	0.202	5.5
23BD6-1	0.351	6.0	0.014	1.2	-	-	-	-
23BD7-4	0.471	4.9	0.016	0.4	-	-	-	-
23BD7-5	0.475	5.3	0.051	0.7	0.687	14.3	0.258	1.5
23BD7-7	0.379	2.9	0.037	0.8	-	-	-	-
23BD8-2	0.417	12.2	0.058	1.5	0.285	10.0	0.249	6.1
23BD8-7	0.507	6.1	0.051	1.4	-	-	-	-

	phase 3			
	Mean		std. error	
Strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	-	-	-	-
23BD1-6	-	-	-	-
23BD1-9	-	-	-	-
23BD2-4	0.396	12.5	0.325	8.3
23BD2-7	0.603	19.6	0.119	1.2
23BD2-9	0.678	20.1	0.135	0.9
23BD3-3	0.478	18.2	-	-
23BD3-4	-	-	-	-
23BD3-9	-	-	-	-
23BD4-3	-	-	-	-
23BD4-4	-	-	-	-
23BD4-7	-	-	-	-
23BD5-1	-	-	-	-
23BD5-7	-	-	-	-
23BD5-10	0.382	12.6	0.386	4.9
23BD6-1	-	-	-	-
23BD7-4	-	-	-	-
23BD7-5	-	-	-	-
23BD7-7	-	-	-	-
23BD8-2	-	-	-	-
23BD8-7	-	-	-	-

Table 7. *Growth rates and lag times of re-sequenced 1,2-propanediol evolved isolates in M9 + 8% (v/v) 1,2-propanediol.*

phase 1		phase 2	
Mean	std. error	mean	std. error

strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0.352	6.2	0.078	1.5	0.336	10.9	0.071	1.1
12PD1-2	0.703	5.0	0.169	1.8	-	-	-	-
12PD1-4	0.692	6.1	0.085	5.1	1.069	5.6	0.069	0.5
12PD1-10	0.754	3.0	0.030	0.2	-	-	-	-
12PD2-8	0.598	9.3	0.138	5.0	-	-	-	-
12PD2-9	0.328	5.8	0.005	1.1	-	-	-	-
12PD3-7	0.584	6.7	0.039	1.5	-	-	-	-
12PD3-8	0.588	3.9	0.060	1.2	0.801	11.6	0.268	1.7
12PD3-10	0.532	3.9	0.054	0.6	0.468	9.7	0.175	1.2
12PD4-6	0.749	6.7	0.209	3.8	-	-	-	-
12PD4-8	0.566	3.5	0.073	0.3	-	-	-	-
12PD4-9	0.677	4.2	0.062	0.3	-	-	-	-
12PD5-1	0.668	1.9	0.013	0.1	-	-	-	-
12PD5-3	0.499	1.5	0.150	4.5	-	-	-	-
12PD6-3	0.432	0.5	0.023	0.5	0.351	2.9	0.329	11.7
12PD6-9	0.546	1.3	0.146	0.4	-	-	-	-
12PD7-5	0.629	5.3	0.234	5.8	-	-	-	-
12PD7-6	0.737	2.7	0.060	0.6	0.376	8.0	0.106	1.4
12PD8-6	0.589	6.0	0.007	2.9	-	-	-	-
12PD8-7	0.502	5.0	0.025	2.5	-	-	-	-
12PD8-10	0.455	1.6	0.089	0.2	-	-	-	-

Knockout strain growth performance

Probable loss-of-function mutations were identified from re-sequencing results as described in methods and the section on resequencing of selected isolates. Initially, single gene knockouts of *metJ*, *relA*, *purT*, *fabR*, *clsA*, *yfgF*, *treA*, and *acrB* were constructed and tested with a selection of evolved isolates in 7% (v/v) 2,3-butanediol (Table 8) or 8% (v/v) 1,2-propanediol (Table 9). The wild-type strain and the majority of single knockout strains did not grow in 7% 2,3-butanediol. Only the *metJ* knockout, and to a much lesser extent the *purT* knockout, exhibited detectable growth phases. For growth in 8% 1,2-propanediol, only the *metJ* knockout and the *acrB* knockout (with more variability) exhibited primary growth phases with higher growth rates than wild-type K-12 MG1655.

Because *metJ* losses-of-function always co-occurred with probable *relA* losses-of-function in nearly every resequenced evolved isolate, and most other apparent loss-of-function mutations co-occurred with other mutations, double knockouts were next tested and screened in the Biolector test format for co-occurring combinations (Table 10). For growth in 2,3-butanediol, the only double knockout with improved growth over K-12 MG1655

metJ::kan was K-12 MG1655 Δ*metJ acrB::kan*. Other knockout combinations with *metJ* exhibited abolished growth relative to the *metJ* knockout alone.

The same double gene knockouts were also tested with 8% 1,2-propanediol (Table 11). In contrast to growth in 2,3-butanediol, K-12 Δ*metJ acrB::kan* did not exhibit improved growth
5 relative to the single knockout K-12 *metJ::kan*, nor did any other knockout combination with *metJ*. K-12 Δ*fabR yfgF::kan* exhibited an increased growth rate in the primary growth phase and reduced lag times relative to the *fabR* and *yfgF* single deletion strains, as well as an increased secondary growth phase (which was present but not automatically detected for at least 2 out of 3 replicates for the *fabR* and *yfgF* single deletion strains). It also had a reduced
10 lag time relative to K-12 MG1655 and a higher secondary phase growth rate than K-12 MG1655.

Finally, triple gene deletions were also constructed and tested with 7% 2,3-butanediol (Table 12) and 8% 1,2-propanediol (Table 13), with the single knockout strain K-12 MG1655 *acrB::kan* also added. Additional genes were also tested in combination with deletions in
15 *metJ* and *relA*, including *rnb* (co-occurring mutations in population 23BD1 and 23BD4-3), *treR* (co-occurring mutations in 23BD7-4 and 23BD7-7), and *yeaR* (co-occurring mutations in 23BD4-3, 23BD4-4, and 23BD5-1).

Table 8. *Growth rates and lag times of single gene knockouts in M9 + 7% (v/v) 2,3-butanediol as measured in the Biolector testing format.*

| strain | phase 1 | | | | phase 2 | | | |
| | Mean | | std. error | | mean | | std. error | |
	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	-	-	-	-	-	-	-	-
23BD2-4	0.501	7.0	0.035	0.5	0.323	12.6	0.006	0.6
23BD4-3	0.341	6.5	0.027	0.8	0.589	15.2	0.027	0.2
23BD6-1	0.367	6.0	0.014	0.5	-	-	-	-
23BD7-4	0.478	4.7	0.024	0.3	-	-	-	-
MG1655 metJ::kan	0.162	4.9	0.017	2.4	-	-	-	-
MG1655 relA::kan	-	-	-	-	-	-	-	-
MG1655 purT::kan	0.093	11.5	0.017	3.6	-	-	-	-
MG1655 fabR::kan	-	-	-	-	-	-	-	-
MG1655 clsA::kan	-	-	-	-	-	-	-	-
MG1655 yfgF::kan	-	-	-	-	-	-	-	-
MG1655 treA::kan	-	-	-	-	-	-	-	-
MG1655 acrB::kan	-	-	-	-	-	-	-	-

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Table 9. *Growth rates and lag times of single gene knockouts in M9 + 8% (v/v) 1,2-propanediol as measured in the Biolector testing format.*

| | phase 1 | | | | phase 2 | | | |
| | mean | | std. error | | mean | | std. error | |
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0.271	8.5	0.030	4.0	0.279	11.6	0.018	0.5
12PD3-8	0.594	3.1	0.037	2.4	0.312	5.5	0.053	2.9
12PD4-6	0.531	14.6	0.012	3.7	0.309	12.4	0.290	8.3
12PD6-3	0.436	1.0	0.012	0.2	0.098	-15.0	0.038	13.5
12PD7-6	0.834	4.1	0.010	0.2	0.543	10.9	0.112	1.0
MG1655 metJ::kan	0.346	4.1	0.030	0.7	0.667	13.6	0.079	0.5
MG1655 relA::kan	0.150	3.4	0.014	2.5	-	-	-	-
MG1655 purT::kan	0.245	3.9	0.054	1.9	0.264	1.3	0.256	12.1
MG1655 fabR::kan	0.133	-3.4	0.036	10.1	0.452	13.3	0.185	3.3
MG1655 clsA::kan	0.186	2.9	0.039	1.6	-	-	-	-
MG1655 yfgF::kan	0.258	3.4	0.011	0.9	0.477	12.0	0.068	0.7
MG1655 treA::kan	0.295	5.0	0.083	2.9	0.420	12.4	0.066	1.3
MG1655 acrB::kan	0.369	4.9	0.196	1.2	0.373	11.1	0.159	1.7

Table 10. *Growth rates and lag times of single and double gene knockouts in M9 + 7% (v/v) 2,3-butanediol as measured in the Biolector testing format.*

| | phase 1 | | | | phase 2 | | | |
| | mean | | std. error | | mean | | std. error | |
Strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	-	-	-	-	-	-	-	-
23BD2-4	0.396	6.2	0.026	0.7	0.465	14.4	0.127	1.4
23BD4-3	0.300	5.4	0.097	2.7	0.605	15.5	0.064	0.1
23BD6-1	0.264	9.6	0.005	1.3	-	-	-	-
23BD7-4	0.492	6.6	0.045	0.3	-	-	-	-
MG1655 metJ::kan	*	*	*	*	-	-	-	-
MG1655 relA::kan	-	-	-	-	-	-	-	-
MG1655 purT::kan	-	-	-	-	-	-	-	-
MG1655 fabR::kan	-	-	-	-	-	-	-	-
MG1655 yfgF::kan	-	-	-	-	-	-	-	-
MG1655 ΔmetJ relA::kan	-	-	-	-	-	-	-	-

MG1655 ΔmetJ purT::kan	-	-	-	-	-	-	-	-
MG1655 ΔrelA purT::kan	-	-	-	-	-	-	-	-
MG1655 ΔmetJ acrB::kan	0.254	9.5	0.035	0.2	-	-	-	-
MG1655 ΔfabR yfgF::kan	-	-	-	-	-	-	-	-

* growth was apparent but phase not detected in 2 out of 3 replicates

Table 11. *Growth rates and lag times of single and double gene knockouts in M9 + 8% (v/v) 1,2-propanediol as measured in the Biolector testing format.*

	phase 1				phase 2			
	mean		std. error		mean		std. error	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0.323	11.7	0.092	1.4	0.256	17.1	0.121	4.8
12PD3-8	0.556	7.9	0.020	2.7	0.314	11.9	0.018	3.1
12PD4-6	0.482	20.4	0.040	0.8	0.123	12.1	0.017	1.1
12PD6-3	0.399	0.2	0.025	0.8	0.128	-1.0	0.022	5.4
12PD7-6	0.761	3.5	0.149	0.5	0.475	8.2	0.236	3.6
MG1655 metJ::kan	0.314	4.8	0.022	1.0	0.483	12.7	0.092	0.7
MG1655 relA::kan	-	-	-	-	-	-	-	-
MG1655 purT::kan	0.381	11.8	0.036	0.9	-	-	-	-
MG1655 fabR::kan	0.229	13.5	0.030	2.0	-	-	-	-
MG1655 yfgF::kan	0.260	12.5	0.039	2.3	-	-	-	-
MG1655 ΔmetJ relA::kan	-	-	-	-	-	-	-	-
MG1655 ΔmetJ purT::kan	0.275	7.3	0.070	5.8	0.304	10.3	0.002	0.1
MG1655 ΔrelA purT::kan	0.156	6.2	0.046	5.9	-	-	-	-
MG1655 ΔmetJ acrB::kan	0.196	5.2	0.016	0.9	0.221	9.8	0.053	3.5
MG1655 ΔfabR yfgF::kan	0.334	5.8	0.030	0.2	0.342	11.7	0.047	0.5

Table 12. *Growth rates and lag times of selected single, double, and triple gene knockouts in M9 + 7% (v/v) 2,3-butanediol as measured in the Biolector testing format.*

	phase 2				phase 3			
	mean		std. error		Mean		std. error	
Strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	-	-	-	-	-	-	-	-
23BD2-4	0.495	14.4	0.049	0.4	0.618	17.6	0.030	0.7

Strain								
23BD4-3	0.831	14.3	0.070	0.2	-	-	-	-
23BD6-1	-	-	-	-	-	-	-	-
23BD7-4	-	-	-	-	-	-	-	-
MG1655 metJ::kan	-	-	-	-	-	-	-	-
MG1655 acrB::kan	-	-	-	-	-	-	-	-
MG1655 ΔmetJ acrB::kan	-	-	-	-	-	-	-	-
MG1655 ΔmetJ ΔrelA acrB::kan	-	-	-	-	-	-	-	-
MG1655 ΔmetJ ΔrelA purT::kan	0.244	12.0	0.006	0.5	0.395	31.4	0.036	0.2
MG1655 ΔmetJ ΔrelA clsA::kan	-	-	-	-	-	-	-	-
MG1655 ΔmetJ ΔrelA rnb::kan	-	-	-	-	-	-	-	-
MG1655 ΔmetJ ΔrelA yeaR::kan	-	-	-	-	-	-	-	-
MG1655 ΔmetJ ΔrelA treR::kan	-	-	-	-	-	-	-	-
MG1655 ΔmetJ ΔrelA treA::kan	-	-	-	-	-	-	-	-

	phase 3			
	Mean		std. error	
Strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	-	-	-	-
23BD2-4	0.618	17.6	0.030	0.7
23BD4-3	-	-	-	-
23BD6-1	-	-	-	-
23BD7-4	-	-	-	-
MG1655 metJ::kan	-	-	-	-
MG1655 acrB::kan	-	-	-	-
MG1655 ΔmetJ acrB::kan	-	-	-	-
MG1655 ΔmetJ ΔrelA acrB::kan	-	-	-	-
MG1655 ΔmetJ ΔrelA purT::kan	0.395	31.4	0.036	0.2
MG1655 ΔmetJ ΔrelA clsA::kan	-	-	-	-
MG1655 ΔmetJ ΔrelA rnb::kan	-	-	-	-
MG1655 ΔmetJ ΔrelA yeaR::kan	-	-	-	-
MG1655 ΔmetJ ΔrelA treR::kan	-	-	-	-
MG1655 ΔmetJ ΔrelA treA::kan	-	-	-	-

For 2,3-butanediol, it was found that K-12 ΔmetJ ΔrelA purT::kan had an increased growth rate vs. K-12 metJ::kan, indicating a positive epistatis between these three loss-of-function mutations. The acrB single knockout strain did not have a detectable growth phase, also demonstrating that metJ and acrB losses-of-function have a synergetic effect when combined. None of the other triple knockout strains exhibited a detectable growth phase in at least 2 out of 3 replicates, with substantially lower growth than K-12 metJ::kan observed.

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For 1,2-propanediol, K-12 Δ*metJ* Δ*relA purT::kan* had a higher average growth rate than K-12 *metJ::kan* alone although with higher variability (individual replicates had growth rates of 0.39, 0.64, and 0.32 h^{-1}). The K-12 Δ*metJ acrB::kan* did not have an increased growth rate over K-12 *metJ::kan* alone (again), and no other triple knockout combination with *metJ* and

5   *relA* exhibited a higher growth rate than K-12 *metJ::kan*.

The Keio collection of gene knockouts is a commercially available collection of knockouts in nearly all non-essential genes and ORFs in *E. coli* strain BW25113. This strain is a K-12 derivative and possesses known mutations relative to the K-12 MG1655 background. All Keio collection strains with knockouts in genes that were found to be mutated in Tables 4 and 5

10   were screened for growth against the BW25113 control in M9 + 1% glucose + 6% (Table 14) or 7% (v/v) 2,3-butanediol, and 6% or 8% (v/v) 1,2-propanediol (Table 15). In 6% 2,3-butanediol, the *yhjA*, *rzpD*, *ycdU*, *iscR*, and *gtrS* knockout strains exhibited improved growth rates compared to the wild-type. In 7% 2,3-butanediol, growth was minimal and it was not possible to automatically calculate growth parameters for any strain except BW25113

15   *rzpD::kan*, which exhibited a growth rate of 0.065 h^{-1}. This strain also visually exhibited the strongest growth in this condition. Other strains which qualitatively had improved growth over K-12 MG1655 were the same as those with higher growth rates in 6% 2,3-butanediol, minus K-12 *iscR::kan*. Corresponding knockouts in K-12 MG1655 remain to be tested in the Biolector.

20   **Table 13.** *Growth rates and lag times of selected single, double, and triple gene knockouts in M9 + 8% (v/v) 1,2-propanediol as measured in the Biolector testing format.*

| | phase 1 | | | | phase 2 | | | |
| | mean | | std. error | | mean | | std. error | |
Strain	μ (h^{-1})	t$_{lag}$ (h)	μ (h^{-1})	t$_{lag}$ (h)	μ (h^{-1})	t$_{lag}$ (h)	μ (h^{-1})	t$_{lag}$ (h)
MG1655	0.236	1.0	0.067	3.3	0.578	11.5	0.057	0.4
12PD3-8	0.661	1.4	0.130	0.5	0.389	3.3	0.242	4.9
12PD4-6	0.915	3.7	0.006	0.2	0.832	5.7	0.012	0.2
12PD6-3	0.409	-0.8	0.055	1.4	0.127	-0.2	0.016	3.6
12PD7-6	0.849	3.6	0.017	0.2	0.468	9.7	0.087	0.6
MG1655 metJ::kan	0.328	3.3	0.038	1.2	0.704	13.1	0.042	0.3
MG1655 acrB::kan	0.296	2.1	0.015	1.1	0.584	9.9	0.095	0.3
MG1655 ΔmetJ acrB::kan	0.342	4.0	0.005	0.8	0.631	13.2	0.084	0.4
MG1655 ΔmetJ ΔrelA acrB::kan	0.198	26.9	0.028	11.0	-	-	-	-
MG1655 ΔmetJ ΔrelA purT::kan	0.452	10.4	0.169	1.5	-	-	-	-
MG1655 ΔmetJ ΔrelA clsA::kan	0.259	7.0	0.081	3.6	-	-	-	-
MG1655 ΔmetJ ΔrelA rnb::kan	0.208	19.4	0.081	5.0	-	-	-	-

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MG1655 ΔmetJ ΔrelA yeaR::kan	0.163	18.6	0.100	3.3	-	-	-	-
MG1655 ΔmetJ ΔrelA treR::kan	0.222	14.4	0.033	1.0	-	-	-	-
MG1655 ΔmetJ ΔrelA treA::kan	0.224	11.7	0.098	9.6	-	-	-	-

	phase 3			
	Mean		std. error	
Strain	μ (h^{-1})	t$_{lag}$ (h)	μ (h^{-1})	t$_{lag}$ (h)
MG1655	-	-	-	-
12PD3-8	-	-	-	-
12PD4-6	0.218	7.3	0.033	2.8
12PD6-3	-	-	-	-
12PD7-6	-	-	-	-
MG1655 metJ::kan	0.121	11.1	0.030	9.0
MG1655 acrB::kan	-	-	-	-
MG1655 ΔmetJ acrB::kan	0.125	15.5	0.005	1.1
MG1655 ΔmetJ ΔrelA acrB::kan	-	-	-	-
MG1655 ΔmetJ ΔrelA purT::kan	-	-	-	-
MG1655 ΔmetJ ΔrelA clsA::kan	-	-	-	-
MG1655 ΔmetJ ΔrelA rnb::kan	-	-	-	-
MG1655 ΔmetJ ΔrelA yeaR::kan	-	-	-	-
MG1655 ΔmetJ ΔrelA treR::kan	-	-	-	-
MG1655 ΔmetJ ΔrelA treA::kan	-	-	-	-

Table 14. *Growth rates and lag times of Keio collection knockouts in M9 + 6% (v/v) 2,3-butanediol as measured in the Growth Profiler testing format. The growth of BW25113 nanK::kan was too low for automatic calculation.*

	Mean		std. error	
Strain	μ (h^{-1})	t$_{lag}$ (h)	μ (h^{-1})	t$_{lag}$ (h)
BW25113	0.191	6.1	0.014	0.5
BW25113 fadB::kan	0.225	5.6	0.031	0.6
BW25113 ybhP::kan	0.108	20.3	0.008	0.4
BW25113 yhjA::kan	0.295	8.0	0.025	1.9
BW25113 ybeT::kan	0.166	16.3	0.003	5.3
BW25113 rhsA::kan	0.169	10.5	0.029	3.2
BW25113 rzpD::kan	0.261	5.8	0.031	1.5
BW25113 nanK::kan	ND	-	-	-
BW25113 ycdU::kan	0.285	4.0	0.020	1.4
BW25113 iscR::kan	0.240	3.7	0.008	0.7
BW25113 gtrS::kan	0.250	2.0	0.007	2.4

For Keio mutants tested in 6% and 8% 1,2-propanediol (Table 13), only minor growth differences were observed, with the *sspA* knockout strain, and to a lesser extent the *rph* knockout strain (8% 1,2-propanediol only) exhibiting increased growth rates over wild-type BW25113. Corresponding knockouts in K-12 MG1655 are to be tested in the Biolector.

5   **Table 15.** *Growth rates of Keio collection knockouts in M9 + 6% and 8% (v/v) 1,2-propanediol as measured in the Growth Profiler testing format.*

strain	6% 1,2-propanediol μ (h^{-1})	std. error	8% 1,2-propanediol μ (h^{-1})	std. error
BW25113	0.498	0.008	0.289	0.011
BW25113 frdA::kan	0.474	0.007	0.250	0.054
BW25113 yfgF::kan	0.405	0.055	0.173	0.021
BW25113 ade::kan	0.508	0.037	0.271	0.028
BW25113 dusA::kan	0.516	0.035	0.303	0.017
BW25113 yagE::kan	0.399	0.024	0.253	0.016
BW25113 ecpC::kan	0.378	0.037	0.138	0.029
BW25113 yraQ::kan	0.423	0.014	0.229	0.003
BW25113 sspA::kan	0.462	0.042	0.346	0.016
BW25113 rph::kan	0.438	0.030	0.313	0.008
BW25113 ycdU::kan	0.389	0.029	0.287	0.006
BW25113 ypjA::kan	0.381	0.001	0.260	0.007

Tabular summaries of knockout strains exhibiting improved growth in 2,3-butanediol and 1,2-propanediol as compared to the wild-type strain are shown in Tables 16 and 17

10   **Table 16:** *Summary of knockout strains with improved growth over the wild-type strain in 2,3-butanediol*

	Growth rate effect vs. K-12 MG1655 or BW25113	
Strain genotype	**6% 2,3-butanediol**	**7% 2,3-butanediol**
K-12 MG1655 *metJ::kan*	not tested	moderate increase
K-12 MG1655 Δ*metJ acrB::kan*	not tested	large increase
K-12 MG1655 Δ*metJ* Δ*relA purT::kan*	not tested	large increase
BW25113 *rzpD::kan*	small increase	moderate increase
BW25113 *yhjA::kan*	small increase	small increase
BW25113 *gtrS::kan*	small increase	small increase
BW25113 *ycdU::kan*	small increase	small increase
BW25113 *iscR::kan*	small increase	None

Table 17: *Summary of knockout strains with improved growth over the wild-type strain in 1,2-propanediol*

Strain genotype	Growth rate effect vs. K-12 MG1655 or BW25113	
	6% 1,2-propanediol	8% 1,2-propanediol
K-12 MG1655 *metJ::kan*	not tested	moderate increase
K-12 MG1655 Δ*metJ* Δ*relA purT::kan*	not tested	large increase (variable)
K-12 MG1655 Δ*fabR yfgF::kan*	not tested	moderate increase (secondary phase)
BW25113 *sspA::kan*	small increase	small increase
BW25113 *rph::kan*	None	small increase

A few of the knockouts identified in the Keio collection screens were additionally constructed
5   as mutants in K-12 MG1655 and tested in the Biolector format. Results for the *rzpD* and *sspA* knockouts grown in 7% v/v 2,3-butanediol are shown in Table 18, and results for the *rph* and *sspA* knockouts grown in 8% v/v 1,2-propanediol (first phase) are shown in Table 19. The *sspA* knockout exhibited a significantly increased growth rate and reduced lag time in 2,3-butanediol, however its improvement was less significant in 1,2-propanediol.

10   **Table 18:** *Growth rates and lag times of additional single gene knockouts in M9 + 7% (v/v) 2,3-butanediol as measured in the Biolector testing format.*

strain	mean		std. error	
	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0.019	-	0.017	-
MG1655 rzpD::kan	0.036	19.9	0.063	-
MG1655 sspA::kan	0.213	17.4	0.056	4.5

Table 19: *Growth rates and lag times of additional single gene knockouts in M9 + 8% (v/v) 1,2-propanediol as measured in the Biolector testing format.*

strain	mean		std. error	
	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0.219	2.0	0.066	1.2
MG1655 sspA::kan	0.259	2.0	0.023	0.5
MG1655 rph::kan	0.249	1.3	0.050	2.2

Methionine feeding reveals insights into mechanisms

A strain evolved for high ethanol concentrations in the literature also exhibited a mutation in *metJ*, and it was shown that deletion of *metJ* or addition of excess methionine improved ethanol tolerance in wild-type cells (Haft *et al.*, 2014). Without being limited to theory, as
5   MetJ is a repressor controlling expression of several genes involved in methionine biosynthesis, a similar effect can occur with toxic concentrations of diols. The wild-type strain and a selection of evolved strains were first tested for growth with and without supplementation of the medium containing 6% (v/v) 2,3-butanediol with 0.3 g/L L-methionine (Table 20). Robust growth of K-12 MG1655 in 6% 2,3-butanediol was
10   significantly restored by the addition of methionine, with a growth rate approaching that of evolved strains in 6% 2,3-butanediol. Evolved strains did not have a significantly enhanced growth rate increase in 2,3-butanediol with the addition of methionine.

Methionine supplementation was also tested for its ability to restore growth in the presence of 8% (v/v) 1,2-propanediol. Wild-type and a selection of evolved strains were tested (Table
15   21), and methionine was again found to restore growth of the wild-type strain, with minimal effect on evolved strains.

Based on these results, many of the causative mutations in evolved strains can be involved in either improving intracellular methionine supply, or allowing the cells to grow despite a condition of methionine starvation. This is clearly the case for loss-of-function mutations in
20   *metJ*, which encodes a transcriptional repressor (MetJ) of methionine biosynthesis and transport genes and acts when it binds S-adenosyl-L-methionine (SAM), for which L-methionine is a precursor in the SAM cycle. Inactivating mutations in *metJ* have previously been seen to result in increased biosynthesis of methionine (Nakamori *et al.*, 1999).

Table 20. *Growth rates and lag times of the wild-type strain, selected 2,3-butanediol*
25   *evolved strains, and K-12 MG1655 metJ::kan in M9 supplemented with 0.3 g/L methionine,*
6% (v/v) 2,3-butanediol, or 6% (v/v) 2,3-butanediol and 0.3 g/L methionine, as measured in
the Biolector testing format.

| | M9 + methionine | | | | M9 + 23BDO | | | |
| | mean | | std. error | | Mean | | std. error | |
Strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0.805	1.3	0.033	0.2	0.194	9.3	0.010	1.0
23BD2-4	0.726	1.9	0.020	0.1	0.393	2.4	0.063	1.0
23BD6-1	0.997	2.5	0.034	0.2	0.541	4.7	0.005	0.3

23BD7-4	0.741	1.2	0.011	0.1	0.572	3.4	0.034	0.5
MG1655 metJ::kan	0.466	1.0	0.011	0.1	0.211	3.8	0.017	0.7

	M9 + 23BDO + methionine			
	mean		std. error	
strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0.381	2.2	0.005	0.3
23BD2-4	0.345	1.0	0.031	0.7
23BD6-1	0.578	4.9	0.078	1.1
23BD7-4	0.609	4.0	0.023	0.2
MG1655 metJ::kan	0.202	-0.7	0.021	1.1

Table 21. *Growth rates and lag times of the wild-type strain and selected 1,2-propanediol evolved strains in M9 supplemented 8% (v/v) 1,2-propanediol, or 8% (v/v) 1,2-propanediol and 0.3 g/L methionine, as measured in the Biolector testing format.*

	M9 + 12PDO							
	phase 1				phase 2			
	mean		std. error		Mean		std. error	
Strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0.321	5.3	0.042	0.5	0.394	10.8	0.024	0.1
12PD3-8	0.523	19.0	0.037	0.7	0.282	20.9	0.036	1.7
12PD4-6	0.481	25.9	0.015	1.6	-	-	-	-
12PD6-3	0.470	1.8	0.057	1.2	0.780	11.7	0.094	0.6
12PD7-6	0.474	12.8	0.009	1.9	-	-	-	-

	M9 + 12PDO + methionine							
	phase 1				phase 2			
	mean		std. error		Mean		std. error	
Strain	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0.441	3.1	0.053	0.2	0.810	8.5	0.026	0.3
12PD3-8	0.344	27.8	0.053	3.9	0.427	35.7	0.100	4.4
12PD4-6	0.323	18.5	0.125	1.9	-	-	-	-
12PD6-3	0.473	1.5	0.024	1.4	-	-	-	-
12PD7-6	0.492	5.5	0.106	0.5	-	-	-	-

For the case of ethanol toxicity in *E. coli*, it was postulated that methionine starvation could be responsible for the observed ribosome stalling at non-start AUG codons, at which methionine is incorporated into translating proteins (Haft *et al.*, 2014). Additionally, the stringent response alarmone guanosine tetraphosphate/guanosine pentaphosphate

5   ((p)ppGpp) has been observed to accumulate as a consequence of growth in toxic concentrations of ethanol (Van Bogelen *et al.*, 1987). (p)ppGpp is largely synthesized by RelA, which associates with with the ribosome and is activated by binding of uncharged tRNAs. (p)ppGpp regulates numerous gene products required for cell growth, with the net effect being the induction of a growth arrest (stringent response) when (p)ppGpp

10 accumulates. If the toxicity mechanism of diols is similar to that of ethanol, then it would be expected that (p)ppGpp also accumulates in diol-stressed cells, and that this occurs via either the sensing of uncharged tRNAs in general by RelA (Hauryiuk *et al.*, 2015), or detection of ribosome stalling by RelA due to lack of methionyl-tRNAs (Haft *et al.*, 2014), or indirectly due to iron starvation (Miethke *et al.*, 2006; Vinella *et al.*, 2005) induced by toxic concentrations

15 of diols, as elaborated on below.

Loss-of-function of RelA, which would prevent cells from entering the stringent response, was found in both 2,3-butanediol and 1,2-propanediol evolved strains, providing a functional linkage between the *metJ* and *relA* mutations. However these two mutations by themselves abolished growth, and growth was only rescued further by the additional *purT* deletion. PurT

20 is one of two transformylases in purine biosynthesis, with the other being PurN. PurT utilizes the formyl group from formate, whereas PurN utilizes the formyl group from formyltetrahydrofolate (formyl-THF), which is also the formyl donor for generating initiator formylmethionine-tRNA (tRNA^{fMet}) that is required for initiating translation of AUG start codons. So, without being limited by theory, by deleting *purT*, competition for the formyl-THF

25 pool between purine biosynthesis and tRNA^{fMet} biosynthesis results in overall reduced levels of tRNA^{fMet}, and can enable the cells to better cope with methionine starvation by having a more balanced ratio between initiator and non-initiator methionyl-tRNAs. This explanation provides a functional linkage between the *metJ*, *relA*, and *purT* genes that all involve coping strategies for methionine starvation, and could explain the negative epistasis in the *metJ relA*

30 double knockout and the positive epistasis in the *metJ relA purT* triple knockout.

Methionine supplementation can thus be a strategy for improving endogenous production of diols in diol-overproducing strains during fermentation, since it is expected that growth would be inhibited by secreted diols at high concentrations due to the same mechanisms of toxicity observed here.

35

The combination of the presence of the *iscR* loss-of-function, which de-represses genes involved in iron-sulfur cluster biosynthesis when bound to free iron-sulfur clusters resulting from iron-sulfur protein degradation (Santos *et al.*, 2015), in addition to the *relA* loss-of-function as well as SpoT coding mutations (present in some isolates) additionally indicates a

5   role of modulation of levels of (p)ppGpp in relation to iron starvation. Iron starvation is known to trigger the stringent response and SpoT-dependent accumulation of (p)ppGpp in *E. coli* and other bacterial species (Miethke *et al.*, 2006; Vinella *et al.*, 2005), which is believed to help stimulate expression of iron uptake systems, thereby alleviating iron starvation conditions (Vinella *et al.*, 2005). Thus the loss-of-function in *relA*, optionally in combination

10   with a SpoT coding mutation such as SpoT-I213L or conservative substitutions thereof, may stimulate SpoT-dependent accumulation of (p)ppGpp and the increased expression of one or more iron uptake systems. Iron starvation could potentially arise from either direct chelation of iron by diols, or from diols interfering with chelation of iron by siderophores such as enterobactin. Derepression of iron-sulfur cluster biosynthesis and assembly enzymes via

15   knockdown or knockout of *iscR* likely enables the more efficient use of cellular ferric iron for this critical function, as iron-sulfur clusters serve as catalytic cores of cytochromes involved in cellular respiration and in glutamate synthase.Furthermore, Miethke *et al.* (2006) speculated on the existence of a link between iron starvation and methionine and cysteine biosynthesis pathways, due to observance of up-regulation of several methionine and

20   cysteine biosynthetic genes during iron starvation of *B. subtilis*. It was noted that in *B. subtilis*, L-threonine is a precursor for production of a catecholic trilactone siderophore that is utilized for ferric iron uptake, and that the threonine, serine/glycine, and cysteine/methionine biosynthetic pathways are interdependent. Conversely, in *E. coli*, L-serine is a precursor for the the production of enterobactin, another siderophore involved in ferric iron uptake. As L-

25   cysteine is synthesized from L-serine, a reduction in levels of L-serine could lead to L-cysteine starvation and thus also L-methionine starvation, as L-cysteine is also a precursor for biosynthesis of L-methionine. Thus the combination of the *metJ* deletion and mutations that alleviate iron starvation, such as knockdown or knockout of *relA* and/or *iscR*, and optionally coding mutations in SpoT, may serve to restore cellular homeostasis at large.

30   Cross-compound tolerance testing

Every secondary screened evolved isolate from the 2,3-butanediol and 1,2-propanediol evolutions was grown in the presence of every other compound in the study as indicated in the Methods. The normalized $t_{OD1(evolved\ strain)}/t_{OD1(wild-type)}$ are shown in Table 21 (for 2,3-butanediol evolved strains) and Table 22 (for 1,2-propanediol evolved strains). Lower values

35   are indicative a larger improvement in growth of the evolved isolate (left column) in that chemical condition (top row), whereas higher values are indicative of a lower improvement or decrease in growth compared to the wild-type. Averaged ratios across conditions and strains

shown at the right and bottom of the plot allow for overall by-chemical and by-strain trends to be observed. Strain names that are followed by an asterisk (*) were not re-sequenced, and strain names in italics were found to be hypermutator strains.

All 2,3-butanediol evolved strains exhibit cross-tolerance to 1,2-propanediol, and isolates from populations 12PD5, 12PD6, 12PD7, and 12PD8, plus several isolates from the other populations, exhibit cross-tolerance to 2,3-butanediol. Isolates from populations 23BD1, 23BD8, 12PD5, 12PD6, and non-mutator isolates from 12PD8 all exhibit significant cross-tolerance to hexanoate, and 23BD8 isolates additionally have strong cross-tolerance to *p*-coumarate (this could be due to the mutation in *ygaH* having a pleiotropic effect on the neighboring *mprA* gene, which has been observed to improve *p*-coumarate tolerance when knocked out, or due to a broader effect from the mutation in *rpoB*). Several 12PD isolates also exhibit cross-tolerance toward coumarate, however the only non-hypermutator strain is 12PD6-9. This strain has non-coding mutations in *ypjA*, and mutations in *ypjA* thought to be inactivating were also found in *p*-coumarate evolved isolates. The 2,3-butanediol evolved strain with the best overall tolerance toward the range of chemical stressors was 23BD8-7. The majority of isolates being hypermutators was likely responsible for highly variable cross-tolerance between compounds in the 1,2-propanediol evolved strains, however the best-performing isolate was 12PD4-9.

Table 21: *Normalized $t_{OD1(evolved)}/t_{OD1(wild-type)}$ values for 2,3-butanediol-evolved isolates grown in the presence of inhibitory concentrations of 12 different chemicals.*

	butanol	glutarate	coumarate	2,3-butanediol	putrescine	HMDA	adipate	isobutyrate	Hexanoate	octanoate	1,2-propanediol	NaCl	average
23BD1-6	0.71	0.77	1.00	2.05	1.34	3.00	0.82	2.88	0.72	1.00	0.80	1.06	1.35
23BD1-8*	0.98	1.89	1.00	0.50	1.26	1.50	0.80	2.87	0.76	1.00	0.73	1.21	1.21
23BD1-9	0.94	0.89	1.00	0.52	1.21	1.78	0.90	2.80	0.74	1.11	0.75	1.15	1.15
23BD2-4	1.20	1.89	1.00	2.05	1.67	1.06	2.03	1.80	1.09	1.70	0.89	1.75	1.51
23BD2-5*	0.86	1.89	1.00	0.53	1.48	1.14	2.03	1.64	1.09	1.50	0.86	1.60	1.30
23BD2-7	0.86	1.89	1.00	0.53	1.42	1.02	2.03	1.80	1.02	1.51	0.80	1.74	1.30
23BD2-9	*0.80*	*1.89*	*1.00*	*1.74*	*1.58*	*1.12*	*2.03*	*2.01*	*1.09*	*1.54*	*0.80*	*2.05*	*1.47*
23BD3-3	1.20	1.48	1.00	0.42	3.10	3.00	1.82	1.62	3.56	1.70	0.77	2.35	1.84
23BD3-4	1.04	1.35	1.00	0.46	3.10	3.00	2.01	3.04	3.56	1.70	0.80	2.35	1.95
23BD3-9	1.20	1.26	1.00	0.55	3.10	3.00	1.81	2.10	3.16	1.70	0.91	2.35	1.85

23BD4-3	0.96	0.89	1.00	0.50	1.16	1.16	0.87	3.43	0.98	1.17	0.80	1.13	1.17
23BD4-4	1.01	1.25	1.00	1.74	1.56	1.76	1.17	3.43	0.93	1.70	0.84	1.38	1.48
23BD4-7	1.12	1.55	1.00	0.52	1.82	1.76	2.03	2.55	1.46	1.70	0.91	1.91	1.53
23BD5-1	1.15	1.00	0.86	2.05	1.43	1.33	1.17	2.51	0.93	0.99	0.80	1.38	1.30
23BD5-7	1.16	1.14	0.99	0.51	1.42	1.37	1.22	2.30	0.82	0.98	0.80	1.38	1.17
23BD5-10	1.14	1.05	0.77	0.62	1.53	1.42	1.13	2.37	0.85	1.00	0.77	1.38	1.17
23BD6-1	1.12	1.03	1.00	0.61	1.61	1.05	0.96	3.43	0.94	0.89	0.77	1.35	1.23
23BD7-4	1.02	1.52	0.79	0.38	1.63	1.56	2.03	1.84	0.89	1.70	0.71	1.78	1.32
23BD7-5	1.06	1.33	0.58	0.44	1.39	1.16	1.19	1.64	0.82	1.00	0.73	1.35	1.06
23BD7-7	0.99	1.43	1.00	0.38	1.56	1.58	2.03	1.85	0.80	1.70	0.73	1.80	1.32
23BD7-10*	0.95	1.31	1.00	0.49	1.67	1.51	2.03	2.07	0.93	1.70	0.71	1.60	1.33
23BD8-2	1.20	0.84	0.55	0.58	1.79	3.00	1.01	0.82	0.67	0.94	0.77	1.53	1.14
23BD8-5*	0.83	0.93	0.62	0.60	1.87	1.40	1.17	1.84	0.71	1.00	0.77	1.63	1.11
23BD8-7	0.77	0.74	0.30	0.47	1.21	0.91	0.71	1.62	0.67	0.89	0.73	0.82	0.82
Average	1.01	1.30	0.89	0.80	1.70	1.69	1.46	2.26	1.22	1.33	0.79	1.58	1.34
# >wt	11	6	8	19	0	1	6	1	16	5	24	1	
% >wt	45.8	25.0	33.3	79.2	0.0	4.2	25.0	4.2	66.7	20.8	100.0	4.2	

Table 22: Normalized $t_{OD1(evolved)}/t_{OD1(wild-type)}$ values for 1,2-propanediol-evolved isolates grown in the presence of inhibitory concentrations of 12 different chemicals.

	Butanol	glutarate	coumarate	2,3-butanediol	putrescine	HMDA	adipate	isobutyrate	hexanoate	octanoate	1,2-propanediol	NaCl	average
12PD1-2	0.94	1.42	1.42	1.86	3.06	2.84	1.52	2.14	0.98	1.44	1.15	2.24	1.75
12PD1-4	1.14	1.18	1.18	0.46	3.06	2.57	0.94	2.97	0.72	1.44	0.72	2.24	1.55
12PD1-10	1.26	1.36	1.36	0.71	3.06	2.84	1.56	1.63	1.51	1.44	1.15	2.24	1.68
12PD2-5*	1.26	1.42	1.42	1.86	3.06	2.84	1.51	2.55	0.77	1.44	0.89	2.24	1.77
12PD2-8	1.26	1.52	1.52	0.62	3.06	2.84	1.48	2.77	1.15	1.44	1.00	2.24	1.74
12PD2-9	1.26	1.02	1.02	1.86	3.06	2.84	1.27	2.32	0.72	1.44	0.76	2.24	1.65
12PD3-7	*1.26*	*1.02*	*1.02*	*0.39*	*3.06*	*2.84*	*1.71*	*0.77*	*0.70*	*0.84*	*0.65*	*2.24*	*1.38*
12PD3-8	1.20	1.20	1.20	1.86	3.06	2.84	1.39	2.02	1.70	1.44	1.15	2.24	1.77
12PD3-10	1.26	1.36	1.36	0.74	3.06	2.84	1.54	2.20	1.21	0.99	0.96	2.24	1.65
12PD4-6	1.22	1.50	1.50	0.64	3.06	2.57	1.61	2.97	1.02	0.75	1.22	2.24	1.69
12PD4-8	1.12	0.83	0.83	1.86	1.44	0.99	0.76	0.72	0.91	0.75	0.65	0.80	0.97
12PD4-9	1.20	0.77	0.77	0.40	1.17	0.94	0.73	0.80	0.83	1.08	0.70	0.84	0.85
12PD5-1	1.26	1.84	1.84	0.41	3.06	2.84	1.70	2.97	0.72	1.01	0.59	2.24	1.71
12PD5-3	1.26	1.84	1.84	0.31	3.06	2.84	1.61	2.97	0.66	1.44	0.59	2.24	1.72
12PD5-9*	1.26	1.84	1.84	0.41	3.06	2.84	1.46	1.08	0.70	0.80	0.57	2.24	1.51
12PD6-3	1.26	1.03	1.03	0.38	2.65	2.84	0.92	2.97	0.66	1.44	0.57	1.97	1.48

12PD6-6*	1.26	0.73	0.73	0.41	2.29	2.53	0.76	2.97	0.66	0.73	0.63	1.62	1.28
12PD6-9	1.08	0.86	0.86	0.53	2.71	2.29	0.82	2.97	0.66	0.88	0.63	1.36	1.30
12PD7-5	1.15	0.59	0.59	0.35	1.17	1.12	0.56	2.97	0.70	0.71	0.59	0.66	0.93
12PD7-6	1.26	0.81	0.81	0.33	3.06	0.97	0.92	1.14	3.64	1.44	0.54	0.91	1.32
12PD7-7*	1.18	1.30	1.34	0.34	1.76	1.09	1.87	1.02	0.74	1.44	0.59	1.72	1.20
12PD8-6	1.26	1.57	1.57	0.56	3.06	2.84	1.61	2.82	1.21	1.44	0.76	2.24	1.75
12PD8-7	1.26	0.73	0.73	0.31	3.06	2.34	0.68	1.89	0.49	0.84	0.54	1.14	1.17
12PD8-10	1.26	0.55	0.55	0.35	3.06	2.84	0.67	1.46	0.58	0.75	0.59	1.78	1.20
average	1.21	1.18	1.18	0.75	2.72	2.38	1.23	2.13	0.98	1.14	0.76	1.84	1.46
# >wt	1	8	8	19	0	3	10	3	17	10	19	4	
% >wt	4.2	33.3	33.3	79.2	0.0	12.5	41.7	12.5	70.8	41.7	79.2	16.7	

Additionally, each evolved isolate was tested for cross-tolerance toward other aliphatic diols of potential biotechnological interest. First, K-12 MG1655 was tested in the Growth Profiler screening format for growth in the presence of a range of concentrations of each compound (note that this had been done in the Biolector format previously for 1,2-pentanediol and 1,5-pentanediol thus was not repeated here): 1,3-propanediol and 1,4-butanediol. Variable concentrations of these compounds elicited growth inhibition in *E. coli* K-12 MG1655 (Table 23). Based on these results, a screening concentration was selected for the evolved isolates for which wild-type cells could achieve at a growth rate of 0.15-0.3 h^{-1} (versus uninhibited growth at 0.7-0.9 h^{-1} in M9 glucose minimal medium). These concentrations were: 5.5% (v/v) 1,3-propanediol, 5.5% (v/v) 1,4-butanediol, 1.25% (v/v) 1,2-pentanediol, and 3.5% (v/v) 1,5-pentanediol. The results of 2,3-butanediol-evolved isolates grown in these concentrations of alternative diols are shown in Table 24. All evolved isolates exhibited marked reductions in lag time in all tested diols. Additionally, all evolved isolates exhibited increased growth rates in 1,3-propanediol, 1,4-butanediol, and 1,5-pentanediol. Smaller numbers of isolates exhibited significantly improved growth rates in 1,2-pentanediol, however this included 23BD3-3, isolates from population 23BD4, 23BD6-1, isolates from population 23BD7 (with the most notable tolerance observed in 23BD7-5), and 23BD8-5 and 23BD8-7.

Table 23. *Growth rates and lag times of K-12 MG1655 in varying concentrations of 1,3-propanediol and 1,4-butanediol, as measured in the Growth Profiler testing format.*

diol % (v/v)	1,3-propanediol				1,4-butanediol			
	mean		std. error		mean		std. error	
	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
0	0.747	5.1	0.030	0.2	0.747	5.1	0.030	0.2

1	0.670	5.3	0.093	0.1	0.672	5.3	0.018	0.1
2	0.686	6.1	0.010	0.3	0.591	5.9	0.024	0.1
3	0.603	7.3	0.057	0.5	0.531	7.1	0.033	0.1
4	0.463	9.6	0.037	0.6	0.434	9.0	0.009	0.2
5	0.277	12.5	0.002	0.6	0.303	11.7	0.042	0.2
6	0.201	14.7	0.012	0.7	0.183	17.6	0.000	0.4
7.5	0.118	24.9	0.029	0.6	-	-	-	-

Table 24. *Growth rates and lag times of K-12 MG1655 and 2,3-butanediol-evolved isolates in specified inhibitory concentrations of diols, as measured in the Growth Profiler testing format.*

| strain | 5.5% (v/v) 1,3-propanediol | | | | 5.5% (v/v) 1,4-butanediol | | | | 1.25% (v/v) 1,2-pentanediol | | | | 3.5% (v/v) 1,5-pentanediol | | | |
| | mean (2) | | std. error (2) | | mean (2) | | std. error (2) | | mean (2) | | std. error (2) | | mean (2) | | std. error (2) | |
	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)	μ (h^{-1})	t_{lag} (h)
MG1655	0.395	11.7	0.020	0.2	0.192	13.2	0.001	0.2	0.366	28.1	0.011	0.9	0.247	17.1	0.008	0.4
23BD1-6	0.639	6.2	0.020	0.1	0.501	6.9	0.011	0.1	0.336	9.6	0.018	0.7	0.384	10.5	0.011	1.8
23BD1-8	0.586	6.3	0.011	0.0	0.440	6.7	0.016	0.2	0.341	10.1	0.015	1.0	0.315	10.2	0.021	0.1
23BD1-9	0.578	6.4	0.013	0.1	0.471	7.0	0.013	0.1	0.314	9.4	0.045	0.0	0.326	10.7	0.007	1.1
23BD2-4	0.500	7.6	0.017	0.1	0.414	8.0	0.011	0.2	0.338	10.3	0.031	0.3	0.352	9.8	0.028	0.2
23BD2-7	0.529	7.2	0.015	0.1	0.418	7.3	0.004	0.0	0.367	9.7	0.030	0.4	0.315	14.0	0.014	1.3
23BD2-9	0.514	7.3	0.015	0.3	0.427	7.5	0.012	0.3	0.377	9.0	0.030	0.3	0.361	9.5	0.005	0.2
23BD3-3	0.647	6.4	0.012	0.2	0.565	6.8	0.009	0.2	0.422	8.0	0.006	0.4	0.379	8.6	0.010	0.2
23BD3-4	0.589	8.0	0.011	1.6	0.361	7.8	0.049	0.3	0.361	9.9	0.078	3.2	0.413	8.1	0.012	0.1
23BD3-9	0.681	7.2	0.008	0.3	0.523	7.6	0.026	0.2	0.409	8.7	0.023	0.9	0.279	10.1	0.015	0.7
23BD4-3	0.646	6.1	0.023	0.2	0.510	6.7	0.047	0.3	0.417	7.2	0.005	1.1	0.315	10.2	0.004	0.3
23BD4-4	0.644	6.5	0.013	0.6	0.508	7.8	0.046	1.0	0.459	8.5	0.015	2.5	0.373	8.4	0.024	0.7
23BD4-7	0.626	7.7	0.019	0.1	0.477	8.3	0.013	0.8	0.443	10.6	0.024	0.3	0.334	10.2	0.022	0.1
23BD5-1	0.651	6.8	0.018	0.2	0.474	7.9	0.013	0.1	0.340	9.9	0.010	0.8	0.380	8.1	0.004	0.1
23BD5-7	0.658	6.3	0.013	0.3	0.499	7.4	0.032	0.3	0.366	9.8	0.018	0.5	0.387	8.0	0.001	0.1
23BD5-10	0.666	6.4	0.019	0.2	0.524	7.7	0.007	0.3	0.348	9.7	0.021	0.7	0.392	8.2	0.014	0.0
23BD6-1	0.656	6.0	0.025	0.2	0.515	6.6	0.023	0.0	0.443	9.0	0.021	0.4	0.390	8.2	0.012	0.3
23BD2-5	0.520	6.7	0.005	0.6	0.437	7.2	0.010	0.2	0.357	9.6	0.027	0.3	0.351	9.1	0.009	0.2
23BD7-10	0.609	5.7	0.031	0.3	0.473	6.4	0.017	0.2	0.453	11.0	0.012	0.8	0.390	12.0	0.014	3.1
23BD7-4	0.617	5.9	0.018	0.1	0.481	6.5	0.005	0.1	0.462	11.1	0.011	0.1	0.310	10.0	0.017	0.4
23BD7-5	0.620	6.0	0.009	0.0	0.503	6.5	0.008	0.1	0.535	9.3	0.013	0.5	0.322	9.4	0.005	0.3
23BD7-7	0.629	5.7	0.012	0.1	0.497	6.3	0.020	0.1	0.461	10.8	0.008	0.2	0.316	10.8	0.033	0.5
23BD8-2	0.714	6.1	0.029	0.2	0.470	7.7	0.007	0.3	0.225	12.4	0.086	1.5	0.412	9.6	0.010	0.1
23BD8-5	0.625	5.6	0.061	0.2	0.434	6.7	0.071	0.3	0.493	5.5	0.043	0.3	0.411	9.6	0.015	0.4
23BD8-7	0.623	5.4	0.009	0.2	0.415	6.8	0.066	0.4	0.486	7.4	0.010	0.5	0.424	9.9	0.010	0.1

Biological production of 1,2-propanediol and 2,3-butanediol

Known biological pathways for the production of 1,2-propanediol (S or R isomers) from various sugars or glycerol are shown in Figure 8 of Dabra *et al.* (2016), hereby specifically incorporated by reference, where the pathway on the left is native to *E. coli*. In one pathway, the sugars L-rhamnose or L-fucose are catabolized to (S)-lactaldehyde and the glycolytic intermediate dihydroxyacetone phosphate (DHAP). Depending on redox conditions, S-lactaldehyde can either be oxidized to lactic acid, or reduced to (S)-1,2-propanediol. Another pathway, which is much more versatile in that any carbon feedstock can be utilized where DHAP can be readily generated (*e.g.* glucose, glycerol, or xylose), involves a methylglyoxal intermediate, which depending on the choice of reducing enzyme, can generate either (S)- or (R)-lactaldehyde, or acetol. These can then be further reduced to (S)- or (R)-1,2-propanediol, or acetol can be reduced to a racemic mixture of both isomers. The highest reported titer of 1,2-propanediol in *E. coli* is 5.6 g/L, and this was obtained using glycerol as a carbon source in a strain with inactivations of *ackA-pta* (acetate formation), replacement of the native PEP-dependent dihydroxyacetone kinase with an ATP-dependent enzyme from *Citrobacter freundii*, and overexpression of native methylglyoxal synthase (MgsA), L-1,2-propanediol dehydrogenase (GldA), and NADPH-dependent aldehyde reductase YqhD (Clomburg *et al.*, 2011). The highest reported titer of (R)-1,2-propanediol from glucose in *E. coli* is 5.13 g/L, obtained using a similar strategy aimed at improving DHAP availability and overexpressing a combination of native genes to covert DHAP to methylglyoxal (MgsA) and subsequently to lactaldehyde (GldA) and 1,2-propanediol (FucO) (Jain *et al.*, 2015). This pathway is shown in Figure 1 of Jain *et al.* (2015), which is hereby specifically incorporated by reference in its entirety. Various alternative production pathways for 1,2-propanediol, either natively in natural 1,2-propanediol fermenting microorganisms or in recombinant strains with different combinations of enzymes, producing different enantiomers, and utilizing different carbon feedstocks, are known in the art.

Biological pathways for production of 2,3-butanediol in bacteria from glucose, CO2, and CO are shown in Figure 2 of Sabra *et al.* (2016), which is hereby specifically incorporated by reference in its entirety. Generally (R)-acetoin is produced from acetolactate, however it is possible to isomerize acetoin to the (S)-isomer or to produce either isomer from diacetyl. Different acetoin reductases can then be utilized to generate 2,3-butanediol stereoisomers, or diacetyl can be used to generate acetylacetoin, from which different stereoisomers of 2,3-butanediol can ultimately derive. Many organisms natively ferment 2,3-butanediol, however most organisms are pathogenic and are not generally recognized as safe. Up to 119 g/L of 2,3-butanediol has been produced in *Enterobacter cloacae* subsp. *dissolvens* SDM utilizing lignocellulosic hydrolysates by simply deleting byproduct producing genes (Li *et al.*, 2015). The best demonstrated production in recombinant *E. coli* from glucose is 73.8 g/L using *E.*

coli BL21(DE3) containing a plasmid (pET-RABC) overexpressing a gene cluster from *Enterobacter cloacae* subsp. *dissolvens* SDM (*lysR-budABC*) with no other modifications (Xu *et al.*, 2014). This is well above the toxicity threshold for this chemical (Table 1; 7.5% (v/v) = 74.0 g/L), and a relatively low cell density was reached during their fed-batch fermentation

5   despite continued production from glucose after cessation of cell growth (Xu *et al.*, 2014), suggesting that higher productivities could be reached by increasing biomass through the use of evolved strains.

Endogenous production of 2,3-butanediol using the overproduction pathway described by Xu *et al.*, 2014, was achieved by transforming plasmid pET-RABC into evolved isolates, plus

10   wild-type K-12 MG1655 as a control, that all harbored inactivation of the EcoKI restriction system (due to the presence of restriction sites on the plasmid). Strains were cultured for production in a screening format as described in the Methods. It was found that cultures would not grow when harboring pET-RABC in a minimal medium without a complex nitrogen source, likely due to branched-chain amino acid starvation (*e.g.*, isoleucine) due to

15   introduction of the heterologous acetolactate synthase, thus yeast extract was utilized in the screen. Results are shown in Table 25. Perhaps unexpectedly due to the use of yeast extract, which was not employed in the evolutions, the majority of evolved isolates did not exhibit improved endogenous production of 2,3-butanediol as compared with wild-type K-12 MG1655 harboring the same modifications. However two isolates, 23BD7-5 and 23BD8-2,

20   exhibited significantly increased titers of 2,3-butanediol, with up to a 67% improvement over the wild-type background. 23BD7-5 is notable in possessing a loss-of-function mutation in *acrB* that the other isolates from population 23BD7 do not possess, however it also lacks mutations in *tolC*, *treR*, and *yhjA* that the other 23BD7 isolates possess. 23BD8-2 is notable in being the only resequenced evolved isolate lacking a mutation in *metJ*. It instead harbors a

25   probable loss- or reduction-of-function mutation in *iscR* (inferred by Keio screening results described above) as well as mutations in *relA*, *rpoB*, *lon*, *ygaH*, and a mutation that increases the expression of PyrE.

Table 25: *2,3-butanediol titers in background strains harboring the ΔhsdR mutation and plasmid pET-RABC, measured from screening in a minimal medium containing 5% (w/v)*

30   *glucose and 1% (w/v) yeast extract after 48 hours at 30°C.*

	48 hour titer	
background strain	average (g/L)	std. error (g/L)
K-12 MG1655	10.24	2.49
23BD1-6	9.48	0.34
23BD1-9	9.34	0.42

23BD2-4	5.54	0.77
23BD2-7	6.17	0.23
23BD2-9	5.60	0.19
23BD3-3	9.01	0.47
23BD3-4	7.92	2.13
23BD3-9	9.22	0.67
23BD4-3	9.10	0.05
23BD4-4	7.33	0.22
23BD4-7	9.34	0.21
23BD5-1	10.00	0.09
23BD5-7	10.09	0.35
23BD5-10	10.02	0.36
23BD6-1	6.75	0.08
23BD7-4	7.81	0.18
23BD7-5	17.12	0.12
23BD7-7	7.98	0.10
23BD8-2	13.14	0.34
23BD8-7	8.70	0.22

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Yim H, *et al.* Metabolic engineering of *Escherichia coli* for direct production of 1,4-butanediol. *Nat. Chem. Biol.* 7:445-452 (2011).

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US 2012/0282661 A1 (Genomatica Inc.)

WO 2009/086075 A1 and US 2009/0162911 A1 (E I Du Pont De Nemours and Company)

US 2003/0068611 A1 (E I DuPont De Nemours and Company)

WO 2009/143455 A2 (Microbia Inc.)

WO 2011/154503 A1 (Evonik Degussa GmbH)

US 2014/0135526 A1 (OPX Biotechnologies Inc.)

WO 2014/029592 A1 (Evonik Degussa GmbH)

EP 2 032 711 A1 (TNO)

EP 2 580 315 A2 (Cobalt Technologies Inc.)

CLAIMS

1.   A bacterial cell comprising a biosynthetic pathway for producing an aliphatic polyol
and at least one genetic modification which reduces expression of an endogenous gene
selected from the group consisting of *metJ, iscR, yhjA, gtrS, ycdU, rzpD, sspA* and *rph*, or a
combination of any thereof, optionally wherein the cell further comprises a genetic
modification which increases the expression of PyrE.

2.   The bacterial cell of claim 1, comprising at least one genetic modification which
reduces expression of *metJ, iscR*, or both.

3.   A bacterial cell comprising at least one genetic modification which reduces expression
of
            (a) *metJ, relA* and *purT*;
            (b) *metJ* and *acrB, acrA* or both;
            (c) *fabR* and *ygfF*; or
            (d) *iscR* and *relA*;
            optionally in combination with a genetic modification which increases the
expression of PyrE.

4.   The bacterial cell of any one of the preceding claims, wherein the genetic modification
comprises a knock-down or knock-out of the endogenous gene or genes.

5.   The bacterial cell of any one of the preceding claims, further comprising an
upregulation of, and/or one or more mutations in, at least one protein selected from NanK
(SEQ ID NO:19), RpsA (SEQ ID NO:37), RpoA (SEQ ID NO:21); RpoB (SEQ ID NO:23), RpoC
(SEQ ID NO:25), SpoT (SEQ ID NO:27), NusG (SEQ ID NO:29, Flu (SEQ ID NO:31), Lon
(SEQ ID NO:33), and YgaH (SEQ ID NO:35), wherein the one or more mutations are selected
from RpoC-L268K, RpoC-L268N, RpoC-L268Q, RpoC-L268R, RpoC-N309F, RpoC-N309S,
RpoC-N309T, RpoC-N309W, RpoC-N309Y, RpoC-Y75A, RpoC-Y75C, RpoC-Y75S, RpoC-
ΔTPVIE(822-827), RpoB-D549A, RpoB-D549G, RpoB-H447F, RpoB-H447S, RpoB-H447T,
RpoB-H447W, RpoB-H447Y, RpoB-I1112S, RpoB-I1112T, RpoB-V931A, RpoB-V931I, RpoB-
V931L, NanK-T128S,Flu-L642E, Flu-L642N, Flu-L642Q, Lon-I716S, Lon-I716T, YgaH-V39A,
YgaH-V39I,YgaH-V39L,NusG-F144A, NusG-F144I, NusG-F144L, NusG-F144M, NusG-F144V,
RpoA-D305A, RpoA-D305G, RpoA-G279A, RpoA-G279F, RpoA-G279I, RpoA-G279L, RpoA-
G279M, RpoA-G279V, RpsA-D310A, RpsA-D310F, RpsA-D310I, RpsA-D310L, RpsA-D310M,

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RpsA-D310V, RpsA-G21A, RpsA-G21F, RpsA-G21I, RpsA-G21L, RpsA-G21M, RpsA-G21V, SpoT-I213A, SpoT-I213F, SpoT-I213L, SpoT-I213M, and SpoT-I213V.

6.   The bacterial cell of any one of the preceding claims, comprising

(a) a mutant RpoC comprising a RpoC-L268R, RpoC-L268K, RpoC-L268Q or RpoC-L268N mutation and at least one genetic modification which reduces the expression of *metJ, relA* and *purT*;

(b) a mutant RpoC comprising a RpoC-L268R, RpoC-L268K, RpoC-L268Q, or RpoC-L268N mutation and at least one genetic modification which reduces the expression of *metJ* and *acrB, acrA* or both;

(c) a mutant RpoC comprising a RpoC-L268R, RpoC-L268K, RpoC-L268Q or RpoC-L268N mutation and at least one genetic modification which reduces the expression of *metJ, relA, purT,* and *acrB, acrA* or both;

(d) a mutant RpoC comprising a RpoC-L268R, RpoC-L268K, RpoC-L268Q, or RpoC-L268N mutation and a mutant NanK comprising a NanK-T128S mutation, and at least one genetic modification which reduces the expression of *metJ, relA,* and *purT,* and *acrB, acrA* or both;

(e) a mutant RpoC comprising a RpoC-L268R, RpoC-L268K, RpoC-L268Q, or RpoC-L268N mutation and a mutant NanK comprising a NanK-T128S mutation, and at least one genetic modification which reduces the expression of *metJ* and *acrB, acrA* or both;

(f) a mutant RpoC comprising a RpoC-L268R, RpoC-L268K, RpoC-L268Q, or RpoC-L268N mutation and a mutant NanK comprising a NanK-T128S mutation, and at least one genetic modification which reduces the expression of *metJ, relA, purT,* and *acrB, acrA* or both;

(g) a mutant RpoC comprising a RpoC-L268R, RpoC-L268K, RpoC-L268Q or RpoC-L268N mutation, a mutant NanK comprising a NanK-T128S mutation, and a mutant Flu comprising a Flu-L642Q, Flu-L642N, or Flu-L642E mutation, and at least one genetic modification which reduces the expression of *metJ, relA, purT, elfD and acrB, acrA* or both;

(h) a mutant RpoB comprising a RpoB-I1112S or RpoB-I1112T mutation and at least one genetic modification which reduces the expression of *iscR, relA,* and *acrB, acrA* or both;

(i) a mutant RpoB comprising a RpoB-I1112S or RpoB-I1112T mutation and at least one genetic modification which reduces the expression of *iscR, relA,* and *acrB, acrA* or both;

(j) a mutant RpoB comprising a RpoB-I1112S or RpoB-I1112T mutation, and a mutant Lon comprising a Lon-I716S or Lon-I716T mutation, and at least one genetic modification which reduces the expression of *iscR, relA,* and *acrB, acrA* or both;

(k) a mutant RpoB comprising a RpoB-I1112S or RpoB-I1112T mutation, a mutant Lon comprising a Lon-I716S or Lon-I716T mutation, and a mutant YgaH comprising a YgaH-V39A, YgaH-V39L, or YgaH-V39I mutation, and at least one genetic modification which reduces the expression of *iscR, relA,* and *acrB, acrA* or both; or

(l) a mutant RpoB comprising a RpoB-I1112S or RpoB-I1112T mutation, a mutant Lon comprising a Lon-I716S or Lon-I716T mutation, a mutant YgaH comprising a YgaH-V39A, YgaH-V39L, or YgaH-V39I mutation, a genetic modification that increases the expression of PyrE, and at least one genetic modification which reduces the expression of *iscR, relA,* and *acrB, acrA* or both.

7.   The bacterial cell of any one of the preceding claims, wherein the at least one genetic modification provides for an increased growth rate, a reduced lag time, or both, of the cell in at least one of 2,3-butanediol and 1,2-propanediol, as compared to the parent bacterial cell.

8.   The bacterial cell of any one of the preceding claims, comprising a recombinant biosynthetic pathway for producing at least one of a propanediol, butanediol, pentanediol and a hexanediol.

9.   The bacterial cell of any one of the preceding claims, which is of the *Escherichia, Enterobacter, Klebsiella, Lactobacillus, Lactococcus, Bacillus, Pseudomonas, Corynebacterium, Ralstonia, Paenibacillus, Clostridia or Citrobacter sp* genera, such as of the *Escherichia coli* species.

10.   A process for preparing a recombinant *E. coli* cell for producing an aliphatic polyol, comprising genetically modifying an *E. coli* cell to introduce a recombinant biosynthetic pathway for producing an aliphatic polyol, and

(a) knock-down or knock-out at least one endogenous gene selected from the group consisting of *metJ, iscR, yhjA, gtrS, ycdU, rzpD, sspA* and *rph;* or a combination of endogenous genes selected from *metJ, relA* and *purT; metJ* and *acrB, acrA* or both; *iscR* and *relA;* and *fabR* and *ygfF;* and

(b) optionally, upregulating and/or introducing one or more mutations in at least one protein selected from NanK (SEQ ID NO:19), RpsA (SEQ ID NO:37), RpoA (SEQ ID NO:21); RpoB (SEQ ID NO:23), RpoC (SEQ ID NO:25), SpoT (SEQ ID NO:27), NusG (SEQ ID NO:29, Flu (SEQ ID NO:31), Lon (SEQ ID NO:33), and YgaH (SEQ ID NO:35), optionally wherein the one or more mutations are selected from RpoC-L268K, RpoC-L268N, RpoC-L268Q, RpoC-L268R, RpoC-N309F, RpoC-N309S, RpoC-N309T, RpoC-N309W, RpoC-N309Y, RpoC-Y75A, RpoC-Y75C, RpoC-Y75S, RpoC-ΔTPVIE(822-827), RpoB-D549A, RpoB-D549G, RpoB-H447F, RpoB-H447S, RpoB-H447T, RpoB-H447W, RpoB-H447Y, RpoB-I1112S, RpoB-I1112T, RpoB-V931A, RpoB-V931I, RpoB-V931L, NanK-T128S,Flu-L642E, Flu-L642N, Flu-L642Q, Lon-I716S, Lon-I716T, YgaH-V39A, YgaH-V39I,YgaH-V39L,NusG-F144A, NusG-F144I, NusG-F144L, NusG-F144M, NusG-F144V, RpoA-D305A, RpoA-D305G, RpoA-G279A, RpoA-G279F, RpoA-G279I, RpoA-G279L, RpoA-G279M, RpoA-G279V, RpsA-D310A, RpsA-D310F, RpsA-D310I, RpsA-D310L, RpsA-D310M, RpsA-D310V, RpsA-G21A, RpsA-G21F, RpsA-G21I, RpsA-G21L, RpsA-G21M, RpsA-G21V, SpoT-I213A, SpoT-I213F, SpoT-I213L, SpoT-I213M, and SpoT-I213V.

11.   A process for improving the tolerance of a bacterial cell to an aliphatic polyol, comprising genetically modifying the bacterial cell to

(a) knock-down or knock-out at least one endogenous gene selected from the group consisting of *metJ, iscR, yhjA, gtrS, ycdU, rzpD, sspA* and *rph*; or a combination of endogenous genes selected from *metJ, relA* and *purT; metJ* and *acrB, acrA* or both; *iscR* and *relA*; and *fabR* and *ygfF*;

(b) optionally introducing one or more mutations in one or more endogenous genes selected from NanK (SEQ ID NO:19), RpsA (SEQ ID NO:37), RpoA (SEQ ID NO:21); RpoB (SEQ ID NO:23), RpoC (SEQ ID NO:25), SpoT (SEQ ID NO:27), NusG (SEQ ID NO:29, Flu (SEQ ID NO:31), Lon (SEQ ID NO:33), and YgaH (SEQ ID NO:35) or the *pyrE/rph* intergenic region;

(c) preparing a population of the genetically modified bacterial cell,; and

(d) selecting from the population in (c) any bacterial cell which has an improved tolerance to the aliphatic polyol.

12.   A method for producing an aliphatic polyol, comprising culturing the bacterial cell of any one of claims 1 to 9 or the bacterial cell obtained by the process of claim 10 or 11, in the presence of a carbon source, and, optionally, isolating the aliphatic polyol.

13.   A composition comprising a propanediol or a butanediol at a concentration of at least 6% v/v and a plurality of bacterial cells according to any one of claims 1 to 9, or the bacterial cell obtained by the process of claim 10 or 11.

14.   A bacterial cell comprising a biosynthetic pathway for producing an aliphatic polyol and at least one genetic modification which increases one or more of

     (a) the biosynthesis of methionine in the bacterial cell;

     (b) growth of the bacterial cell during polyol-induced methionine starvation;

     (c) intracellular iron levels during polyol-induced growth inhibition;

     (d) biosynthesis of iron siderophores during polyol-induced growth inhibition; and

     (e) biosynthesis of iron-sulfur clusters during polyol-induced growth inhibition,

     optionally wherein the bacterial cell is the bacterial cell of any one of claims 1 to 9 or the bacterial cell obtained by the process of claim 10 or 11.

15.   A method for producing an aliphatic diol, comprising

     (a) culturing a plurality of bacterial cells capable of producing the aliphatic diol in a medium, the medium comprising methionine at a concentration of from about 0.004 g L^{-1} gDCW^{-1} to about 0.2 g L^{-1} gDCW^{-1} and at least one carbon source, wherein the medium comprises no more than 4 other natural amino acids at a concentration of at least 0.002 g L^{-1} gDCW^{-1}; and

     (b) optionally, isolating the aliphatic diol,

     wherein, optionally, the bacterial cell is the bacterial cell of any one of claims 1 to 9 or the bacterial cell obtained by the process of claim 10 or 11.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P7/18   C12N1/20
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)
C12P C12N

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, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2007/072279 A1 (MEYNIAL-SALLES ISABELLE [FR] ET AL) 29 March 2007 (2007-03-29) claim 1; examples 1,2,4-7 -----	1,2,4-13
A	WO 2012/078311 A1 (DU PONT [US]; VAN DYK TINA K [US]) 14 June 2012 (2012-06-14) examples 1,2 ----- -/--	1,2,4-13

[X] Further documents are listed in the continuation of Box C.   [X] 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	Date of mailing of the international search report
1 September 2017	30/10/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Bucka, Alexander

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	E. CELINSKA: "Klebsiella spp as a 1, 3-propanediol producer - the metabolic engineering approach", CRC CRITICAL REVIEWS IN BIOTECHNOLOGY, vol. 32, no. 3, 13 October 2011 (2011-10-13), pages 274-288, XP055355343, US ISSN: 0738-8551, DOI: 10.3109/07388551.2011.616859 page 282 - page 283 -----	1,2,4-13
X	T. A. SEIFLEIN ET AL: "Two Transsulfurylation Pathways in Klebsiella pneumoniae", JOURNAL OF BACTERIOLOGY, vol. 188, no. 16, 15 August 2006 (2006-08-15), pages 5762-5774, XP055355922, US ISSN: 0021-9193, DOI: 10.1128/JB.00347-06 page 5770; figure 7; tables 1,3 -----	1,2,4,9
Y	US 2005/054060 A1 (CHATEAU MICHEL [FR] ET AL) 10 March 2005 (2005-03-10) page 10, right-hand column page 15, left-hand column -----	1,2,4-13
A	ZIJUN XIAO ET AL: "Thermophilic fermentation of acetoin and 2,3-butanediol by a novel Geobacillus strain", BIOTECHNOLOGY FOR BIOFUELS, BIOMED CENTRAL LTD, GB, vol. 5, no. 1, 6 December 2012 (2012-12-06), page 88, XP021137745, ISSN: 1754-6834, DOI: 10.1186/1754-6834-5-88 figure 7; table 3 -----	1,2,4-13
X	MARION F. CUBITT ET AL: "A Metabolic Regulator Modulates Virulence and Quorum Sensing Signal Production in Pectobacterium atrosepticum", MOLECULAR PLANT-MICROBE INTERACTIONS, vol. 26, no. 3, March 2013 (2013-03), pages 356-366, XP055355504, US ISSN: 0894-0282, DOI: 10.1094/MPMI-09-12-0210-R figure 3 -----	1,2,4

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C(Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GÉRALDINE EFFANTIN ET AL: "Massive production of butanediol during plant infection by phytopathogenic bacteria of the genera Dickeya and Pectobacterium", MOLECULAR MICROBIOLOGY., vol. 82, no. 4, 27 October 2011 (2011-10-27), pages 988-997, XP055356162, GB ISSN: 0950-382X, DOI: 10.1111/j.1365-2958.2011.07881.x table 2 -----	1,2,4-13
Y	R. J. F. HAFT ET AL: "Correcting direct effects of ethanol on translation and transcription machinery confers ethanol tolerance in bacteria", PROCEEDINGS NATIONAL ACADEMY OF SCIENCES PNAS, vol. 111, no. 25, 9 June 2014 (2014-06-09) , pages E2576-E2585, XP055355335, US ISSN: 0027-8424, DOI: 10.1073/pnas.1401853111 page E2577; table 2 -----	1,2,4-13
A	WO 2009/086075 A1 (DU PONT [US]; LAROSSA ROBERT A [US]; SMULSKI DANA R [US]) 9 July 2009 (2009-07-09) example 7 -----	1,2,4-13
Y	ADITYA M. KUNJAPUR ET AL: "Deregulation of S-adenosylmethionine biosynthesis and regeneration improves methylation in the E. coli de novo vanillin biosynthesis pathway", MICROBIAL CELL FACTORIES, vol. 15, no. 1, 11 April 2016 (2016-04-11) , XP055355500, DOI: 10.1186/s12934-016-0459-x figures 4,5 -----	1,2,4-13
A	D.-H. LEE ET AL: "Adaptive Evolution of Escherichia coli K-12 MG1655 during Growth on a Nonnative Carbon Source, L-1,2-Propanediol", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 76, no. 13, 30 April 2010 (2010-04-30), pages 4158-4168, XP055355784, US ISSN: 0099-2240, DOI: 10.1128/AEM.00373-10 table 1 -----	1,2,4-13

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C(Continuation).   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BURGARD ANTHONY P ET AL: "OptKnock: A bilevel programming framework for identifying gene knockout strategies for microbial strain optimization", BIOTECHNOLOGY AND BIOENGINEERING, WILEY ETC, vol. 84, no. 6, 7 October 2003 (2003-10-07), pages 647-657, XP002489685, ISSN: 0006-3592, DOI: 10.1002/BIT.10803 [retrieved on 2003-10-07] table I	1,2,4-13
A	WO 2015/141886 A1 (CJ CHEILJEDANG CORP [KR]) 24 September 2015 (2015-09-24) example 1	1,2,4-13
A	P. NAWABI ET AL: "Engineering Escherichia coli for Biodiesel Production Utilizing a Bacterial Fatty Acid Methyltransferase", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 77, no. 22, 16 September 2011 (2011-09-16), pages 8052-8061, XP055111317, ISSN: 0099-2240, DOI: 10.1128/AEM.05046-11 figure 8	1,2,4-13
A	Y. USUDA ET AL: "Effects of Deregulation of Methionine Biosynthesis on Methionine Excretion in Escherichia coli", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 71, no. 6, June 2005 (2005-06), pages 3228-3234, XP055039220, ISSN: 0099-2240, DOI: 10.1128/AEM.71.6.3228-3234.2005 table 3	1,2,4-13
A	HARRY YIM ET AL: "Metabolic engineering of Escherichia coli for direct production of 1,4-butanediol", NATURE CHEMICAL BIOLOGY, NATURE PUBLISHING GROUP, UNITED KINGDOM, vol. 7, no. 7, 22 May 2011 (2011-05-22), pages 445-452, XP002690293, ISSN: 1552-4469, DOI: 10.1038/NCHEMBIO.580 [retrieved on 2011-05-22] page 448	1,2,4-13

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C(Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SABRA W ET AL: "Microbial cell factories for diol", BIOREACTOR ENGINEERING RESEARCH AND INDUSTRIAL APPLICATIONS I : CELL FACTORIES; [ADVANCES IN BIOCHEMICAL ENGINEERING/BIOTECHNOLOGY; ISSN 0724-6145 , VOL. 155], BERLIN ; HEIDELBERG : SPRINGER, [2016], DE, 17 October 2015 (2015-10-17), pages 165-197, XP008181563, ISBN: 978-3-662-49159-1 [retrieved on 2015-10-17] cited in the application the whole document -----	1,2,4-13

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INTERNATIONAL SEARCH REPORT

Box No. II   Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such
   an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III   Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

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see additional sheet
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1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable
   claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of
   additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers
   only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is
   restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

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1, 2, 4-13(all partially)
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Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the
                             payment of a protest fee.

                           ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest
                             fee was not paid within the time limit specified in the invitation.

                           ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of)
inventions in this international application, as follows:

1. claims: 1, 2, 4-13(all partially)

> A bacterial cell comprising a biosynthetic pathway for
> producing an aliphatic polyol and at least one genetic
> modification which reduces expression of the endogenous gene
> metJ; related methods and products.
> ---

2-8. claims: 1, 2, 4-13(all partially)

> A bacterial cell comprising a biosynthetic pathway for
> producing an aliphatic polyol and at least one genetic
> modification which reduces expression of an endogenous gene,
> wherein in each separate invention the gene is selected from
> the group consisting of iscR, yhjA, gtrS, ycdU, rzpD, sspA
> and rph; related methods and products.
> ---

9. claims: 3-13(partially)

> A bacterial cell comprising genetic modifications which
> reduce expression of metJ, relA and purT.
> ---

10. claims: 3-13(partially)

> A bacterial cell comprising genetic modifications which
> reduce expression of metJ and acrB, acrA or both.
> ---

11. claims: 3-13(partially)

> A bacterial cell comprising genetic modifications which
> reduce expression of fabR and ygfF.
> ---

12. claims: 3-13(partially)

> A bacterial cell comprising genetic modifications which
> reduce expression of iscR and relA.
> ---

13. claim: 14

> A bacterial cell comprising a biosynthetic pathway for
> producing an aliphatic polyol and at least one genetic
> modification which increases one or more of(a) the
> biosynthesis of methionine in the bacterial cell;(b) growth
> of the bacterial cell during polyol-induced methionine
> starvation;(c) intracellular iron levels during
> polyol-induced growth inhibition;(d) biosynthesis of iron
> siderophores during polyol-induced growth inhibition; and(e)

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

biosynthesis of iron-sulfur clusters during polyol-induced
growth inhibition.
---

14. claim: 15

A method for producing an aliphatic diol, comprising(a)
culturing a plurality of bacterial cells capable of
producing the aliphatic diol in a medium, the medium
comprising methionine at a concentration of from about 0.004
g L-1 gOCW-1 to about 0.2 g L-1 gDCW"1 and at least one
carbon source, wherein the medium comprises no more than 4
other natural amino acids at a concentration of at least
0.002 g L-1 gDCW"1 ; and(b) optionally, isolating the
aliphatic diol.

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