Construction of a synthetic metabolic pathway for biosynthesis of the non-natural methionine precursor 2,4-dihydroxybutyric acid

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2,4-Dihydroxybutyric acid (DHB) is a molecule with considerable potential as a versatile chemical synthon. Notably, it may serve as a precursor for chemical synthesis of the methionine analogue 2-hydroxy-4-(methylthio)butyrate, thus, targeting a considerable market in animal nutrition. However, no natural metabolic pathway exists for the biosynthesis of DHB. Here we have therefore conceived a three-step metabolic pathway for the synthesis of DHB starting from the natural metabolite malate. The pathway employs previously unreported malate kinase, malate semialdehyde dehydrogenase and malate semialdehyde reductase activities. The kinase and semialdehyde dehydrogenase activities were obtained by rational design based on structural and mechanistic knowledge of candidate enzymes acting on sterically cognate substrates. Malate semialdehyde reductase activity was identified from an initial screening of several natural enzymes, and was further improved by rational design. The pathway was expressed in a minimally engineered Escherichia coli strain and produces 1.8 g l⁻¹ DHB with a molar yield of 0.15.
The growing shortage of fossil raw materials has prompted an increasing economic and ecological interest in replacing petrol-based chemical syntheses by biochemical processes that rely on the utilization of renewable resources. The amino-acid product family provides a particularly striking example of our growing technological ability to replace petrol-based syntheses of bulk chemicals by microbial production processes. Most of the 20 proteinogenic amino acids are currently synthesized by fermentation processes using renewable glucose as the primary carbon source. A notable exception is the essential amino-acid methionine and its analogue 2-hydroxy-4-methylthio-butyrate (HMTB), which are still exclusively produced from petrol. The cumulated annual volume of methionine and HMTB production reached ~1 million tons in 2014, the largest part of which is used as a supplement in animal diet, greatly increasing the nutritional value of feed stocks. Methionine production is thus an important cornerstone in the satisfaction of the ever-growing demand of the human population for animal protein. The development of sustainable means of methionine or HMTB production from renewable resources is therefore of significant importance.

Economically viable methionine and HMTB fermentation processes are currently lacking, which is mainly due to the extremely high metabolic cost of incorporating sulfur into these molecules. A promising alternative to the microbial production of HMTB is to envisage a two-stage process whereby the fermentative production of a functionalized carbohydrate precursor is followed by the chemical incorporation of sulfur. For example, by employing established chemistry, it is possible to convert 2,4-dihydroxybutyric acid (DHB) and methanethiol into HMTB with 100% carbon yield. However, while the chemical synthesis of DHB from petrol-derived γ-butyrolactone is feasible, it is not economically viable.

Although trace amounts of DHB have been identified in human patients suffering from succinic semialdehyde dehydrogenase deficiency, no natural metabolic pathway to access DHB has ever been described. Thus, DHB is a metabolite that cannot be efficiently synthesized via currently annotated natural metabolic networks. These considerations have motivated our exploration of plausible non-natural pathways leading to DHB that take advantage of synthetic biology approaches. We herein present a new synthetic DHB pathway whose chemical logic was inspired by the biosynthesis of homoserine. This amino acid is naturally produced by the successive action of aspartate kinase (AK), aspartate-β-semialdehyde dehydrogenase (ASD), and homoserine dehydrogenase (HSD). Homoserine and DHB are structurally very similar molecules and differ only in the presence of an amino or hydroxyl group, respectively, on the carbon 2 position. Since the amino groups of the homoserine pathway intermediates are not involved in the catalytic reaction mechanism of the individual enzymes, we considered it feasible to apply the (bio)chemical principle of the natural homoserine pathway to convert malate into DHB. However, the existence of neither the required malate kinase (MK), malate-β-semialdehyde dehydrogenase (MSD), and malate semialdehyde reductase (MSR) enzymatic activities, nor the malyl-4-phosphate (malyl-P) and malate-4-semialdehyde pathway intermediates has been previously reported. Thus, it was not possible to use established approaches in which the synthetic pathway is assembled by harvesting genes encoding the required activities from different biological sources and expressing them together in a single production organism.

We therefore set out to create the required enzymatic activities by computer-aided engineering of template enzymes acting on sterically cognate substrates but which displayed little or no activity towards the synthetic pathway intermediates. We used structural analysis and molecular modelling methods to identify target residue sites for mutation and designed mutant libraries of moderate size that were screened for the isolation of mutants with the requisite enzymatic properties. We have obtained all three enzymatic activities and demonstrated the production of 1.8 g l\(^{-1}\) DHB from an initial glucose concentration of 20 g l\(^{-1}\) by expressing the synthetic pathway in an E. coli strain.

**Results**

**Thermodynamic feasibility and maximum DHB yield.** The proposed pathway proceeds through the activation of the malate β-carboxylate group by phosphorylation followed by two successive rounds of reduction to yield DHB (Fig. 1). The negative standard Gibbs free energy for the pathway (Supplementary Note 1; Supplementary Table 1) attests to its thermodynamic viability. Stoichiometric analysis of the metabolic network in E. coli shows that DHB can be produced from glucose with a theoretical maximum yield of 1.5 mol mol\(^{-1}\) (Supplementary Note 2; Supplementary Fig. 1). Given that DHB can be converted into the methionine-analogue HMTB without carbon loss, the production of methionine via DHB increases the theoretical yield by ~100% compared to the conventional one-step biosynthesis of methionine from glucose and sulfate, and by ~30% when compared to the biosynthesis of methionine from glucose and methanethiol.

Given the structural similarity of DHB and homoserine pathway intermediates, we identified three homoserine pathway enzymes as prototype candidates for screening and engineering of enzyme activity in the DHB pathway. We chose AK, encoded by *lysC*, and ASD, encoded by *asd*, from *E. coli*, and HSD, encoded by *HOM6* (ref. 19), from *Saccharomyces cerevisiae* since the HSD enzymes in *E. coli* are bifunctional enzymes with an associated AK activity. We found that Ec-Asd had only trace activity on the synthetic substrate \(k_{cat} = 0.13 \text{ s}^{-1}\) on malyl-P versus 36 s\(^{-1}\) on aspartyl-4-phosphate (aspartyl-P), whereas Ec-LysC and Sc-Hom6 had no detectable activity on malate and malate semialdehyde, respectively (Supplementary Table 2; Supplementary Note 3). These findings indicated that the feasibility of the envisaged *de novo* synthetic pathway necessitated the engineering of all three enzyme activities.

**Engineering of malate kinase activity.** We first set out to engineer MK activity into aspartate kinase III from *E. coli* (Ec-LysC). Binding interactions of the (L)-aspartate natural substrate in the enzyme active site are revealed in the 2.5 Å X-ray crystallographic structure of the dimeric wild-type *L. casei* abortive ternary complex with (L)-aspartate and the Mg-ADP reaction co-product in the R-state. The amino-acid substrate is anchored in position via water-mediated interactions of the β-carboxylate group with the metal ion in Mg-ADP, and by respective salt bridge electrostatic interactions of the (L)-aspartate α-amino and α-carboxylate groups with charged enzyme side-chain functional groups of Glu119 and Arg198 as shown in Supplementary Fig. 2. Productive binding of the (L)-aspartate substrate is further supported by a network of van der Waals and hydrogen bonding interactions with other residues in the active site.

(L)-Malate is an isostere of (L)-aspartate in which the positively charged α-amino group is replaced by an uncharged hydroxyl group. In common with other (succinate and malonate) structural analogues of (L)-aspartate that do not contain an α-amino group, and which therefore cannot form a salt-bridge with the side-chain of Glu119, (L)-malate has been reported to be a weak competitive inhibitor of Ec-LysC with a \(K_i\) of 53 mM (ref. 23). These observations suggest that (L)-malate binds non-productively to the wild-type enzyme in the absence of additional binding energy provided by the salt-bridge interaction.
To investigate the impact of Glu119 substitution by other amino acids, we carried out saturation mutagenesis at this residue position and analysed the enzymatic activities of the resulting mutants. Trace but measurable enzymatic activity on (l)-malate could be detected with the Ec-LysC E119Q and Ec-LysC E119N mutants, in which the Glu119 side-chain carboxylate group is replaced by an uncharged carbamoyl group (Supplementary Table 3). Significant increases of up to 200-fold in the $k_{cat}/K_m$ value for (l)-malate were obtained in E119G, E119A, E119S and E119C variants with shorter side-chains at residue position 119 (Fig. 2a; Supplementary Table 3).

To further improve the catalytic efficiency towards (l)-malate, the construction of a small combinatorial mutant library was undertaken. Analysis of binding interactions of Ec-LysC in the experimental complex with (l)-aspartate enabled the selection of eight amino-acid residue positions including Glu 119, highlighted in Supplementary Fig. 2. Of these eight positions, five make direct contact with the (l)-aspartate substrate (Ala40, Thr45, Glu119, Phe184 and Ser201), and three others (Val115, Thr195 and Thr359) are located within a second residue shell not in direct contact with the substrate. The design of the library, described in the Supplementary Note 4, took account of natural sequence variation in the active-site region of Ec-LysC homologues and the results of computational re-design at nine residue positions in the vicinity of residue position 119. Restrictions placed on the number of permitted mutations at each of the eight residue positions in the library constrained its overall size to 2,160 positions in the library constrained its overall size to 2,160 possible theoretical combinations (Supplementary Table 5). A miniaturized screening protocol (see Methods) was devised to permit the direct measurement of (l)-malate kinase activity by a single end-point measurement with an accuracy of ±8% in microtiter plates. To ensure adequate sequence space coverage, 6,720 clones were tested by this method leading to the identification of nine positive variants which were confirmed by enzymatic assay of sequenced and individually purified clones. The best mutant Ec-LysC V115A:E119S:E434V exhibited a $k_{cat}/K_m$ value of 0.82 s$^{-1}$ mM$^{-1}$ on (l)-malate, approximately only twofold lower than that of the wild-type enzyme acting on the (l)-aspartate natural substrate. In addition, the mutant retained very little activity towards (l)-aspartate, resulting in a marked change in enzyme specificity (Fig. 2b). It is of note that the Glu434 position at the enzyme surface was not targeted in the construction of the library, but an E434V mutation was unexpectedly found in the best malate kinase mutant. A molecular model of (l)-malate bound in the active-site of the ternary complex with the Ec-LysC V115A:E119S double mutant and Mg-ADP is shown in Fig. 2c.

To render the malate kinase enzyme more efficient for in vivo applications, we individually tested E250K, T344M, S345L and T352I mutations previously shown to alleviate feedback inhibition by lysine in the wild-type enzyme$^{25}$. We found that all these mutations strongly decreased the inhibitory effect of lysine on malate kinase activity (Supplementary Fig. 4). The quadruple mutant, Ec-LysC V115A:E119S:E250K:E434V was therefore selected for implementation of the DHB pathway.

**Engineering of malate semialdehyde dehydrogenase activity.** The aspartate semialdehyde dehydrogenase from *E. coli* (Ec-Asd) enzyme was found to possess only trace activity on malyl-P in the reductive (biosynthetic) reaction direction, and on malate semialdehyde (MSA) in the reverse oxidative phosphorylation reaction (Supplementary Table 6). We first sought to engineer increased activity towards the malyl-P/MSA substrate/product couple through site-directed mutagenesis of the Ec-Asd Gram-negative bacterial enzyme. Active site residues involved in the binding of aspartate semialdehyde (ASA) to Ec-Asd have been previously identified in an X-ray crystal structure of a covalent complex formed as the reaction product of Cys135 thiol group attack on the substrate analogue S-methylcysteine sulfoxide in the presence of NADP$^+$ (ref. 26). The binding of the ASA $\alpha$-amino and $\alpha$-carboxylate groups in the Ec-Asd active-site occurs via salt-bridge interactions with oppositely charged Glu241 and Arg267 residue side-chains. This salt bridging arrangement is similar to that of the (l)-aspartate substrate $\alpha$-amino and $\alpha$-carboxylate groups in the complex with *E. coli* aspartate
kinase III (ref. 22) that catalyses the preceding reaction step in the physiological pathway. The 2-OH group in a malyl-P/MSA substrate/product couple might be expected to hydrogen bond with Glu241 in Ec-Asd thereby providing for substrate binding similar to that of the natural substrate derivative in the experimental complex. However, the poor observed activity of wild-type Ec-Asd on MSA compared to ASA may be in part due to a lowering in the binding affinity for an alternative substrate carrying net negative charge. Replacement of the conserved Glu241 residue in the wild-type E. coli enzyme by residues with uncharged side-chains would then be expected to improve MSA binding affinity and reduce that of ASA.

To test this hypothesis, saturation mutagenesis of Ec-Asd was carried out at residue position 241. Since aspartyl-P and malyl-P are highly unstable molecules, initial kinetic characterization of the wild-type and mutant enzymes was carried out on the MSA substrate of the reverse (oxidative phosphorylation) reaction. The wild-type enzyme and the most promising mutants were examined as alternative enzyme engineering platforms. ASDs from these phylogenetic branches are differentiated by the covalently bound MSA reaction intermediate.

In an attempt to further increase malyl-P reductive dephosphorylation activity, orthologues of the ASD family from the Gram negative, and archaeal and fungal phylogenetic branches were examined as alternative enzyme engineering platforms. The active-site region in a modelled structure of a putative hemithioacetal MSA substrate derivative covalently bound to Cys135 in the Ec-Asd E241Q mutant (see Methods) is shown in Fig. 3c. The figure highlights electrostatic interactions between residues in the mutant enzyme active site and the covalently bound MSA reaction intermediate.

In an attempt to further increase malyl-P reductive dephosphorylation activity, orthologues of the ASD family from the Gram negative, and archaeal and fungal phylogenetic branches were examined as alternative enzyme engineering platforms. ASDs from these phylogenetic branches are differentiated by the presence of characteristic structural insertions and deletions in the co-enzyme binding-site region and at the enzyme homodimer subunit interface. Although a high degree of conservation of
amino-acid residue functional groups exists within the active-site core, shared sequence identities of ASD family enzymes fall to as low as 10% (ref. 27). This structural variation correlates closely with marked differences in ASA oxidative phosphorylation catalytic efficiency, which varies by two orders of magnitude\(^2\). The natural variation in structure and sequence afforded by Gram-positive bacterial and archaeal ASDs may thus provide opportunities to modulate kinetic reaction rates other than through the introduction of additional mutations in the enzyme active-site.

The activities of wild-type and mutant ASDs from the Gram-positive bacterium Bacillus subtilis (Bs), that shares 26% sequence identity with Ec-Asd, and the archaeon Methanocaldococcus jannaschii (Mj) which is 21% identical to Ec-Asd, were assayed on aspartyl-P and malyl-P. Enzymatic activities of wild-type Bs-Asd and Mj-Asd on malyl-P were approximately threefold lower than those observed for Ec-Asd (Fig. 3b; Supplementary Table 6). However, by analogy to the effects observed in Ec-Asd the reaction specificity of these enzymes is significantly improved in favour of malyl-P when the conserved active-site glutamate residues (Glu218 in Bs-Asd, Glu210 in Mj-Asd) are mutated to favour of malyl-P when the conserved active-site glutamate (Glu218 in Bs-Asd, Glu210 in Mj-Asd) are mutated to glutamine or cysteine. The best result was obtained for the Bs-Asd E218Q mutant enzyme, which displayed an almost eightfold increased activity on malyl-P, and a \(k_{cat}/K_{M}\) value of 0.38 s\(^{-1}\). This is comparable with Mj-Asd wild-type activity towards aspartyl-P (\(k_{cat} = 0.38\) s\(^{-1}\)). It is, however, much lower than that of the Ec-Asd wild-type (\(k_{cat} = 14.5\) s\(^{-1}\)). The kinetically efficient alternating-sites mechanism operating in bacterial ASD forms is absent in Mj-ASD and other archaeal ASDs. Comparison of the relative turnover numbers suggests that the conversion of malyl-P to MSA catalysed by the Bs-Asd E218Q mutant may also be rate-limited by decoupled inter-subunit communication. Taking into account the loss in activity on the natural substrate, the specificity of Bs-Asd E218Q was shifted 650-fold towards malyl-P (Fig. 3a,b; Supplementary Table 6). This enzyme variant was therefore chosen for integration in the construction of the DHB pathway.

**Engineering of malate semialdehyde reductase activity.** Having found homoserine dehydrogenase from Saccharomyces cerevisiae (Sc-Hom6) to be inactive on MSA, we sought to identify an alternative enzyme template for the engineering of MSR activity. We experimentally screened for MSA reductase activity in oxidoreductases that act on substrates structurally similar to MSA (Supplementary Table 7). Significant MSA reductase activity was detected for the broad-range aldehyde reductase, Ec-YqhD\(^2\), from *E. coli*, methylbutyraldehyde reductase, Sc-Ypr1 (ref. 31), from *S. cerevisiae*, 4-hydroxybutyrate dehydrogenase, Pg-4hbd\(^3\), from *Porphyromonas gingivalis*, and the succinic semialdehyde dehydrogenase, Ms-Ssr\(^4\), from *Metallosphaera sedula*. Amongst the enzymes that were active on MSA, the NADP\(^+\)-dependent succinic semialdehyde reductase from *Metallosphaera sedula* displayed the highest specific activity.
activity (4.0 μmol min$^{-1}$ mg$^{-1}$) and the best affinity (1.1 mM). However, its $k_{cat}/K_m$ value was 112-fold lower than that of the (Sc-Hom6) homoserine dehydrogenase benchmark enzyme in the natural pathway on aspartate semialdehyde (Supplementary Table 7; Supplementary Note 5). Therefore, a comparative molecular modelling approach based on the exploitation of conserved relational structural and functional features was used to improve the catalytic efficiency of Ms-Ssr towards MSA.

Ms-Ssr is a zinc-dependent alcohol dehydrogenase$^{34}$ belonging to the medium-chain dehydrogenase/reductase (MDR) alcohol dehydrogenase (ADH) superfamily$^{35}$. A molecular model of the Ms-Ssr dimer in a ternary complex with NADP$^+$ and (L)-2,4-dihydroxybutyric acid (DHB) with the atomic co-ordinate data from experimentally determined alcohol dehydrogenase structures (see Methods). Binding interactions of the zinc-coordinated DHB alcholate anion in the active-site region are shown in Fig. 4a. The main contacts of the DHB x-carboxyl and 2-hydroxyl groups in the model are with the Gln108 and Asn305 residues. The presence of bulky Phe85 and Leu281 side-chains lining the substrate binding pocket suggests that the Ms-Ssr enzyme has an intrinsic preference for (unbranched) primary alcohol substrates. Structural analysis furthermore revealed that the enzyme employs a proton relay pathway which is different from the archetypal pathway that is present in most of the ADH family enzymes, and which is considered to be less efficient$^{35-37}$ (Fig. 4b). The engineering of an ADH1E-like proton relay system into Ms-Ssr, comprising a histidine at position 43 and an arginine at position 39, was thus considered to be a promising means of directly improving Ms-Ssr catalytic activity.

The Ms-Ssr H39R:N43H double mutant exhibited an approximately fourfold increased catalytic efficiency on MSA compared to the wild-type enzyme, but still retained a strong preference for SSA over MSA (Fig. 4c, Supplementary Table 8). However, SSA is produced in E. coli only under extreme acid stress conditions, and its supply can be turned off by deleting both glutamate decarboxylases GadA and GadB$^{38}$. Potential competition between SSA and MSA was therefore not considered to be a significant impediment to the efficient biosynthesis of DHB, and the Ms-Ssr H39R:N43H enzyme was thus chosen for incorporation in the DHB pathway.

Minimal metabolic engineering enables biosynthesis of DHB. To assure functional expression of the DHB pathway, we assembled a synthetic operon in the medium copy number pACT3 plasmid$^{39}$, which expressed the three genes encoding the best DHB pathway enzymes (MK: Ec-LysC V115A:E119S:E250K:E434V, MSD: Bs-Asd E218Q, MSR: Ms-Ssr H39R:N43H) under the control of the inducible tac promoter. The resulting plasmid pDHB was transformed into the wild-type E. coli MG1655 strain and produced 60 mg l$^{-1}$ DHB after 24 h of shake-flask cultivation in glucose mineral medium. The wild-type control strain that produced no detectable quantities of DHB (Fig. 5; Supplementary Table 9). These results indicated that cellular DHB production via the synthetic pathway can be achieved, and we set-out to increase DHB production by metabolic engineering of the host strain and by optimization of the expression system.

The DHB-producing strain accumulated 50% more acetate than the control strain (Fig. 5). Therefore, we first tested whether DHB production could be increased by inactivating the pyruvate...
Figure 5 | Production of 2,4-dihydroxybutyrate by engineered *E. coli* strains. Cells were cultivated in shake flasks on mineral medium containing 20 g per l glucose. Values correspond to concentrations after 24 h of cultivation. Error bars represent STDV from at least two replicate experiments. All strains were derived from *E. coli* K-12 substr. MG1655. All plasmids were derived from the pACT3 medium-copy number plasmid. pHOM expresses the genes encoding the homoserine pathway enzymes: AK: Ec-LysC E250K, ASD: Ec-Asd, HSD: Sc-Hom6. pDHB expresses the genes encoding the DHB pathway enzymes: MK: Ec-LysC V115A:E119S:E250K, MSD: Bs-Asd E218Q, MSR: Ms-Ssr V39R:N43H, pDHBopt-ppc* additionally expresses the malate-insensitive PEP carboxylase mutant PpcK620S, pDHBopt-ppc* has optimized ribosome binding sites in front of each DHB pathway gene. pDHBopt-ppc*(Ec-asd*) and pDHBopt-ppc*(Mj-asd*) express, respectively, the Ec-Asd* E241Q or Mj-Asd* E210Q mutant enzymes instead of Bs-Asd E218Q.

Discussion

Biosynthesis of value-added chemicals is typically achieved by metabolic engineering, which relies on alleviating product feedback inhibition of natural metabolic pathways and optimizing cofactor supply to enable overproduction of a target molecule. However, many molecules of chemical interest, such as DHB, are not naturally produced by living cells and annotated metabolic pathways for their biosynthesis do not exist. The development of tools and methodologies for the construction of synthetic pathways that expands the range of biochemically produced compounds or that improves stoichiometric yields in the biosynthesis of natural metabolites is therefore of major interest. However, only a handful of studies have so far succeeded in the creation of pathways for the production of unnatural metabolites, and these largely rely on the recombination of cognate enzymatic activities to build up the desired pathway. While the concept of implementing synthetic pathways through enzyme engineering for the biosynthesis of unnatural metabolites has been formulated, and the industrial potential of this technology has been recognized, experimental studies that demonstrate the actual feasibility of fully synthetic enzyme pathway construction are still lacking.

Our study demonstrates that the implementation of an efficient multistep non-natural DHB pathway is possible by applying design principles of synthetic biology, that is, conceptual pathway design and thermodynamic evaluation, computer-aided enzyme development, and strain engineering, in a straightforward manner. We have built upon the pioneering work of Holbrook et al. who were the first to show that structure-guided rational protein engineering can provide new enzymatic functions, and extended their ideas to the redesign of an entire fully synthetic enzymatic pathway. Our work shows that industrial biosynthesis of non-natural value-added chemicals through synthetic biology is a realizable objective.

The engineering of the individual enzymatic activities was crucial in achieving a significant level of DHB production. This is supported by the fact that the expression of the unmodified template enzymes did not yield any DHB. In line with this result, our group and Li et al. detected only trace amounts of DHB (6.4 mg l⁻¹) when investigating an alternative non-natural DHB pathway, which employs malyl-CoA synthetase, malyl-CoA reductase, and malate semialdehyde reductase activities, and previously reported to be more beneficial for the production of Krebs cycle-derived C4 acids than maximizing Ppc activity. When the optimized pDHBopt-ppc* plasmid was expressed in a wild-type strain, we observed the accumulation of 1.8 g l⁻¹ DHB after 24 h of cultivation, corresponding to a molar yield of 0.15 (Fig. 5; Supplementary Table 9). This nearly twofold improvement can be attributed to a significant increase of MK and MSD activities, whereas the Ppc and MSA reductase activities remained nearly unchanged upon expression of the new plasmid (Supplementary Table 10). Expression of the optimized plasmid in the acetate pathway mutants did not further increase DHB production (Supplementary Table 9), in accord with the absence of acetate accumulation in DHB-producing strains. Furthermore, DHB synthesis dropped to ~0.7 g l⁻¹ when Mj-Asd E210Q or Ec-Asd E241Q were applied as the MSD enzyme, indicating that the superior *in vitro* performance of Bs-Asd E218Q also translates into the best cellular DHB production (Fig. 5). Taken together our results show that overexpression of the anaplerotic Ppc activity is a key factor in enabling cellular DHB production, and that increasing the barely measurable MSD activity is a major requirement for the further improvement of DHB production levels.
which was assembled using natural enzymes with only residual activities on the synthetic substrates.

The activities of the corresponding homoserine pathway enzymes may serve as a benchmark in a quantitative assessment of the synthetic activities obtained. The catalytic performance of the best malate kinase mutant (Ec-LysC V115A:E119S:E250-K/E434V) is only two-fold lower than the activity of the wild-type AK enzyme (LysC) on the natural substrate, and can therefore be considered adequate for the biosynthesis of DHB. In contrast, the best MSD (Bs-Asd E218Q) and MSR (Ms-Ssr H393R/N43H) enzymes have approximately 50- and 25-fold lower activities than their respective ASD and HSD (Bs-Asd, Sc-Hom6) counterparts. Thus, further improvement of these enzymatic activities is necessary to increase DHB production via our pathway.

The engineering of appropriate host strains is another important factor for achieving efficient product formation. In the present work we have shown that the expression of a malate-insensitive anaplerotic enzyme (Ppc K620S) significantly increases DHB production. It is expected that additional metabolic engineering to optimize both the supply of the natural DHB precursor malate and the required cofactors will have an additional positive impact on DHB production.

The biosynthesis of DHB from renewable carbon sources can enable a more sustainable production of methionine, whose annual production volume increases at an average rate of ~5%, to satisfy the increasing demand for this amino acid in the production of animal protein9. In addition, DHB may serve as an intermediate for the (bio)chemical synthesis of other value-added products. Recently, synthetic metabolic pathways have been disclosed that start from DHB to produce 1,3-propanediol52, or 1,2,4-butanetriol52. Furthermore, 2-hydroxy-γ-butyrolactone, which is spontaneously formed from DHB at acidic pH, can be chemically converted to γ-butyrolactone, and 1,4-butanediol53-54. All of these compounds are bulk chemicals with a wide range of applications as precursors for plastics, solvents and propellants55,52,55. Thus, we consider DHB to be a versatile platform molecule with high industrial potential. Our study describes an efficient biosynthetic route for the production of this hitherto inaccessible molecule from renewable resources.

Methods

DNA manipulations. Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs and used according to manufacturer’s instructions. DNA plasmid isolation was performed using GeneJET Plasmid Miniprep Kit (Thermo scientific). DNA extraction from agarose gel was carried out using the GeneJET Gel Extraction Kit (Thermo scientific). DNA sequencing was carried out by Beckman Coulter Genomics (Takley, United Kingdom).

Vectors for protein expression and purification. Genes were amplified from genomic DNA (extracted from Escherichia coli MG1655, Bacillus subtilis BS168 (kindly provided by Dr M. Jules, INRA, Paris), Saccharomyces cerevisiae BY4741, Metallosphaera sedula DSM5348 (kindly provided by Prof. G. Fuchs, University of Freiburg, Germany), using high fidelity DNA polymerase Phusion (Finnzymes) and primer pairs listed in Supplementary Table 11. The genes asd and dhbd from Methanocaldococcus jannaschii DSM2661 and Parophymonas gingivalis W83, respectively, were synthesized by Eurofins. The resulting DNA fragments were digested with suitable restriction enzymes (Supplementary Table 11), cloned into the corresponding sites of PET28a (Novagen) using T4 DNA ligase (Biolabs), thereby adding an N-terminal hexa-His tag. The ligation product was transformed into E. coli DH5α cells (NEB) using standard protocols54. The resulting plasmids were isolated, and shown by DNA sequencing to contain correct sequences.

Protein mutant construction by site-directed mutagenesis. Site-directed mutagenesis was carried out on pET28-derived plasmids using the primer pairs listed in Supplementary Table 12. Point mutations to change the amino-acid sequences were introduced by PCR (Phusion 1U, HF buffer 20% [v/v), DNTPs 2.5 mM, direct and reverse primers 0.1–0.5 μM each, template plasmid ~ 50 ng, water to bring to 50 μl). When possible, plasmids created by PCR contained new restriction sites (introduced by silent mutations) in addition to the functional mutation to facilitate identification of mutated clones. The PCR products were digested by DpnI at 37°C for 1 h to remove template DNA, and transformed into NEB-α-blue competent E. coli cells (NEB). The mutated plasmids were identified by restriction site analysis and verified to carry the desired mutations by DNA sequencing.

Protein expression and purification. Expression of enzymes: E. coli BL21 (DE3) cells were transformed with the appropriate pET28a-derived plasmids using standard protocols56. Strains containing the expression vectors were pre-cultured overnight in Luria-Bertani (LB) medium before they were used to inoculate 50 ml of LB medium at OD600 = 0.2. Protein expression was induced by addition of IPTG and 0.1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) when OD600 reached ~ 0.6. After 3 h of incubation at 37°C in LB containing 50 μg/ml kanamycin, cells were harvested by centrifugation at 13,000 × g for 10 min (Sorvall ST 40R, Thermo) and stored at ~20°C until further analysis.

Purification of enzymes: Frozen cell pellets from expression cultures were suspended in 0.5 ml of breakage buffer (50 mM Hepes, 300 mM NaCl, pH 7.5) and broken open by four successive rounds of sonication (Bioblock Scientific, VibraCell 72,437) with the power output set to 30%. Cell debris was removed by centrifuging the crude extracts for 15 min at 4°C at 13,000 × g (Sorval ST 40R, Thermo) and retaining the clear supernatant. RNA and DNA were removed from the supernatant by adding 15 μg/ml RNase A and 10 μg/ml RNase T1 respectively, and incubating for 30 min at 37°C. The samples were then centrifuged at 13,000 × g for 10 min at 20°C and retaining the supernatant. Clear protein extract was incubated for 1 h at 4°C with 0.75 ml bed volumes of TalonTM Cobalt affinity resin (Clontech). The suspension was centrifuged at 700 × g in a table top centrifuge (Sorval ST 40R, Thermo) and supernatant was removed. The resin was washed with wash buffer (50 mM Hepes, 15 mM Imidazole, pH 7.5) before enzyme elution was done with 0.5 ml of elution buffer (50 mM Hepes, 300 mM NaCl, 250 mM Imidazole, pH 7.5). The eluate was used for the purification of aspartate and aspartate semialdehyde dehydrogenase preparations the eluate was exchanged with wash buffer w/o imidazole using Amicon Ultra-0.5 ml centrifugal filters (cut-off 10 KDa, 2 centrifugations at 13,000 × g for 7 min at 4°C). Purity of eluted enzymes was verified by SDS-PAGE analysis. Protein concentrations were measured with the method of Bradford57.

Enzymatic assays. Malate and aspartate kinase activity: Aspartate or malate kinase activities were assayed by coupling ADP production in the kinase reactions to NADH oxidation in the presence of phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase. The assay mixture contained 50 mM Hepes (pH 7.5), 50 mM KCl, 5 mM MgCl2, 0.5 mM NADH, 2 mM ATP, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when OD600 reached ~ 0.6. The reactions were followed by the characteristic absorption of NADH at 340 nm by restriction site analysis and verified to carry the desired mutations by DNA sequencing.

Malate and aspartate β-semialdehyde dehydrogenase activity: α-semialdehyde dehydrogenase activity was assayed in both the reductive and the oxidative direction were carried out by following oxidation of NADPH during the reduction of malylphosphate or aspartylphosphate. Since both substrates are unstable and not commercially available, they were prepared in situ by the action of a purified malylphosphate or aspartylphosphate (Ec-LysC E119S) or aspartylsemialdehyde (Ec-LysC E119S) to malate or aspartate semialdehyde to 4-phospho-(l)-aspartate or 4-phospho-(l)-malate, respectively58. The assay mixture contained 200 mM Hepes (pH 9), 50 mM K2HPO4, 5 mM MgCl2 and 0.25 mM NADP. Reactions were started by adding (l)-aspartate-β-semialdehyde hydrate trifluoroacetate (maintained at pH 9) to prevent degradation) which is a suitable substrate for enzymatic tests of homoserine dehydrogenase58. MSA was produced freshly prior to the enzymatic tests by the deprotection of the stable MSA derivative 2-[(4S)-2,2-dimethyl-5-oxo-1,3-dioxolan-4-yl]acetaldheyde (DMOADA) (provided by Activation, France).

Malate and aspartate β-semialdehyde dehydrogenase activity: α-semialdehyde dehydrogenase activity was assayed in both the reductive and the oxidative direction were carried out by following the reduction of NADPH during the oxidation of aspartate semialdehyde or malate semialdehyde to 4-phospho-(l)-aspartate or 4-phospho-(l)-malate, respectively58. The assay mixture contained 200 mM Hepes (pH 9), 50 mM K2HPO4, 5 mM MgCl2 and 3 U per ml malate kinase or aspartate kinase. The reaction was started by adding 20 mM malate or aspartate. Assays in the reverse biosynthetic (oxidative) direction were carried out following the oxidation of NADPH during the dehydrogenation of α- and β-methylmalate semialdehyde to malylphosphate or aspartylphosphate. The reactions were started by adding appropriate amounts of purified enzyme or cell extract. Reactions were followed by adding appropriate amounts of malyl semialdehyde, aspartyl semialdehyde, or succinic semialdehyde. All enzymatic assays were carried out at 37°C in 96-well flat bottom microtitre plates in a final volume of 250 μl. The reactions were followed by the characteristic absorption of NADH at 340 nm in a microplate reader (Biotek EON).

Construction of the aspartate kinase mutant libraries. Mutagenesis of the lysC gene was carried out using the ISOR method59. Primers pETseq_for and pETseq_rev (Supplementary Table 13) were used to amplify the gene LysC from
the plasmid pET28-lysC. The purified PCR product was digested with DNase I and endonuclease to obtain fragments having an average size of ~100 bp. Gel-purified fragments (~100 bp) and reaction mix were real-mixed for PCR using the primers Ec_lysC_for and Ec_lysC_rev (Supplementary Table 13). The PCR was gel purified, digested with NdeI and BamHI and ligated into the corresponding sites of plasmid pET28a (Novagen). Commercial NEB 5-α-plasmid competent E. coli cells were transformed with the ligation product and plated on LB agar plates containing 50 μg per ml kanamycin to obtain isolated colonies. A total number of 6,720 colonies was picked and transferred into 96-well microplates (Nunc Brands Products, Roskilde, Denmark) (containing in each well 200 μl of LB medium supplemented with kanamycin and 8% glycerol. The storage microplates were kept at ~8°C after overnight culture at 30°C.

Screening of the asparatase kinase mutant library. Screening of the enzyme variants was carried out using the automated robotics facilities of the ICEO platform (http://iceo.genotoul.fr/index.php?id=172&172=2). Storage microplates containing plasmid pET28-lysC were thawed and inoculated to inoculate cultures into 96-well microplates (Nunc Brands Products, Roskilde, Denmark) filled with 200 μl x 24 wells extract-tryptone (YT) medium (16 g per l tryptone, 10 g per l yeast extract, 5 g per l NaCl) per well supplemented with kanamycin (50 μg per ml). The starter cultures were inoculated from the storage microplates and cultivated in 96-well microplates (200 μl of YT medium and 50 μg per ml kanamycin). After overnight incubation at 30°C under horizontal shaking at 250 rpm (Infors HT, Bottmingen, Switzerland), 50 μl of each starter culture were used to inoculate the expression cultures which were cultivated in 96-deepwell plates (Algene, Epsom, UK) containing 1 ml auto-inducing medium ZYM 5-052 (ref. 60) supplemented with kanamycin (50 μg per ml). Expression cultures were inoculated at 30°C for 24 h at 700 rpm in an incubator-shaker (INFORSH HT, Bottmingen, Switzerland). The supernatant was removed by centrifugation (10 min, 3,700 x g, 4°C) before the cell pellets were suspended in 200 μl of lysozyme solution (0.5 mg per ml), incubated for 30 min at 30°C and stored at ~8°C overnight. Cell pellets were thawed by incubation at room temperature for 1 h and 800 μl of 0.3 M HCl were added to each well to digest DNA. Plates were incubated for 30 min at 30°C before pelleting debris by centrifugation (10 min, 3,700 x g, 4°C). Protein extracts were diluted 250-fold in water by two successive transfers onto new microplates. 10 μl of the diluted enzymatic extracts were transferred onto a new microplate to which 60 μl of reaction mix (115 mM Hepes buffer pH 7.5, 7.5 mM ATP, 5 mM MgCl₂, 3.85 mM phosphoenolpyruvic acid, 2.88 mM NADH, 3.85 U per ml lactate dehydrogenase and 3.85 U per ml pyruvate kinase) was added. The reaction was started by adding 0.5 μl of cell homogenate by adding 150 μl of 0.3 M HCl. After 20 min incubation at room temperature, 600 μl of the reaction mix was transferred from each well onto a new microplate containing 190 μl of the reaction mix (115 mM Hepes buffer pH 7.5, 7.5 mM ATP, 5 mM MgCl₂, 3.85 mM phosphoenolpyruvic acid, 2.88 mM NADH, 3.85 U per ml lactate dehydrogenase and 3.85 U per ml pyruvate kinase)

Construction plasmids for biosynthesis of DHB. Escherichia coli K-12 substr. MG1655 (ATCC 47076) was used as the parental strain for the construction of DHB-producing strains. Deletion of the ackA-pta operon was carried out using the method of Datsenko & Wanner63. The kamycin resistance cassette of pKD4 vector (Thermo-Scientific) was inserted into the genomic target locus (Supplementary Table 16). The resulting DNA fragment was ligated into pENTR(−asd*)-ppc*. The resulting plasmid was transformed into NEB 5-α-plasmid competent E. coli cells (NEB) and verified by DNA sequencing to contain the correctly assembled operon.

Construction of pDHBo-ppc*: The upstream operon comprised of Ec-lysC (V115A:E119S:E250K:E343V) and Ms-ssrH39R N43H was amplified from pDHBo using the primers IF_ppc_for and IF_pACT_ssr_rev (Supplementary Table 15). The resulting PCR fragment was cloned into theXbaI/HindIII digested pACT3-ppc* plasmid using the Clusion HD Cloning kit (Clontech Laboratories, Inc.). The resulting plasmid was transformed into NEB 5-α-plasmid competent E. coli cells (NEB) and verified by DNA sequencing to contain the correctly assembled operon.

Construction of pDHBo(−typ)*-ppc* and pDHBo(Ec-aspd*)-ppc*: TheMs-ssrH39R N43H gene was PCR amplified from pET28-My-ssrH39R (Thermo-Scientific) using the primers Ms-ssrH39R N43H-IF and Ms-ssrH39R N43H referring to Supplementary Table 15, respectively. The forward primer contained an RBS sequence which had been optimized by the RBS calculator web-tool. The resulting DNA fragment was ligated into pENTR(−asd*)-ppc* vector using SpeI and BglII restriction sites. The resulting plasmid was transformed into NEB 5-α-plasmid competent E. coli cells (NEB) and verified by DNA sequencing. pDHBo(Ec-aspd*)-ppc* was constructed analogously using the primers Ec-aspdFor and Ec-aspd_rev (Supplementary Table 15).

Construction of strains for biosynthesis of DHB. Escherichia coli K-12 substr. MG1655 (ATCC 47076) was used as the parental strain for the construction of DHB-producing strains. Deletion of the ackA-pta operon was carried out using the method of Datsenko & Wanner63. The kamycin resistance cassette of pKD4 vector (Thermo-Scientific) was inserted into the genomic target locus (Supplementary Table 16). The PCR product was transformed into the target strain which expressed λ-red recombine from the pKD46 plasmid.

Kamycin-resistant colonies were selected on LB agar plates containing 25 μg per ml of the antibiotic and were verified by PCR. Deletion of pexB was achieved using the phase transduction method adapted from Miller64. The plasmid was prepared from the ApsX strain of KEO collection65. Positive clones were selected on kamycin-containing LB agar plates and verified by PCR analysis. The kanamycin resistance cassette was removed from the genome by expressing FLP recombinase from the pCF19 (Thermo-Scientific) plasmid. The resulting plasmid was transformed into target E. coli strains using standard protocols56, obtaining the strains listed in Supplementary Table 17.

Cultivation conditions for biosynthesis of DHB. All cultivations were carried out at 37°C on an Infors rotary shaker running at 170 r.p.m. Precultures (3 ml LB medium in test tubes) were inoculated from glycerol stocks, cultivated overnight, and used to inoculate a second preculture (25 ml mineral medium in 250 ml baffled shake flasks) at an OD600 of ~0.1. Exponentially growing cells of this preculture were used to inoculate the production culture (25 ml mineral medium in 250 ml baffled shake flasks) at OD600 of ~0.05. IPTG was added at a concentration of 1 mM 1 h after OD600 (~0.5) to induce the production cultures reached ~0.6. One liter mineral medium contained 3 g glucose, 18 g NaHPO₄·12H₂O, 1.3 g KH₂PO₄, 0.9 g NaCl, 0.05 g MgSO₄·7H₂O, 0.015 g CaCl₂·2H₂O, 1 ml of 0.01 mol FeCl₃ stock solution prepared in 100 diluted concentrated HCl, 2 ml of 10 mM thiamine HCl stock solution, 20 g MOPS, (and the antibiotics kanamycin sulfate and chloramphenicol at 50 μg per ml). The pH of the culture medium was maintained at pH 7.0 and the medium was filter-sterilized.

Analytical methods. The concentration of DHB was determined on a Dionex Ultimate 3,000 HPLC system (Thermo scientific, France) using an Aminex HPX-87H column (protected by a ‘Micro-Guard cation H Reilh Cartridge’ precolumn) heated at 68°C. Mobile phase was 0.01% (v/v) formic acid at 0.4 ml min⁻¹ isocratic flow rate. Monitoring on the reversed phase column and quantitation of DHB were performed by a dual-wave UV–vis (200 and 210 nm) detector (Dionex Diode Array Detector) or by a MSQ mass.
spectrometer (Finnigan Surveyor MSQ plus) in electrospray mode (ESI probe at 0.25 kV) with negative ionization (ionization temperature, 450 °C; cone voltage, 50 volts; Glucose and bacitracin were quantified on a Dionex UltiMate 3,000 HPLC system equipped with a UV and a RI (Shodex RI-101) detector using an Aminex HPX-87H column (protected by a Micro-Guard column) at 35 °C, using 0.1% (v/v) sulfuric acid as mobile phase, at 0.5 ml/min isocratic flow rate.

Computational methods. Molecular models of Ec-LysC wild-type and mutant complexes with (l)-malate were built by modification of the X-ray crystal structure of a ternary complex of the wild-type enzyme in the R-state with (l)-aspartate and Mg-ADP. An all-atom starting set of co-ordinates was prepared by stepwise energy minimization of the experimental structure (PDB code 2jol) with harmonic restraints initially placed on all heavy atom positions. Restraining forces on atom subsets comprising the (l)-malate ligand, mutated and neighbouring protein residues and water molecules were then removed before the release of all restraints in the final round of geometry refinement of the mutant enzyme model complexes. Potential energy force field parameters used in minimization are described below.

Computational re-design of the active-site region in Ec-LysC centred around residue position 119 was performed by the application of 30,000 Monte Carlo rounds of random mutagenesis at nine residue positions (Ser39, Ala40, Thr45, Val115, G1u119, Phe184, Thr195, Ser201 and Thr359) in a modelled binary complex of the E119Q mutant with (l)-malate using RosettaDesign software. All possible exchanges with the 20 native amino-acid side-chains were permitted at each of the nine residue positions. Positions of all atoms in the (l)-malate ligand and in the protein main-chain were held fixed, along with side-chain atom positions in non-mutated residues. A molecular model of the Ec-ADH E241Q mutant complex containing the hemithioacetel malate semialdehyde tetrahydrofuran covalent reaction intermediate presumed to be formed, following phosphate release from malyl-P, by hydride transfer to a thioacyl enzyme intermediate, was built from the template X-ray crystal structure (PDB code 1g13) of the Ec-ADH covalent complex formed by reaction of the Cys315 thiol group with the S-methylcysteine sulfoxide substrate analogue inhibitor in the presence of NADP+ (ref. 26). The covalently attached malate semialdehyde substrate derivative was constructed by superposition of a geometry-optimized structure of the model compound (4S)-4-[(2S)-2-amino-3-carboxypropyl] sulfanyl-L-malate in an analogous conformation to the L-cysteine semialdehyde substrate by superposition of atomic coordinates (PDB code 1nx6) from the Haemophilus influenzae ASD covalent complex with a hemithioacetal ASA partial covalent complex with a hemithioacetal ASA partial. Two enzymes possess an overall shared sequence identity of 37%. The structurally equivalent (His47/Asn51) residue pairing to that in Ms-Ssr (His39/Asn43), and the aligned helical region from Cys46 to Ala52 in the P47H ADH2 crystal structure was used as a fragment template for manual re-building of the rigid-body shifted equivalent (Cys46 to Ala52 in the P47H ADH2 crystal structure was used as a fragment template for manual re-building of the rigid-body shifted equivalent (Cys368-Glu46) helical section in the initial Ms-Ssr model. Following a further round of restrained energy minimization of the refined monomeric structure, the Ms-Ssr dimer was regenerated as before, and subjected to a final round of energy minimization in the absence of harmonic restraints.

All energy minimizations were carried out using the f99SBamber molecular mechanics force field for protein atoms and the GAFF force field3 for atoms in the model. Potential charges for ADP– and NADP+ were taken from the CHARMM27 force field. Potential charges for atoms in (l)-malate, (l)-2,4-dihydroxybutyrate (DHb), the negatively charged 4-hydroxy-2-oxobutanoic acid aldehyde (and the (4S)-4-[(2S)-2-amino-3-oxo-propyl] sulfanyl-l-malate model compound used in the parameterization of the putative hemithioacetel (l)-malate semialdehyde Ec-ASD E241Q covalent reaction intermediate were obtained by RESP fitting to the quantum chemical electrostatic potential of geometry-optimized structures.

Initial Fock quantum chemical calculations were carried out with GAMESS using the 6–31G* basis set.

Position-dependent amino-acid residue variation in multiple sequence alignment data was analysed using a normalized measure of the Shannon information entropy (Hs). The relative Shannon entropy is calculated at each residue alignment position (X) as

\[
H_{S} = \frac{20}{\sum_{i=1}^{20} \frac{p_{i}(X) \ln p_{i}(X)}{\sum_{i=1}^{20} p_{i}(X) \ln p_{i}(X)}},
\]

where \(p_{i}(X)\) is the conditional probability of residue type (i) occurrence at alignment position (X), and \(p_{0}(X)\) is the normalized probability of residue type (i) occurrence at any position. To minimize sampling bias, \(p_{0}(X)\) was taken as the globally normalized residue type probability values for all natural proteins tabulated by Ranganathan and co-workers.9

Data availability. The authors declare that all the data supporting the findings of this study are available within the paper and its Supplementary Information Files, as well as from the authors upon reasonable request.

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Acknowledgements

This work was supported by the French National Research Agency (ANR programme d’Investissement d’Avenir, Project SYNTHACS, ANR-10-BTBR-05-01) and by Adisseo. We are grateful to S. Bonnonet and S. Pizzi-Serin for providing support in using the ICEO high-throughput facility at the USBP, devoted to the engineering and screening of new and original enzymes, and to A. Vax, H. Serrano-Bataille, F. Calvayrac, and J. Fredonnet for their technical assistance. This work was granted access to the HPC resources of the Computing Center of Region Midi-Pyrénées (CALMIP, Toulouse, France).

Author contributions

T.W. and J.M.F. originally proposed the novel metabolic pathway for DHB production. T.W., M.M., R.H., I.A., M.R.-S., and J.M.F. conceived the general structure of the project. T.W., R.I. and C.A. carried out the enzyme and strain engineering, A.B., H.C., C.D., I.L.-H., N.M., M.N. and Y.M. contributed to the analysis of the enzymes and engineered strains. C.M.T., N.T., R.I., M.R.S. and I.A. performed the enzyme structural analyses, molecular modelling, design of enzyme libraries and screening assays. T.W., C.M.T., R.I., I.A., M.R.-S. and J.M.F. wrote the manuscript. All authors read and approved the manuscript.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing interests: M.M. and R.H. are employees of the ADISSEO Company that provides financial support to this study. T.W., C.D., H.C. and J.M.F. are coauthors on a patent based upon this work WO2013/160762A2 (2013). In addition, T.W., C.M.T., M.M., R.H., I.A., M.R-S. & J.M.F. are coauthors of a patent that described the present work WO2012/056318A1, that has been granted January 19th, 2016 as US 9,238,829 B2. All remaining authors declare no competing financial interests.

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How to cite this article: Walther, T. et al. Construction of a synthetic metabolic pathway for biosynthesis of the non-natural methionine precursor 2,4-dihydroxybutyric acid. Nat. Commun. 8, 15828 doi: 10.1038/ncomms15828 (2017).

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