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Construction of a synthetic metabolic pathway for the production of 2,4-dihydroxybutyric acid from homoserine

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Abstract

2,4-dihydroxybutyrate (DHB) is a precursor for the chemical synthesis of the methionine analogue 2-hydroxy-4-(methylthio)butyrate. Since no annotated metabolic pathway exists for its microbial production from sugar, we have conceived a two-step synthetic metabolic pathway which converts the natural amino acid homoserine to DHB. The pathway proceeds through the homoserine transaminase-catalyzed deamination of homoserine to obtain 2-oxo-4-hydroxybutyrate (OHB), and continues with the reduction of OHB to DHB, which is catalyzed by an OHB reductase enzyme. We identified homoserine transaminase and OHB reductase activity in several candidate enzymes which act on sterically cognate substrates, and improved OHB reductase activity of lactate dehydrogenase A of *Lactococcus lactis* by structure-based enzyme engineering. Fed-batch cultivation of a homoserine-overproducing *Escherichia coli* strain which expressed homoserine transaminase and OHB reductase enzymes resulted in the production of 5.3 g/L DHB at a yield of 0.1 g/g.

Introduction

The sulfur-containing essential amino acid (L)-methionine and its analogue 2-hydroxy-4-(methylthio)butyrate (HMTB) are important supplements in poultry diets (Dilger and Baker, 2007; Sauer et al., 2008). The cumulated annual production volume of both compounds reached ~1 million tons in 2014 ("Feedinfo News Service"). While most of the 20 proteinogenic amino acids are produced by fermentation processes from renewable sugar, the production of methionine and HMTB is still dominated by petroleum-based syntheses (Faurie et al., 2003; Leuchtenberger et al., 2005; Willke, 2014). This is mainly due to the very high metabolic cost of incorporating sulfur which requires the NADPH-dependent reduction of the standard sulfur source sulfate to sulfide (Krömer et al., 2006). Comparatively low methionine yields of microbial fermentations in combination with the fact that chemically produced racemic (D/L)-methionine has a very similar nutritional value as the pure (L)-enantiomer (Dilger and Baker, 2007) renders methionine fermentation processes at present uncompetitive with chemical production.

Producing methionine or HMTB by using more reduced sulfur sources, or via two-stage processes, where first a functionalized precursor is produced by fermentation and sulfur is subsequently incorporated by (bio)chemical means, is therefore a promising alternative. For example, (Zelder et al., 2009) have shown that methionine yields can be increased in microbial fermentations using engineered *Corynebacterium glutamicum* when the less oxidized dimethyl disulfide is used to replace sulfate as the sulfur source. In addition, an elegant two-stage industrial production process for (L)-methionine was recently developed in which the precursors O-acetylhomoserine or O-succinyl-homoserine are produced by optimized microorganisms through the fermentation of sugars (Hong et al., 2014; Shim et al., 2016). These precursors are then reacted with methanethiol in an enzymatic process stage to yield methionine. It was proposed that acetate or succinate, which is released during the enzymatic conversion of the precursor, can be recycled as substrate or be commercialized (Shim et al., 2016).

An alternative approach was recently proposed by our group and consists in the production of 2,4-dihydroxybutyrate (DHB) by microbial fermentation of sugar (Walther et al., 2017), and the subsequent chemical conversion of DHB and methanethiol to HMTB. The chemical process stage has near 100 % product yield (Deck et al., 2009) and does not produce any by-products, which may simplify process design and reduce manufacturing costs.

Annotated natural DHB-producing metabolic pathways do not exist. Reports on the occurrence of DHB in natural organisms are limited to a study on human patients suffering from succinic semialdehyde dehydrogenase deficiency, where the accumulation of trace amounts of DHB in the patient's urine was observed without revealing the underlying metabolic pathway responsible for DHB production (Shinka et al., 2002). Therefore, we have recently developed a three-step synthetic metabolic pathway which converts the natural precursor malate into DHB via the activation of malate by phosphorylation followed by two successive reduction steps (Walther et al., 2017). The pathway employs previously unknown malate kinase, malate semialdehyde dehydrogenase, and malate semialdehyde reductase enzymatic activities which were obtained by semi-rational engineering of template enzymes which act on sterically cognate substrates. An engineered *Escherichia coli* strain which simultaneously expressed the optimized enzymes and the anaplerotic enzyme phosphoenolpyruvate carboxylase produced 1.8 g/L DHB from 20 g/L glucose in shake flasks (Walther et al., 2017). In addition, Li et al. (2014) and our group (Walther et al., 2013) have constructed an alternative three-step synthetic pathway which converts malate to DHB via the characteristic intermediate malyl-CoA. This pathway, however, was so far reported to yield only trace amounts of DHB (<10 mg/L).

In the present work we describe the design and construction of a two-step synthetic metabolic pathway which converts the natural precursor homoserine into DHB. The pathway relies on the homoserine transaminase-catalyzed deamination of homoserine to produce 2-oxo-4-

hydroxybutyrate (OHB), and the subsequent reduction of OHB to DHB, which is catalyzed by an OHB reductase. Although other aminotransferase-reductase pathways appear to exist in natural organisms, as indicated by the serine-to-glycerate pathway which may operate in mice (Hagopian et al., 2005; Snell, 1986), we found no report on the operation of the homoserine-to-DHB pathway in natural organisms. Our work showed that several natural candidate transaminase and 2-hydroxyacid dehydrogenases had significant homoserine transaminase and OHB reductase activity, respectively. OHB reductase activity of (L)-lactate dehydrogenase A from *Lactococcus lactis* was increased by introducing the Gln85Cys point mutation into the active site of the enzyme. Simultaneous expression of the homoserine transaminase and OHB reductase enzymes in a homoserine-overproducing strain growing on glucose yielded 0.58 g/L DHB after 24 h shake flask cultivation. During a fed-batch cultivation of this strain 5.3 g/L DHB were produced from glucose at a molar yield of 0.1 g/g.

Materials and Methods

Strains and cultivation conditions

All strains were derived from *E. coli* K-12 substr. MG1655. The mutant strains listed in Table 1 were constructed by the phage transduction method (Malke et al., 1992) using single gene deletion mutants of the Keio collection (Baba et al., 2006) as the donor strains. Gene deletions were verified by PCR using primers that hybridized upstream and downstream of the modified genomic locus (Table S1).

All shake flask cultivations including pre-cultures were carried out in M9 mineral medium (Walther et al., 2017) supplemented with 0.2 g/L methionine and threonine to complement strain auxotrophies. Growth cultures for DHB production were carried out in 250 mL baffled shake flasks containing 30 mL medium. Cultures were inoculated at an initial OD₆₀₀ of 0.2 from overnight pre-cultures and expression of homoserine and DHB pathway enzymes was induced by the addition of 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) when culture OD₆₀₀ was approximately 0.6. Growth and pre-cultures were incubated at 37 °C on a rotary shaker (Infors Multitron) running at 200 rpm.

Construction of expression vectors

High fidelity polymerase Phusion^{TM} (Finnzymes) was used in all PCRs. T4 DNA ligase (Fermentas or Biolabs) was used to ligate digested PCR products into the corresponding vectors. Ligation products were transformed into *E. coli* DH5 α cells (NEB) using standard protocols. All constructed plasmids were isolated and shown by DNA sequencing to contain the correct sequences. All plasmids used in this study are listed in Table 2.

Construction of vectors for protein expression and purification: The genes *EC-mdh*, *EC-ldh*, *EC-tyrB*, *EC-aspC*, *EC-ilvE*, and *LL-ldhA*, *LL-panE* were PCR-amplified from genomic DNA of *E. coli* K12-substr. MG1655, or of *L. lactis* subsp. *lactis* Il1403, respectively, using the forward and reverse primers listed in Supplementary Table S1. The resulting DNA fragments were digested with the restriction enzymes listed in Supplementary Table S1 and ligated into the corresponding restriction sites of the pET-28(a)+ vector (Novagen).

Construction of vectors for expression of the homoserine and the DHB pathway:

Plasmids pEXT20-aspC and pEXT20-LLldh_{AQ85C} were constructed by amplifying the *EC-aspC* and *LL-ldhA*_{Q85C} genes by PCR using the forward and reverse primer couples 609/610 and 679/680, respectively, that introduced a ribosome binding site (RBS) in front of each gene, and *BamHI* and *Sall* restriction sites upstream of the RBS and downstream of the stop codon, respectively. Genomic DNA of *E. coli* K-12 substr. MG1655 or the pET28-LL-ldhA_{Q85C} plasmid was used as the template. The PCR products were digested with *BamHI* and *Sall*, ligated into the corresponding sites of the pEXT20 expression vector (Dykxhoorn et al., 1996).

Plasmid pEXT20-ilvE-LLldhA_{Q85C} was constructed by PCR-amplifying the *EC-ilvE* and *LL-ldhA*_{Q85C} genes individually using the forward and reverse primer pairs 820/821 and 822/823, respectively, listed in Table S1, and pET28-ilvE or pET28-LLldhA_{Q85C} as the template. The amplified PCR fragments were fused in an overlap extension PCR by adding 150 ng of each fragment to 50 µL of the reaction mix which contained primers 820 and 823. The resulting PCR fragment was purified, digested with *KpnI* and *XbaI*, and ligated into the corresponding sites of pEXT20.

Plasmid pEXT20-aspC-LLldhA_{Q85C} was obtained by replacing the *ilvE* gene in pEXT20-ilvE-LLldhA_{Q85C} by *aspC*. Specifically, the *aspC* gene was amplified by PCR using the forward and reverse primers 668 and 674 (Table S1) that introduced a RBS in front of the gene, and *SacI* and *BamHI*

restriction sites upstream of the RBS and downstream of the stop codon, respectively. pEXT20-aspC was used as the template. The SacI/BamHI digested PCR fragment was ligated into the corresponding sites of the SacI/BamHI-digested and gel-purified pEXT20-ilvE-LLldh_{AQ85C} vector.

Plasmid pZA23-thrA was constructed by amplifying the *E. coli thrA* gene by PCR using the forward and reverse primers 1250 and 1252 that introduced a RBS in front of *thrA*, and *EcoRI* and *SmaI* restriction sites upstream of the RBS and downstream of the stop codon, respectively. Genomic DNA from *E. coli* MG1655 was used as the template. The PCR product was digested with *EcoRI* and *SmaI*, ligated into the corresponding sites of the pZA23 expression vector (Expressys^{TM}). An aspartate kinase/homoserine dehydrogenase with strongly decreased sensitivity for inhibition by threonine was constructed by site directed mutagenesis which replaced serine in position 345 by phenylalanine (S345F). Site-directed mutagenesis was carried out as described above using the primers listed in Table S1. Plasmids created by PCR contained a new restriction site for *XhoI* which was introduced by silent mutation in addition to the functional mutation to facilitate identification of mutated clones.

Plasmid pZA23-thrA_{S345F}-aspC-LLldhA_{AQ85C} was constructed by PCR-amplifying a DNA fragment containing the *aspC*-*LLldhA*_{AQ85C} operon using pEXT20-aspC-LLldhA_{AQ85C} as the template and forward and reverse primers (1469, 1479, Table S1) which introduced *SmaI* and *KpnI* restriction sites upstream of *aspC* and downstream of *LLldhA*_{AQ85C}, respectively. The PCR product was digested with *SmaI* and *KpnI*, ligated into the corresponding sites of the pZA23-thrA_{S345} plasmid.

Site-directed mutagenesis of OHB reductase LL-LdhA_{Q85}: Point mutations to change the amino acid sequence of LL-LdhA were introduced by PCR (Phusion 1U, HF buffer 20 % (v/v), dNTPs 0.2 mM, direct and reverse primers 0.04 μ M each, template plasmid 30-50 ng, water) using the oligonucleotide pair listed in Supplementary Table S1 and the pET28-LLldhA plasmid as the template. The genes mutated by PCR contained a new restriction site listed in Supplementary Table S1 (introduced using silent mutations) in addition to the functional mutation to facilitate identification

of mutated clones. The PCR product was digested by *DpnI* at 37 °C for 1 h to remove template DNA, and transformed into competent *E. coli* DH5 α cells (NEB). Mutated plasmids were isolated from individual clones, and verified by restriction site analysis and DNA sequencing to carry the desired mutations.

Protein expression and enzymatic assays

Expression of enzymes: *E. coli* BL21 (DE3) star cells were transformed with the appropriate plasmids using standard genetic protocols (Sambrook et al., 1989). Enzymes with an N-terminal hexa-His tag were expressed in 50 mL LB cultures that were inoculated from an overnight culture at OD₆₀₀ of 0.1 and grown to OD₆₀₀ of 0.6 before protein expression was induced by addition of 1 mM IPTG to the culture medium. After 15 h of protein expression at 25 °C, cells were harvested by centrifugation at 4000 g at 4 °C for 10 min and discarding the supernatant. Cell pellets were stored at -20 °C until further analysis. Culture media contained 50 μ g/mL kanamycin.

Purification of enzymes: Frozen cell pellets 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 (sonication interval: 20 s, power output: 30 %, sonicator: Bioblock Scientific, VibraCell™ 72437). Cell debris was removed by centrifuging the crude extracts for 15 min at 4 °C at 4000 g and retaining the clear supernatant. RNA and DNA were removed from the extracts by adding 15 mg/mL streptomycin sulfate (Sigma), centrifuging the samples at 13000 g for 10 min at 4 °C and retaining the supernatant. Clear protein extract was incubated for 1 h at 4 °C with 0.75 mL (bed volume) of Talon™ Cobalt affinity resin (Clontech). The suspension was centrifuged at 700 g in a table top centrifuge and supernatant was removed. The resin was washed with 10 bed volumes of wash buffer (50 mM Hepes, 300 mM NaCl, 15 mM Imidazole, pH 7.5) before proteins were eluted with 0.5 mL of elution buffer (50 mM Hepes, 300 mM NaCl, 250 mM Imidazole, pH 7.5). Purity of eluted enzymes was verified by SDS-PAGE analysis. Protein concentrations were estimated with the method of Bradford

(Bradford, 1976). To stabilize the lactate dehydrogenase enzymes, the elution buffer was systematically exchanged by 100 mM phosphate buffer adjusted to pH 7. The protein sample was transferred to an Amicon™ Ultra centrifugal filter (cut-off 10 kDa), and centrifuged during 8 min at 4000 g at 4 °C to remove the buffer. The protein was diluted into phosphate buffer and the procedure was repeated 4 times.

Estimation of 2-oxo-acid reductase activities: The reaction mixture contained 60 mM Hepes (pH 7), 50 mM potassium chloride, 5 mM MgCl₂, 0.25 mM NADH, (5 mM fructose-1,6-bisphosphate with Ldh enzymes) (all products from Sigma), and appropriate amounts of purified enzymes or cell extract. Reactions were started by adding appropriate amounts of 2-oxo-4-hydroxybutyrate (OHB), pyruvate, or oxaloacetate (OAA). Enzymatic assays were carried out at 37 °C in 96-well flat bottomed microtiter plates in a final volume of 250 µL. The reactions were followed by the absorption of NADH at 340 nm in a microplate reader (BioRad 680XR). OHB was synthesized by incubating 125 mM homoserine with snake venom (L)-amino acid oxidase (1.25 U/mL, Sigma) and catalase (4400 U/mL, Sigma) in 100 mM Tris buffer at pH 7.8 for 90 min at 37 °C. Subsequently, the reaction mixture was purified on an Amicon™ Ultra centrifugal filter with a cut-off of 10 kDa to eliminate the enzymes (method adapted from (Wellner and Lichtenberg, 1971)). OHB was quantified by mixing 100 µL of the tested solution with 1 mL of a solution containing 1 M sodium arsenate and 1 M boric acid at pH 6.5. The mixture was incubated at room temperature for 30 min and the absorbance at 325 nm was used to quantify OHB. The relation between absorbance and concentration of the ketone was calibrated using pyruvate solutions of known concentrations (method adapted from (Wellner and Lichtenberg, 1971)). The typical OHB yield of the method was 90 %.

Estimation of transaminase activities: Transaminase activity of several candidate aminotransferases on their preferred substrate and homoserine was quantified with 2-oxoglutarate as the amino group acceptor. Reaction rates were measured by monitoring the NADH-dependent reduction of the 2-

oxoacid products of the transaminase reaction. The reaction mixture contained 60 mM Hepes (pH 7), 50 mM potassium chloride, 5 mM MgCl₂, 4 mM 2-oxoglutarate, 0.1 mM pyridoxal-5'-phosphate (PLP), 0.25 mM NADH, (optionally 5 mM fructose-1,6-bisphosphate) (all products from Sigma), 4 Units/mL of auxiliary 2-hydroxyacid dehydrogenase, and appropriate amounts of purified aminotransferase or cell extract. The auxiliary dehydrogenase enzyme was purified PanE from *L. lactis* in case of the amino acids phenylalanine and leucine (Chambellon et al., 2009), malate dehydrogenase (Sigma) in case of aspartate, and rabbit muscle (L)-lactate dehydrogenase (Sigma) when homoserine was used as the starting substrate. Reactions were started by adding appropriate amounts of the amino acid. The assays were carried out at 37 °C in 96-well flat bottomed microtiter plates in a final volume of 250 µL. NADH consumption was monitored in a microplate reader (BioRad 680XR).

Analytical methods

Quantification of glucose and metabolites was carried out by High Performance Liquid Chromatography (HPLC) on an Ultimate 3000 system (Thermo scientific, France) equipped with an autosampler (WPS-3000RS, Dionex), which was connected to a RI detector (RID 10A, Shimadzu), a UV/VIS detector (SPD-20A, Shimadzu), and/or a MSQ mass spectrometer (Finnigan Surveyor MSQ plus) depending on the measured compounds.

Sugars and organic acids were measured with the UV and RI detectors and by using a cation-exchange column (Aminex HPX-87H - 300 x 7.8 mm, 9 µm, protected by a "Micro-Guard cation H Refill Cartridge" precolumn, Biorad). Mobile phase was 1.25 mM H₂SO₄ at a flow rate of 0.5 ml/min. Column temperature was held at 35 °C. Homoserine, methionine and threonine concentrations were determined on the RI detector and by using a Chirex 3126 column (Phenomenex) with an aqueous mobile phase containing 2mM CuSO₄ and 15 % methanol. Flow rate was 1 ml/min. The column was held at 22°C. DHB concentrations were measured using an Aminex HPX-87H column (protected by a

“Micro-Guard cation H Refill Cartridge” precolumn) held at 48 °C. Mobile phase was 0.01 % (v/v) formic acid at 0.4 ml/min isocratic flow rate. Depending on the concentration range, detection and quantification of DHB were performed by a dual-wave UV-VIS (200 nm and 210 nm) detector (Dionex Diode Array Detector) or by a MSQ mass spectrometer in electrospray mode (ESI probe at 0.25 kV) with negative ionization (ionization temperature, 450 °C; cone voltage, 50 volts).

Exhaust gas of the bioreactor culture was analyzed by a Thermo Scientific Prima Pro mass spectrometer. Total Organic Carbon (TOC) was measured using a Shimadzu TOC-L analyzer with ASI-L auto sampler. Preparation of standard solutions was made with potassium hydrogen phthalate (28420-82, Nacalai Tesque Inc). TOC of a sample was calculated from three replicate measurements.

Bioreactor cultures

The pre-cultures for the inoculation of the bioreactors were cultivated in 1000 mL shake flasks containing 150 mL mineral medium (composition as indicated above) until exponential phase. The cells were harvested and used to inoculate a 2 L bioreactor (Biostat B Sartorius) that initially contained 1500 mL medium adjusting an OD_{600} of ~0.3. The composition of the fermentation medium was similar to the mineral medium used in the shake flask experiments, except that it additionally contained 6 g/l $(NH_4)_2HPO_4$, 0.4 g/l $(NH_4)_2SO_4$, 1 g/L threonine and methionine, 6 g/L citrate and no MOPS. Initial glucose concentration was 45 g/L. Glucose concentration was monitored during the fermentation and a concentrated glucose stock solution (500 g/L) was manually added to assure non-limiting glucose concentrations during the first 45 h of cultivation. The pH of the cultures was kept at 7.0 by the addition of 5 M KOH, and reactors were aerated with air at 0.3 to 1.5 vvm. Dissolved oxygen tension was maintained above 30 % of the saturating oxygen concentration by adjusting the appropriate agitation speed (300-1500 rpm, Rushton rotor, 28 mm diameter) and aeration rate. Polypropylene glycol (P 2,000) was used as antifoaming agent during the culture.

Results

Identification of enzymes with OHB reductase and homoserine transaminase activity

We first set out to identify enzymes with homoserine transaminase activity. It has been previously reported that transaminases frequently have significant activity on sterically and electrostatically different substrates, which explains that alternative transaminases could take over the metabolic function of a deleted transaminase enzyme (Berg et al., 1988; Jensen and Calhoun, 1981). We intended to make use of this pronounced promiscuity of transaminases and characterized enzymes from three different classes of aminotransferases, namely the aspartate (EC-AspC), phenylalanine (EC-TyrB), and branched-chain amino acid (EC-IlvE) transaminases of *E. coli* on homoserine and their corresponding preferred substrate. The genes encoding these enzymes were amplified from genomic DNA and cloned into the pET-28(a)+ expression vector. The His-tagged enzymes were expressed in *E. coli* BL21(DE3) cells, and purified using affinity chromatography. The purity of the purified protein fraction was verified by SDS-PAGE (not shown) prior to enzymatic tests.

The estimated K_m values of the three aminotransferases EC-AspC, EC-IlvE and EC-TyrB for their respective natural substrates aspartate, leucine, and phenylalanine were very similar to previously reported results (Lee-Peng et al., 1979; Mavrides and Orr, 1975; Yano et al., 1998) (Table 3) indicating that our assay conditions were appropriate for the characterization of these enzymes. The three tested transaminases had only residual activity on homoserine which was at least more than 450-fold lower than on their natural substrate (Table 3). Initial reaction velocities of the enzymes on homoserine did not saturate at substrate concentrations of up to 50 mM, which was an additional indicator for their comparatively poor activity on homoserine. Since EC-AspC had the highest activity on homoserine with $0.082 \mu\text{mol}/(\text{mg}_{\text{prot}} \text{ min})$ we chose this enzyme for the implementation of the synthetic homoserine-DHB pathway.

We continued our work with the identification of enzymes with 2-oxo-4-hydroxybutyrate (OHB) reductase activity. Our screen comprised four 2-hydroxy acid dehydrogenases which are active on substrates that are sterically cognate to OHB. Specifically, we estimated the kinetic parameters of lactate dehydrogenase (Ldh), malate dehydrogenase, and branched-chain 2-hydroxyacid dehydrogenase enzymes on OHB and their natural substrates. The selected enzymes included (L)-Ldh from *Lactococcus lactis*, which is encoded by *LL-ldhA* (Llanos et al., 1992), (D)-Ldh from *E. coli*, which is encoded by *EC-ldhA* (Bunch et al., 1997), (L)-malate dehydrogenase from *E. coli*, which is encoded by *EC-mdh* (Sutherland and McAlister-Henn, 1985), and the branched-chain 2-hydroxyacid dehydrogenase from *L. lactis*, which is encoded by *LL-panE* (Chambellon et al., 2009). As for the characterization of the aminotransferases, the genes encoding these enzymes were cloned into pET-28(a)+ expression vectors, and the His-tagged proteins were expressed and purified to homogeneity prior to enzymatic assays.

High OHB reductase activity of 11 $\mu\text{mol}/(\text{mg}_{\text{prot}} \text{ min})$ was found for LL-LdhA (Table 4), which was in qualitative agreement with an earlier work reporting OHB reductase activity for rabbit muscle (L)-lactate dehydrogenase (Lane and Dekker, 1969). Our results showed furthermore that the (D)-lactate dehydrogenase EC-Ldh (0.56 $\mu\text{mol}/(\text{mg}_{\text{prot}} \text{ min})$) and the branched chain 2-hydroxyacid dehydrogenase LL-PanE (2.07 $\mu\text{mol}/(\text{mg}_{\text{prot}} \text{ min})$) were also active on OHB. In contrast, the EC-Mdh enzyme had only hardly measurable activity on OHB (Table 4). Substrate affinities of the enzymes for OHB could not be estimated since initial reaction velocities did not saturate at substrate concentrations of up to 20 mM OHB. Therefore, we used the ratio of the reaction rates attained at saturating concentrations of the natural substrate or at 20 mM OHB as a rough estimate for the substrate specificities of the enzymes. We found that LL-LdhA had not only the highest activity on OHB, but also the smallest specificity for its natural substrate (Table 4). Therefore, we decided to continue our work with this enzyme.

Engineering of (L)-lactate dehydrogenase to improve OHB reductase activity

Key amino acid residues that provide substrate specificity to LL-LdhA were identified by protein sequence alignment of several (L)-lactate dehydrogenases of different biological origin, and visual inspection of a previously published crystal structure from *Geobacillus stearothermophilus* bound to its activator fructose-1,6-bisphosphate, the NAD cofactor, and the substrate analogue oxamate (Wigley et al., 1992). Figure 2 shows key residues in the active site of GS-Ldh. The negative charge of the substrate carboxylic acid group is neutralized by electrostatic interaction with the positively charged side chain of Arg155. Thr233 and Asn126 contribute to correct substrate positioning by undergoing additional polar interactions with the oxamate carboxyl and oxo functions, and Gln86 restricts the size of the active site pocket. The catalytic residues His179 and Arg92 serve as the acid/base catalyst and to stabilize the developing charge, respectively, during product formation. The pivotal role of these residues in substrate binding and catalysis has been reported in previous studies ((Dunn et al., 1991) and references therein).

Manual docking of OHB into the active site configuration of Gs-Ldh revealed a steric clash between Gln86 (which aligns to Gln85 in LL-LdhA) and the larger synthetic substrate (Figure 2). Therefore, the impact of mutations in position Gln85 on OHB reductase activity was characterized. Out of the 19 alternative amino acid residues assayed in position 85, only cysteine and asparagine significantly increased OHB reductase activity (not shown). In a refined analysis of both mutants we found that the best variant LL-LdhA Gln85Cys had a 4-fold increased activity on OHB and a more than 4.5-fold decreased activity on the natural substrate pyruvate (Table 5). Contrary to the wild-type enzyme, the Gln85Cys mutant was not saturated by pyruvate concentrations of up to 20 mM thus exhibiting an almost equal affinity for both substrates (Table 5). Therefore, the LL-LdhA_{Q85C} mutant enzyme was used as the OHB reductase in the synthetic DHB pathway.

Optimization of DHB production by engineered *E. coli*

To achieve high DHB production via the above described synthetic pathway, both homoserine production and homoserine-to-DHB conversion needed to be optimized. Homoserine production was increased by expressing the threonine-insensitive bifunctional aspartate kinase/homoserine dehydrogenase mutant, ThrA_{S345F}, from a medium-copy plasmid. To further increase homoserine availability in the production strain, the homoserine-consuming homoserine kinase and homoserine O-succinyltransferase enzymes, which are encoded by *thrB* and *metA*, respectively, were deleted. The soluble lactate dehydrogenase of *E. coli*, which is encoded by EC-*ldhA* and which also bears some OHB reductase activity (Table 4), was deleted from the genome of the production strain to properly demonstrate the positive contribution of the optimized OHB reductase enzyme LL-LdhA_{Q85C} to DHB production. In addition, alcohol dehydrogenase, encoded by *adhE*, was deleted to increase DHB production under oxygen-limited conditions (not shown in this paper).

The resulting quadruple mutant *E. coli* $\Delta adhE \Delta ldhA \Delta thrB \Delta metA$ (strain Eco4) was transformed with plasmids that expressed ThrA_{S345F}, the homoserine transaminase-bearing EC-AspC, and the OHB reductase LL-LdhA_{Q85C} alone or in combination. DHB and homoserine production by these strains were determined after 24 h of cultivation under aerobic conditions on a defined mineral medium which contained 20 g/L glucose as the carbon source and which was supplemented with 0.5 g/L methionine and threonine to complement the auxotrophies of the strains. Strain Eco4 which harbored no plasmids produced 477 mg/L homoserine indicating that the deletion of *metA* and *thrB* effectively blocked homoserine consumption by the methionine and threonine pathways (Table 6). The production of 59 mg/L DHB by strain Eco4 showed that *E. coli* possesses residual endogenous homoserine transaminase and OHB reductase activities which lead to the production of small amounts of DHB under conditions where homoserine was overproduced. The transformation of empty control plasmids into Eco4 yielded strains Eco5-7 which exhibited impaired growth and reduced homoserine and DHB production (Table 6) indicating that these plasmids represent a significant metabolic burden.

Strain Eco10 expressed both DHB pathway enzymes EC-AspC and LL-LdhA_{Q85C} but had no increased ThrA_{S345F} activity. Although growth of the strain was restored, it only produced 48 mg/L DHB and 329 mg/L homoserine which was less than the Eco4 strain. Similarly, strain Eco10 which expressed ThrA_{S345F} but no DHB pathway enzyme produced only 48 mg/L DHB and thus less than Eco4. In contrast, strains Eco11 and Eco12, which expressed EC-AspC or LL-LdhA_{Q85C} in addition to ThrA_{S345F}, exhibited approximately 2-fold increased DHB production when compared to Eco4 and Eco10. As expected, DHB production was further increased to 190 mg/L when both DHB pathway enzymes, EC-AspC and LL-LdhA_{Q85C}, were expressed simultaneously with ThrA_{S345F} in strain Eco13. Taken together, these results showed that each individual enzyme of the synthetic DHB pathway positively contributed to DHB production (compare Eco10, 11, 12, 13), and that increased homoserine production was required to achieve significant DHB titers (compare Eco13 and Eco9, Table 6). To further optimize DHB production we decreased the metabolic burden on the strain by expressing ThrA_{S345F} and the two DHB pathway enzymes from the same medium-copy number plasmid. DHB production by the resulting strain Eco18 was increased to 410 mg/L (Table 6).

Fed-batch cultivation of DHB-producing strain

DHB production by strain Eco19 was analyzed in a fed-batch bioreactor cultivation. The strain was grown on the same mineral medium as during the shake flask experiments except that the medium was not buffered by MOPS, ammonium (and phosphate) concentrations were increased by the addition of 6 g/L diammonium phosphate, initial threonine and methionine concentrations were increased to 1 g/L, and 6 g/L citrate were added to stabilize the iron ions in the medium. HPLC analyses confirmed that citrate was not consumed during the cultivation. Controlled addition of KOH was used to maintain the culture medium at pH 7. Glucose concentration was monitored throughout the fermentation, and a concentrated glucose stock solution was manually added to the reactor to

assure non-limiting carbon source concentrations during the entire cultivation. The dissolved oxygen concentration was maintained above 40 % of the saturating concentration to assure aerobic conditions.

Under these conditions, strain Eco19 ceased exponential growth after approximately 9 h of cultivation due to the depletion of threonine in the culture medium (Figure 2). The onset of DHB production coincided with the growth arrest. The strain continued to consume glucose during the following 39 h of cultivation and DHB concentration increased linearly up to a final concentration of approximately 5.3 g/L corresponding to a product yield of 0.1 g/g. Interestingly, homoserine concentration peaked at only 0.3 g/L at the end of the exponential growth phase and decreased during the DHB production phase indicating that the amino acid was directly converted into DHB (Figure 2).

A carbon balance of the fed-batch fermentation revealed that only 59 % of the consumed carbon could be recovered in the form of biomass (9 %), soluble fermentation products identified by HPLC (11 %), and CO₂ (39 %) (Table 7). In an attempt to identify unknown carbon sinks we measured the Total Organic Carbon (TOC) content of the fermentation medium after 48 h with and without cells and compared these results with our HPLC analyses and spectrophotometric biomass measurements. We found that 0.8 C-mol in the medium could not be accounted for by HPLC analyses (Table S2, Please note that concentrations of OHB and lysine were found to be zero.). Since TOC and spectrophotometric measurements of the biomass-bound carbon gave identical results (0.3 C-mol, Table S2), we concluded that the apparent lack of carbon could not be explained by unexpected alterations of the biomass composition.

When the carbon balance was complemented by TOC measurements, 86 % of the total consumed carbon could be recovered (Table 7). From these results we conclude that 27 % of the total consumed carbon were bound in one or several unidentified products which have accumulated

in the medium as the result of unexpected metabolic activities and/or cell lysis, and that 14 % of the carbon most likely left the system in the form of one or several unidentified volatile products.

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Discussion

Direct cost-efficient microbial synthesis of methionine or its analogue HMTB from sugar is hampered by both the high metabolic cost of sulfur incorporation (Kromer et al., 2006) and the comparatively low solubility of methionine which limits product concentrations at physiological temperatures and near neutral pH to approximately 50 g/L (Fuchs et al., 2006). To tackle these technological problems, we have recently proposed a two-stage process which includes the fermentative production of DHB from sugar (Walther et al., 2017), and the subsequent carbon-conserving chemical incorporation of methanethiol to produce HMTB (Deck et al., 2009). In contrast to the method described by Shim and coworkers (Shim et al., 2016), where methionine is enzymatically produced from the activated precursors O-acetylhomoserine or O-succinylhomoserine, the DHB-based process, at least in theory, does not generate any metabolic by-products and eventually yields the water soluble methionine analogue HMTB thus permitting high product titers.

Since annotated DHB-producing metabolic pathways do not exist, we and others have recently conceived three-step synthetic metabolic pathways which produce DHB from malate (Li et al., 2014; Walther et al., 2013, 2017). By describing a pathway which derives DHB from homoserine in the present work, we extend the panel of DHB-producing metabolic routes. The synthetic pathway is composed of homoserine transaminase and OHB reductase activities, both of which have been identified in several natural transaminase or 2-hydroxyacid dehydrogenase enzymes, respectively. Among the tested enzymes, OHB reductase activity was found to be highest in (L)-LdhA from *L. lactis*. Activity of LL-LdhA on OHB was further enhanced by structure-based engineering of the enzyme, which alleviated a steric clash between the OHB substrate and Gln85 which restricts the size of the active pocket. Mutation of Gln85 to the smaller cysteine yielded an enzyme which accepted OHB and the natural substrate pyruvate with almost equal specificity thus supporting efficient OHB to DHB conversion. In contrast, homoserine transaminase activities of the tested enzymes were rather small compared to the preferred substrates. Nevertheless, we found that expression of the EC-AspC

enzyme which bears homoserine transaminase activity significantly increased DHB production suggesting that intracellular homoserine concentrations were sufficiently high to compete with the natural substrate aspartate.

A homoserine-overproducing *E. coli* strain which expressed the homoserine transaminase and OHB reductase enzymes EC-AspC and LL-LdhA_{Q85C}, respectively, produced 5.3 g/L DHB at a yield of 0.1 g/g after 48 h fed-batch cultivation. This result shows that significant DHB production can be achieved via the synthetic pathway. However, much work is necessary to reach industrially relevant productivities and yields. The development of more efficient enzymes represents the major lever to increase the performance of the homoserine-dependent DHB pathway. In particular, the very small activities of all tested transaminases on homoserine appear to limit pathway flux. It is therefore interesting that Bouzon and coworkers have very recently shown that the alanine aminotransferase double mutant AlaC A142P:Y275D has strongly increased activity on homoserine (Bouzon et al., 2017). These authors used long-term cultivations of *E. coli* cells under conditions that were selective for homoserine transaminase activity and subsequent genetic analysis of fast growing cells to obtain the new activity. The AlaC A142P:Y275D mutant enzyme was shown to have a K_m of 1.7 mM for homoserine (Bouzon et al., 2017), which makes it an attractive candidate to improve the performance of the synthetic DHB pathway. In addition, substrate affinity of the OHB reductase enzyme, which currently cannot be saturated by OHB concentrations of up to 20 mM, needs to be strongly improved. The intracellular OHB concentration is expected to be in the sub-millimolar range since the mass action ratio of aminotransferase reactions which use 2-oxoglutarate or pyruvate as amino group acceptor is commonly close to unity (Jankowski et al., 2008), and because the concentrations of the corresponding substrate/product couple 2-oxoglutarate (or pyruvate)/glutamate in *E. coli* are typically in the range of 0.4 mM and 90 mM (Bennett et al., 2008), respectively. Thus, only an OHB reductase enzyme with very high substrate affinity can “pull” carbon flux through the homoserine transaminase reaction thereby enabling efficient *in vivo* operation of the DHB pathway.

The optimization of the producer strain is another important factor to increase DHB yields. Notably, strain auxotrophies for methionine and threonine which were caused by the deletions of MetA and ThrB should be removed in an industrial strain. This could be achieved, for instance, by decreasing the transcription of the corresponding genes rather than by deleting them. Furthermore, improving homoserine production appears to be a promising approach to increase DHB production, as shown for other homoserine-derived products like threonine and O-succinylhomoserine (Debabov, 2003; Hong et al., 2014; Lee et al., 2007). In this context, the results of Li et al. (2016) are particularly interesting in that they have demonstrated production of 40 g/L by an engineered *E. coli* strain. Their initial strain harbored the same effective homoserine-enhancing deletions ($\Delta metA$, $\Delta thrB$) as our strain and overexpressed a bifunctional aspartate kinase/homoserine dehydrogenase (MetL). This strain produced only 6.8 g/L homoserine at a yield of 0.13 g/g from glucose after 48 h of a fed-batch cultivation, which is very similar to the 5.3 g/L DHB and 0.1 g/g product yield which have been produced by our strain. Li et al. (2016) reached much higher homoserine titers of 33.4 g/L and 40 g/L when the producer strain was further optimized by additionally overexpressing the homoserine-exporting permease RhtA, and by deleting the homoserine-importing permease TdcC, respectively. From the observations that homoserine inhibits aspartate kinase activity in vitro, and that overexpression of RhtA decreases intracellular homoserine concentrations, Li et al. (2016) conclude that accumulation of intracellular homoserine strongly inhibits its own biosynthesis. In line with these arguments we suggest that elevated intracellular homoserine concentrations, which build up as a consequence of the very small homoserine transaminase activities, are a major cause for the rather limited production of DHB by our strain. Increasing the efficiency of the synthetic DHB pathway enzymes can be expected to decrease homoserine accumulation and is, therefore, required to prevent product-feedback inhibition on the homoserine-producing pathway. Furthermore, strain optimization must tackle the problem of the formation of unknown fermentation products. Strain Eco19 transformed 41 % of the consumed carbon into unidentified soluble (27 %) and volatile (14 %)

products. Identifying these carbon sinks and preventing the formation of these by-products is a major requirement to improve DHB production.

In summary, we have demonstrated DHB production at a yield of 0.1 g/g via a two-step synthetic metabolic pathway which derives from homoserine. Further enzyme engineering to increase the catalytic capacity of the synthetic pathway, and strain engineering to avoid excessive accumulation of unknown products and to redirect carbon flux towards DHB synthesis are necessary to achieve industrially relevant DHB productivities and yields.

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Figure and Table Legends

Table 1: Strains used in this study.

Table 2: Plasmids used in this study.

Table 3: Kinetic parameters of several transaminases on homoserine and their natural substrates.

Subtext: Data are presented as means $\pm$ STDV calculated from at least three replicates. (*) Natural substrates (each at 20 mM): Ec-IlvE – leucine, Ec-TyrB – phenylalanine, Ec-AspC – aspartate. (**) activity was estimated at a homoserine concentration of 50 mM. ns – Not saturated at up to 50 mM substrate concentration.

Table 4: Kinetic parameters of candidate α -oxo acid reductases on 2-oxo-4-hydroxybutyrate and their natural substrates.

Subtext: Data are presented as means $\pm$ STDV calculated from at least three replicates. (*) Ratio of activities measured on the natural substrate (at 20 mM pyruvate or 0.05 mM oxaloacetate) and OHB (at 20 mM). ns – not saturated at substrate concentrations of up to 20 mM. nc – not calculated. Natural substrates: Pyruvate for Ec-Ldh, Ll-LdhA; Oxaloacetate for Ec-Mdh.

Table 5: Impact of mutations in (L)-lactate dehydrogenase A from *L. lactis* on its activity on pyruvate and 2-oxo-4-hydroxybutyrate.

Subtext: Data are presented as means $\pm$ STDV calculated from at least three replicates. (*) Ratio of activities measured on pyruvate and OHB at 20 mM substrate concentration. ns – not saturated at substrate concentrations of up to 20 mM. nc – not calculated.

Table 6: Product formation by *E. coli* strains expressing the synthetic DHB pathway.

Subtext:

- (1) wt – wild-type strain, $\Delta 4 - \Delta adhE \Delta ldhA \Delta thrB \Delta metA$
- (2) *E. coli* genes *aspC*, and *thrA*_{S345F} encode aspartate aminotransferase, and bifunctional aspartate kinase-homoserine dehydrogenase enzymes, respectively. Serine 345 was replaced by phenylalanine to alleviate threonine inhibition of ThrA. The LldhA gene is from *Lactococcus lactis* and encodes (L)-lactate dehydrogenase. In this enzyme variant, glutamine 85 was replaced by cysteine to increase OHB reductase activity.
- (3) Data from a single experiment.

Table 7: Carbon balance of the fed-batch fermentation of strain Eco19.

Figure 1: Synthetic metabolic pathway for production of 2,4-dihydroxybutyrate from homoserine. Synthetic reactions are shown in red, natural homoserine pathway reactions are shown in black. Names of the enzymes catalyzing reactions in the natural homoserine pathway are shown in blue. OHB – 2-oxo-4-hydroxybutyrate, DHB – 2,4-dihydroxybutyrate.

Figure 2: Active site region in the crystal structure of (L)-lactate dehydrogenase of *G. stearothermophilus*. The synthetic 2-oxo-4-hydroxybutyrate (OHB) substrate shown in green was manually docked into the experimental X-ray structure of the enzyme complex with NAD and the substrate analogue oxamate (PDB code: 1LDN). Atoms of key amino acid residues and substrates are colored according to element type: carbon, grey; nitrogen, blue; oxygen, red. Polar interactions between the oxamate and enzyme amino acid residues are shown as dashed red lines. The overall structure is shown in pale brown.

Figure 3: Fermentation profile of the DHB-producing Eco18 strain. The strain was cultivated on defined mineral medium which was supplemented with 1 g/L threonine and methionine to complement strain auxotrophies. Glucose was manually added to assure non-limiting carbon source concentrations of > 10 g/L during the fermentation.

Table 1: Strains used in this study.

Strain name	Genotype	Reference
MG1655	F ⁻ λ ⁻ ilvG ⁻ rfb-50 rph-1	ATCC 47076
NEB5-α	<i>fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80Δ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	NEB™
BL21(DE3)	<i>huA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS</i>	NEB™
JW1228-1	λ DE3 = λ <i>sBamHlo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5</i> F ⁻ , Δ(<i>araD-araB</i>)567, Δ(<i>lacZ4787::rrnB-3</i>), λ ⁻ , <i>rph-1</i> , Δ(<i>rhaD-rhaB</i>)568, <i>hsdR51</i> , Δ <i>adhE748::kan</i>	(Baba et al., 2006)
JW1375-1	F ⁻ , Δ(<i>araD-araB</i>)567, Δ(<i>lacZ4787::rrnB-3</i>), λ ⁻ , <i>rph-1</i> , Δ(<i>rhaD-rhaB</i>)568, <i>hsdR514</i> , Δ <i>ldhA744::kan</i>	(Baba et al., 2006)
JW0002-3	F ⁻ , Δ(<i>araD-araB</i>)567, Δ(<i>lacZ4787::rrnB-3</i>), λ ⁻ , <i>rph-1</i> , Δ(<i>rhaD-rhaB</i>)568, <i>hsdR514</i> , Δ <i>thrB723::kan</i>	(Baba et al., 2006)
JW3973-1	F ⁻ , Δ(<i>araD-araB</i>)567, Δ(<i>lacZ4787::rrnB-3</i>), λ ⁻ , <i>rph-1</i> , Δ(<i>rhaD-rhaB</i>)568, <i>hsdR514</i> , Δ <i>metA780::kan</i>	(Baba et al., 2006)
Eco4	MG1655 Δ <i>adhE</i> Δ <i>ldhA</i> Δ <i>thrB</i> Δ <i>metA</i>	This study
Eco5	Eco4 harboring empty pZA23	This study
Eco6	Eco4 harboring empty pEXT20	This study
Eco7	Eco4 harboring empty pZA23 and pEXT20	This study
Eco9	Eco4 harboring empty pZA23 and pEXT20-aspC-LLdhA _{Q85C}	This study
Eco10	Eco4 harboring pZA23-thrA _{S345F} and empty pEXT20	This study
Eco11	Eco4 harboring pZA23-thrA _{S345F} and pEXT20-aspC	This study
Eco12	Eco4 harboring pZA23-thrA _{S345F} and pEXT20-LLdhA _{Q85C}	This study
Eco13	Eco4 harboring pZA23-thrA _{S345F} and pEXT20-aspC-LLdhA _{Q85C}	This study
Eco18	Eco4 harboring pZA23-thrA _{S345F} -aspC-LLdhA _{Q85C}	This study

Table 2: Plasmids used in this study.

Name	Relevant characteristics	Reference
pCP20	<i>ori</i> pSC101, Amp ^R , plasmid expressing Flp recombinase to remove Kan cassette	(Cherepanov and Wackernagel, 1995)
pEXT20	<i>ori</i> colE1, Amp ^R , tac promoter	(Dykxhoorn et al., 1996)
pZA23	<i>ori</i> p15A, Kan ^R , P _{A1lacO-1} promoter	(Lutz and Bujard, 1997)
pET-28(a)+	<i>ori</i> f1, Kan ^R , T7 promoter	Novagen ^{TM}
pZA23-thrA	pZA23 derivative carrying <i>thrA</i>	This study
pZA23-thrA _{S345F}	pZA23 derivative carrying <i>thrA</i> _{S345F}	This study
pEXT20-aspC	pEXT20 derivative carrying <i>aspC</i>	This study
pEXT20-LLldhA _{Q85C}	pEXT20 derivative carrying <i>LLldhA</i> _{Q85C}	This study
pEXT20-aspC-LLldhA _{Q85C}	pEXT20 derivative carrying <i>aspC</i> and <i>LLldhA</i> _{Q85C}	This study
pEXT20-ilvE-LLldhA _{Q85C}	pEXT20 derivative carrying <i>ilvE</i> and <i>LLldhA</i> _{Q85C}	This study
pZA23-thrA _{S345F} -aspC-LLldhA _{Q85C}	pZA23 derivative carrying <i>thrA</i> _{S345F} , <i>aspC</i> , <i>LLldhA</i> _{Q85C}	This study

Table 3: Kinetic parameters of several transaminases on homoserine and their natural substrates.

Enzyme	Vmax [$\mu\text{mol}/(\text{min} \cdot \text{mg prot})$]		Km [mM]	
	Homoserine**	Natural substrate*	Homoserine	Natural substrate*
Ec-IlvE	0.077 $\pm$ 0.02	35.5 $\pm$ 3.2	ns	0.49 $\pm$ 0.21
Ec-TyrB	0.057 $\pm$ 0.01	86.4 $\pm$ 2.8	ns	1.33 $\pm$ 0.84
Ec-AspC	0.082 $\pm$ 0.01	74.0 $\pm$ 5.7	ns	1.59 $\pm$ 0.69

Data are presented as means $\pm$ STDV calculated from at least three replicates. (*) Natural substrates (each at 20 mM): Ec-IlvE– leucine, Ec-TyrB – phenylalanine, Ec-AspC – aspartate. (**) activity was estimated at a homoserine concentration of 50 mM. ns – Not saturated at up to 50 mM substrate concentration.

Table 4: Kinetic parameters of candidate α -oxo acid reductases on 2-oxo-4-hydroxybutyrate and their natural substrates.

Enzyme	Natural substrate			2-oxo-4-hydroxybutyrate			Specificity*
	Vmax [$\mu\text{mol}/(\text{mg min})$]	Km [mM]	Vmax/Km	Vmax [$\mu\text{mol}/(\text{mg min})$]	Km [mM]	Vmax/Km	
Ec-Ldh	405 $\pm$ 129	2.86 $\pm$ 0.47	141	0.56 $\pm$ 0.01	ns	nc	718
LI-LdhA	466 $\pm$ 72	1.39 $\pm$ 0.2	335	11.33 $\pm$ 4.1	ns	nc	41
Ec-Mdh	105 $\pm$ 22	0.03 $\pm$ 0.02	3500	0.02 $\pm$ 0.005	ns	nc	4748
LI-PanE	-	-	-	2.07 $\pm$ 0.69	ns	nc	-

Data are presented as means $\pm$ STDV calculated from at least three replicates. (*) Ratio of activities measured on the natural substrate (at 20 mM pyruvate or 0.05 mM oxaloacetate) and OHB (at 20 mM). ns – not saturated at substrate concentrations of up to 20 mM. nc – not calculated. Natural substrates: Pyruvate for Ec-Ldh, LI-LdhA; Oxaloacetate for Ec-Mdh.

Table 5: Impact of mutations in (L)-lactate dehydrogenase A from *L. lactis* on its activity on pyruvate and 2-oxo-4-hydroxybutyrate.

Mutation	Pyruvate			2-oxo-4-hydroxybutyrate			Specificity*
	Vmax [$\mu\text{mol}/(\text{mg min})$]	Km [mM]	Vmax/Km	Vmax [$\mu\text{mol}/(\text{mg min})$]	Km [mM]	Vmax/Km	
wt	466 $\pm$ 72	1.39 $\pm$ 0.2	335	11 $\pm$ 4	ns	nc	41
Q85C	101 $\pm$ 11	ns	nc	47 $\pm$ 7	ns	nc	2
Q85N	216 $\pm$ 29	ns	nc	16 $\pm$ 3	ns	nc	13

Data are presented as means $\pm$ STDV calculated from at least three replicates. (*) Ratio of activities measured on pyruvate and OHB at 20 mM substrate concentration. ns – not saturated at substrate concentrations of up to 20 mM. nc – not calculated.

Table 6: Product formation by *E. coli* strains expressing the synthetic DHB pathway.

Strain No	Strain Genotype ⁽¹⁾	Plasmids ⁽²⁾	OD ₆₀₀	Homoserine [mg/L]	DHB 24h [mg/L]
MG1655	wt	none	7.2 ±0.42	0 ±0	0 ±0
Eco4	Δ4 ⁽¹⁾	none	5.6 ±0.45	477 ±27	59 ±4
Eco5 ⁽³⁾	Δ4	pZA23-empty	5.5	396	45
Eco6 ⁽³⁾	Δ4	pEXT20-empty	1.6	36	11
Eco7	Δ4	pZA23-empty + pEXT20-empty		no growth	
Eco9	Δ4	pZA23-empty + pEXT20-aspC-LLldhA _{Q85C}	5.3 ±0.1	329 ±5.8	48 ±2
Eco10 ⁽³⁾	Δ4	pZA23-thrA _{S345F} + pEXT20-empty	4.0	581	48
Eco11	Δ4	pZA23-thrA _{S345F} + pEXT20-aspC	6.1 ±0.1	703 ±20	87 ±8
Eco12	Δ4	pZA23-thrA _{S345F} + pEXT20-LLldhA _{Q85C}	5.5 ±0.1	747 ±53	81 ±1
Eco13	Δ4	pZA23-thrA _{S345F} + pEXT20-aspC-LLldhA _{Q85C}	5.2 ±1	1040 ±350	190 ±17
Eco18	Δ4	pZA23-thrA _{S345F} -aspC-LLldhA _{Q85C}	5.4 ±0.1	1110 ±12	439 ±29

(4) wt – wild-type strain, Δ4 - Δ*adhE* Δ*ldhA* Δ*thrB* Δ*metA*

(5) *E. coli* genes *aspC*, and *thrA*_{S345F} encode aspartate aminotransferase, and bifunctional aspartate kinase-homoserine dehydrogenase enzymes, respectively. Serine 345 was replaced by phenylalanine to alleviate

threonine inhibition of ThrA. The LldhA gene is from *Lactococcus lactis* and encodes (L)-lactate dehydrogenase. In this enzyme variant, glutamine 85 was replaced by cysteine to increase OHB reductase activity.

(6) Data from a single experiment.

Table 7: Carbon balance of the fed-batch fermentation of strain Eco19.

Compound	C-mol	% Carbon
Substrates		
Glucose	2.906	
Thr	0.037	
Met	0.034	
Sum	2.977	100
Products		
Biomass	0.268	9.0
DHB	0.280	9.4
Homoserine	0.003	0.1
Lactate	0.030	1.0
Acetate	0.019	0.7
CO ₂	1.149	38.6
Sum	1.749	59
Undetected C in medium	0.800	26.9
Sum	2.549	86

Highlights:

- A new synthetic metabolic pathway for production of 2,4-dihydroxybutyrate is presented
- The pathway employs homoserine transaminase and OHB reductase activities
- Homoserine transaminase activity was identified by screening of natural transaminases
- OHB reductase activity was improved by rational engineering of lactate dehydrogenase
- An engineered *E. coli* strain expressing the pathway produces DHB at a yield of 0.1 g/g

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