Biotin-independent Strains of *Escherichia coli* for Enhanced Streptavidin Production

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Abstract

Biotin is an archetypal vitamin used as cofactor for carboxylation reactions found in all forms of life. However, biotin biosynthesis is an elaborate multi-enzymatic process and metabolically costly. Moreover, many industrially relevant organisms are incapable of biotin synthesis resulting in the requirement to supplement defined media. Here we describe the creation of biotin-independent strains of *Escherichia coli* and *Corynebacterium glutamicum* through installation of an optimized malonyl-CoA bypass, which re-routes natural fatty acid synthesis, rendering the previously essential vitamin completely obsolete. We utilize biotin-independent *E. coli* for the production of the high-value protein streptavidin which was hitherto restricted because of toxic effects due to biotin depletion. The engineered strain revealed significantly improved streptavidin production resulting in the highest titers and productivities reported for this protein to date.

Keywords: streptavidin production, biotin-independent, bioprocess, Escherichia coli

1. Introduction

Vitamins play an important role as cofactors in enzymes and fulfill a multitude of other essential biological functions including signaling, regulation, electron transfer, oxidation protection and radical scavenging[1]. Correspondingly, vitamin deprivation or inadequate intake cause severe metabolic disorders such as cardiovascular disease, increased risk for cancer and miscarriage, and osteoporosis to name but a few[2]. Biotin (or vitamin B7/H) constitutes an archetypal representative for vitamins since it is inevitably required by most independently living organisms distributed over the three domains of life[1] with some exceptions in archaeal clades[3]. While only synthesized by some bacteria, yeasts, molds, algae and plants, mammals rely on dietary uptake of the vitamin or its supply from the intestinal microflora[1,4]. Biotin serves as enzymatic cofactor in carboxylation reactions in fatty acid biosynthesis, amino acid metabolism and gluconeogenesis where it activates CO_2 for the carboxyltransfer domains of the respective enzymes[4,5].

Several industrially relevant microorganisms lack the ability to independently synthesize biotin including *Saccharomyces cerevisiae*[6], *Pichia pastoris*[7] and *Corynebacterium glutamicum*[8,9]. In the case of *P. pastoris*, for instance, high amounts of the cofactor are added to defined media and process complications are frequently associated with poor quality of the supplemented biotin[7]. Similarly, biotin has to be added in serum-free cell culture medium formulations[10]. In order to overcome this limitation, several efforts have been undertaken to genetically engineer prototrophic variants of different organisms for industrial applications[6-9]. These works comprised introduction of biotin biosynthesis genes from naturally prototrophic hosts like *Escherichia coli* or *Bacillus subtilis*. An alternative approach to the aforementioned efforts could be the metabolic

engineering of biotin-independent organisms that *a priori* do no longer rely on biotin, which has thus far not been systematically elaborated.

Biotin metabolism has been extensively studied in *E. coli*[4,5,11,12], where it is used for the carboxylation of acetyl-CoA to yield malonyl-CoA which represents the first committed step in fatty acid synthesis[13] (Fig. 1). This reaction catalyzed by the acetyl-CoA carboxylase complex (AccABCD) is the only essential utilization of biotin in *E. coli*. Other than that, only propionate metabolism has been reported to rely on biotin for the carboxylation of propionyl-CoA in some strains but the corresponding genes and gene products remain elusive[14]. Despite its scarce usage, the biosynthesis of biotin is a metabolically costly procedure involving many enzymatic steps (Fig. 1): starting from glucose, a minimum of twelve enzymatic reaction steps is required to yield acetyl-CoA[15], which is then converted into malonyl-CoA by the aforementioned AccABCD reaction[13]. Subsequently, the fatty acid synthesis machinery is employed to successively couple three malonyl-CoA units in ten enzymatic steps to yield pimeloyl-ACP methyl ester[4]. This precursor is processed into biotin by five final biosynthesis enzymes (BioHFADB) before loading the cofactor onto the biotinyl carboxyl carrier protein (BCCP) by the biotin ligase BirA[4]. Hence, at least 29 steps are required to produce biotin including the comparably inefficient, yet catalytic, final biotin synthase (BioB) reaction[16,17] and not to mention involved cofactors (SAM, $NAD(P)^+$/NAD(P)H, CoA, ATP etc.) and the genetics of biotin biosynthesis including regulation[18,19].

In this work we describe the creation of biotin-independent phenotypes by re-wiring initial fatty acid biosynthesis using a malonyl-CoA bypass. The resulting engineered strains of *E. coli* and *C. glutamicum* are able to survive and proliferate in the complete absence of biotin. We apply this concept of biotin-independence to improve the production of the high-affinity biotin binder

streptavidin (SAV hereafter), which was thus far restricted due to toxic biotin depletion in the host cell. The biotin-independent *E. coli* strain revealed a significantly enhanced SAV production as well as improved growth behavior as compared to a conventional strain. Transferring this strategy to an SAV production process in lab-scale bioreactors and fine-tuning of the bypass led to the highest streptavidin titers reported to date (up to 8.3±0.2 g L^{-1}).

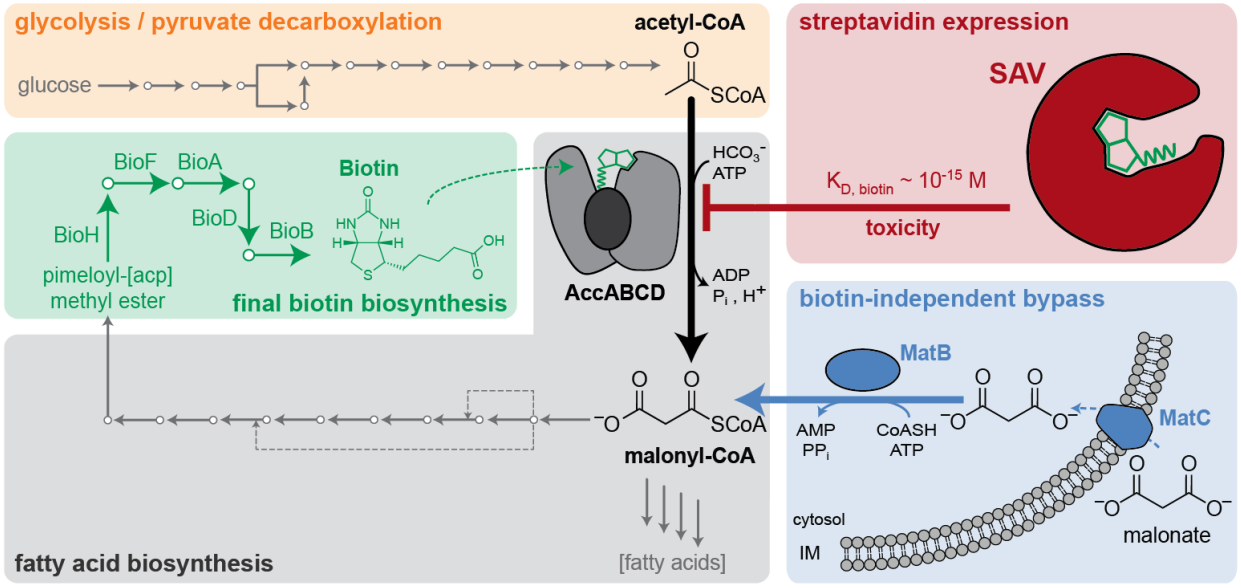


Fig. 1 | A biotin-independent bypass for malonyl-CoA synthesis. Biotin biosynthesis from glucose requires 29 enzymatic steps including synthesis of acetyl-CoA (orange box) and fatty acids (grey box) as well as the final steps exclusive to biotin production (green box). AccABCD requires biotin as cofactor for the essential conversion of acetyl-CoA to malonyl-CoA during fatty acid synthesis in most natural organisms[20]. Bypassing this reaction creates a biotin-independent phenotype achieved by implementing two heterologous proteins from *R. trifolii* (blue box) facilitating uptake of malonate (MatC) and its subsequent conversion to malonyl-CoA (MatB). The resulting strain should be superior to conventional hosts in its capability to produce biotin-binders such as streptavidin (SAV; red box), which is hitherto restricted due to the sequestration of biotin and inhibition of AccABCD[21,22].

2. Materials and methods

2.1 Suppliers

All chemicals were purchased from Sigma Aldrich (Buchs, Switzerland) unless stated otherwise. Enzymes and reagents for cloning were purchased at New England Biolabs (Ipswich, USA). Purified, lyophilized SAV was kindly provided by Prof. Thomas R. Ward (University of Basel, Switzerland).

2.2 Growth media

E. coli strains were grown in Luria–Bertani (LB) liquid medium or agar[23] for maintenance and genetic engineering supplemented with 50 mg L^{-1} kanamycin or 34 mg L^{-1} chloramphenicol where appropriate. The basic M9 medium[23] contained 10 g L^{-1} glucose and 20 mg L^{-1} thiamine. For the experiments with JM83 and its derivatives (Fig. 2(b)-(c); Supplementary Fig. 1; Supplementary Fig. 3) basic M9 was additionally supplemented with 1 mM L-proline, 500 µM isopropyl-β-D-thiogalactopyranosid (IPTG) and 50 mg L^{-1} kanamycin or 34 mg L^{-1} chloramphenicol where appropriate. Additionally 0.2 mg L^{-1} D-biotin or between 0 and 50 mM malonate were added to yield $M9^{BIO+}$ and $M9^{MAL+}$, respectively. For SAV expression with BL21(DE3) in shake flasks (Fig. 3(b)) and for the batch phase of bioreactor cultivations (Fig. 4) a defined mineral medium (pH 7.0) was used containing 3.0 g L^{-1} KH_2PO_4, 4.2 g L^{-1} Na_2HPO_4, 2.3 g L^{-1} $(NH_4)_2SO_4$, 1.9 g L^{-1} NH_4Cl, 1 g L^{-1} citric acid, 10 g L^{-1} glucose, 20 mg L^{-1} thiamine, 55 mg L^{-1} $CaCl_2$, 240 mg L^{-1} $MgSO_4$, 50 mg L^{-1} kanamycin, 34 mg L^{-1} chloramphenicol, and

1 mL L^{-1} trace element solution US [24]. Heterologous gene expression was induced by addition of 500 µM IPTG and where appropriate a concomitant malonate pulse to a final concentration of 5 mM was added.

Glucose feed medium (phase II bioreactor cultivation, Fig. 4(a)) contained 400 g L^{-1} glucose, 13.3 g L^{-1} $MgSO_4 \cdot 7H_2O$, 20 mg L^{-1} thiamine, 50 mg L^{-1} kanamycin, 34 mg L^{-1} chloramphenicol and 1 mL L^{-1} trace element solution US[24]. Glucose-malonate feed medium (phase III bioreactor cultivation, Fig. 4(a)) was prepared likewise and additionally contained between 0 and 560 mM malonate (pH adjusted to 7.0 by addition of sodium hydroxide).

For strain maintenance *C. glutamicum* was grown in LB liquid medium or agar containing 10 g L^{-1} glucose and where appropriate 25 mg L^{-1} kanamycin. Transformation was performed by electroporation as described elsewhere[25]. For biotin complementation experiments with *C. glutamicum* the minimal medium CGXII[26] with 2% glucose (w/v) was used either with (20 mg L^{-1}) or without biotin or supplemented with varying concentrations of malonate. Unless stated otherwise 10 µM IPTG were added for induction of *matBC* genes in this strain.

2.3 Cultivation conditions

E. coli growth experiments in microtiter plates were carried out in an Infinite M200 plate reader (Tecan, Männedorf, Switzerland) at 37°C and under agitation (orbital shaking, 2 mm amplitude) and bacterial growth was monitored by measuring the optical density of the cultures (200 µL total volume) at 600 nm (OD_{600}). Shake flask cultivations were carried out using 1 L Erlenmeyer flasks and a culture volume of 100 mL in a shaking incubator (37°C, 220 r.p.m.) and growth was monitored by OD_{600} determination in a cuvette photometer. SAV concentrations were determined by a fluorescence quenching assay (see section 2.7).

161 *C. glutamicum* was cultivated in 96-deepwell plates in 500 µL culture volume. Wells were
162 inoculated from single colonies from biotin-containing CGXII plates (1.6% agarose) and
163 cultivation was performed in a shaking incubator (30°C, 250 r.p.m.) for 48 h. The final OD_{600}
164 was determined in an Infinite M1000 plate reader (Tecan, Männedorf, Switzerland).
165
166 *2.4 Bioreactor cultivation*
167 Bioreactor cultivations with *E. coli* were carried out with a Labfors-5 benchtop fermenter system
168 (Infors HT, Bottmingen, Switzerland) with 7.5 L vessel volume at 37°C and a pH of 7.0 which
169 was maintained by titration with 10% (v/v) sulfuric acid and 5 M ammonium hydroxide. The
170 dissolved oxygen concentration was maintained above 30% of the saturation level by firstly
171 adjusting the stirrer speed (300 to 1250 rpm, 50 rpm increments; two six-blade Rushton
172 impellers) and secondly the air flow (1 to 5 vvm).
173 The batch phase was started by inoculation of 2 L of defined mineral medium to an initial OD_{600}
174 of 0.15 from an overnight shake flask pre-culture in the same medium. In order to prevent
175 foaming 1 mL of 20% (v/v) of polypropylene glycol was added to the culture and additionally as
176 necessary in 500 µL increments throughout the cultivation. After the consumption of the initial
177 amount of glucose (10 g L^{-1}), as indicated by a sudden rise in the dissolved oxygen signal,
178 glucose feed medium was gradually applied to the culture in an exponential manner (μ_{set} =
179 0.09 h^{-1}) until an approximate OD_{600} of 55 was reached (corresponding to a dry cell weight
180 concentration (DCW) of roughly 21 g L^{-1}). Subsequently, heterologous gene expression was
181 induced by addition of 500 µM IPTG and the administered medium was switched to glucose-
182 malonate feed medium which was applied at a constant rate of 0.53 mL min^{-1} until the end of the
183 process. Cell growth was monitored throughout the process by OD_{600} determination and DCW

184 measurement and SAV concentrations were determined by a fluorescence quenching assay (see
185 section 2.7).
186
187 *2.5 Strain engineering*
188 All strains used in this study are listed in Supplementary Table 1. In order to facilitate
189 transcription using P_{T7} promoters, the T7 RNA polymerase was integrated into the chromosome
190 of strain JM83 using the λDE3 Lysogenization Kit (Merck-Millipore, Darmstadt, Germany) and
191 the resulting strain was designated JM83(DE3). A biotin auxotroph derivative of JM83(DE3)
192 was created by P1 transduction from the Keio collection strain BW25113, which carries an
193 insertional knockout of the biotin synthase gene (*bioB*:*kan*), as described elsewhere[27].
194 Subsequently the kanamycin resistance gene was removed using FRT recombination with
195 plasmid pCP20, which was cured from the resulting strain JM83(DE3)*ΔbioB* by incubation at
196 43°C[28]. Colony PCR was performed to verify both successful transduction and removal of the
197 resistance gene using primers 1 and 2 flanking the *bioB* gene (Supplementary Tab. 2).
198
199 *2.6 Cloning procedures*
200 All plasmids used in this study are listed in Supplementary 1. The natural *matBC* cassette from
201 *R. trifolii* was obtained as a synthetic DNA fragment (Life Technologies, Regensburg, Germany;
202 Supplementary Tab. 3) and PCR-amplified using oligonucleotides 3 and 4 in order to introduce
203 flanking restriction sites for *Bam*HI and *Eco*RV. Accordingly, the plasmid pCK01[29] was PCR-
204 amplified with primers 5 and 6 introducing sites for *Bam*HI and *Eco*RV. Both PCR products
205 were digested (*Bam*HI and *Eco*RV) and joined by ligation resulting in plasmid pCKmatBC. To
206 generate pET30matBC the synthetic DNA construct of *matBC* (Supplementary Tab. 3) was PCR

amplified (primers 7 and 4) and the resulting PCR product was digested and ligated into the backbone of pET-30b(+) treated with the same restriction enzymes (*Nde*I and *Eco*RV).

pET30matBC*, in which the natural GTG start codon of the *matB* gene is replaced by ATG, was constructed by digestion of the PCR product of the *matBC* cassette (Supplementary Tab. 3) and primers 8 and 9 with *Nde*I and *Bam*HI and subsequent ligation into the backbone of pET-30b(+) treated with the same restriction enzymes (*Nde*I and *Bam*HI).

pEKEx2matBC was constructed by PCR amplification of the natural *matBC* cassette (Supplementary Tab. 3) with primers 10 and 11 followed by restriction digest with *BamH*I and *Kpn*I and subsequent ligation into the backbone of pEKEx2[30] treated with the same restriction enzymes.

2.7 Quantification of active SAV

Cell lysates of *E. coli* were produced by spinning down (20'000 rcf, 5 min, 4° C) 1 mL of broth and re-suspending the cell pellet in lysis buffer (10 mM Tris buffer at pH 7.4 containing 1.0 g L^{-1} lysozyme, 1 mM $MgSO_4$ and 10 mg L^{-1} DNAse). Afterwards, three consecutive freeze-thaw cycles were performed and the SAV-containing supernatant was cleared from cell debris by centrifugation (20'000 rcf, 10 min, 4° C). Free biotin binding sites in SAV were then quantified using a fluorescent quenching assay derived from a previously described protocol[31]. For this purpose, a binding site buffer containing 1 µM Atto-565-biotin (Atto-Tec, Siegen, Germany) and 0.1 g L^{-1} bovine serum albumin in phosphate buffered saline [23] was freshly prepared for each measurement. Aliquots of 10 µL of samples (diluted into the linear range of the assay; 0-0.95 µM biotin-binding sites) were mixed with 190 µL of binding site buffer and incubated for 30 minutes at ambient temperature to ensure binding of the dye to SAV. Afterwards a fluorescent

measurement was performed (λ_{Ex} = 563 nm, λ_{Em} = 620 nm) in black 96-well microtiter plates using an Infinite M1000Pro microtiter plate reader (Tecan, Männedorf, Switzerland) and SAV concentrations were calculated by correlation with an SAV standard curve (prepared from purified, lyophilized SAV) similarly prepared as the samples and recorded in the same plate. The cell specific SAV yields were calculated from the measured concentrations of SAV and DCW of the samples assuming a whole cell protein content of 0.5 g per gram DCW. In order to verify integrity of the product and confirm the validity of the optical quantification by a second method SDS-PAGE analysis was performed (Supplementary Fig. 4). Therefore cell lysates from one of the biotin-independent bioreactor processes (Fig. 4(c)) were run in comparison to a purified, lyophilized SAV standard corresponding to a concentration of 4 g L^{-1}.

253

254

255 **3. Results and discussion**

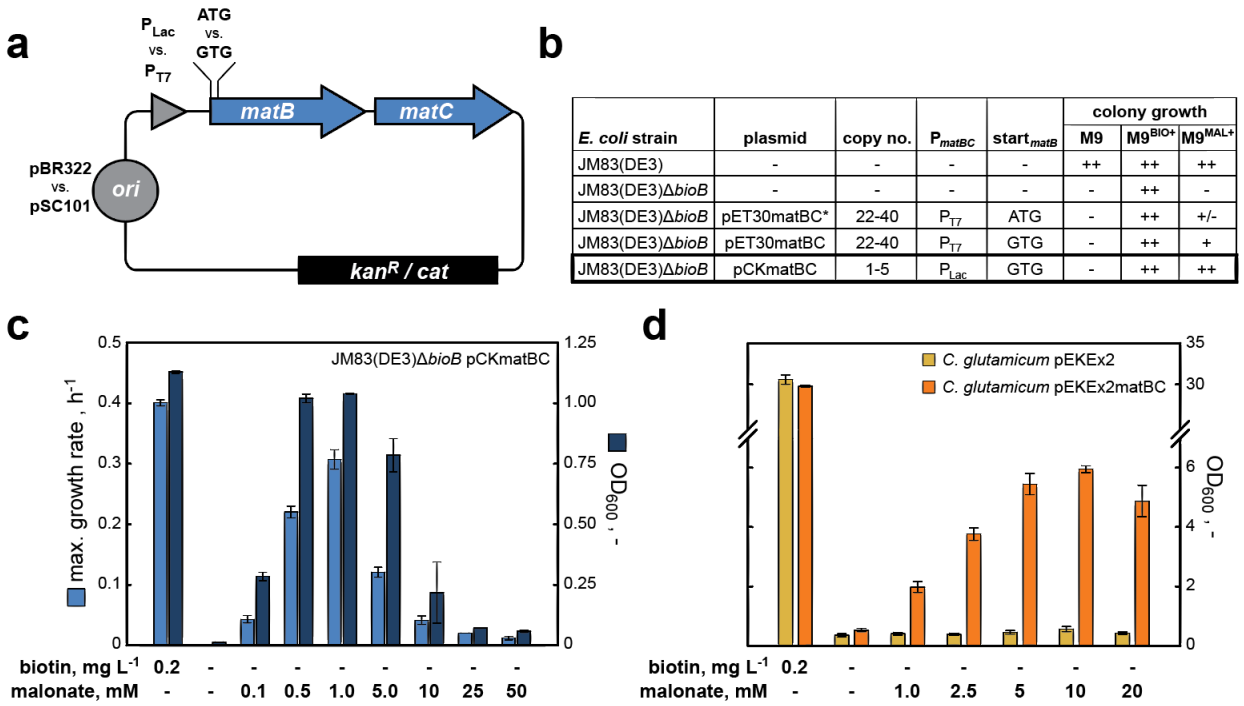
256 *3.1 Construction of biotin-independent Escherichia coli strains*

257 We hypothesized that biotin as such and consequently the complicated associated metabolic
258 machinery (final biotin biosynthesis genes and AccABCD complex) could be rendered
259 superfluous if a biotin-independent route to malonyl-CoA was established. The resulting strain
260 should be able to proliferate independently of biotin.

261 In order to create this biotin-independent bypass (Fig. 1) we selected two heterologous genes
262 (*matBC*) from the *Rhizobium trifolii* malonate utilization operon that are responsible for the
263 uptake of malonate (*matC*, malonate transporter) and its subsequent conversion to malonyl-CoA
264 (*matB*, malonyl-CoA synthetase)[32]. Malonic acid is a cheap bulk chemical readily synthesized by
265 plants but its bulk production mainly relies on chemical synthesis from chloroacetic acid[33,34]. The
266 *matABC* gene cluster has been expressed in *E. coli* previously to improve polyketide
267 synthesis[32,35]. Importantly, James and Cronan elegantly demonstrated that this system can be
268 used to create deletion mutants for various subunits of the essential acetyl-CoA carboxylase[36].
269 Building up on these previous studies, we anticipated that it should be possible to create a biotin-
270 independent phenotype using *matBC* and supplementation of growth media with malonate and
271 thereby insulating fatty acid synthesis from central carbon metabolism. Alternatively, malonate
272 could also be directly synthesized from glucose as very recently demonstrated in genetically
273 engineered *E. coli*[37].

274 We constructed three different *matBC* expression vectors for *E. coli* differing in the anticipated
275 expression levels by using different copy numbers and promoters and varying the start codon

(wild type GTG vs. ATG) of *matB* (Fig. 2(a)). To test whether the only biotin-dependent reaction can be bypassed by MatBC, we constructed a strain with disrupted chromosomal biotin synthase gene *bioB* which reportedly prevents biotin production and thus growth in biotin-free medium[38]. The resulting strain JM83(DE3)*ΔbioB* was transformed with the different *matBC* expression plasmids and plated on selective M9-agar containing either 0.2 mg L^{-1} biotin or 5 mM malonate as well as neither of the two supplements (Fig. 2(b)). Whereas parent strain JM83(DE3) exhibited normal growth even in biotin absence, the *bioB* mutants failed to proliferate on biotin-free agar. As expected, supplementation of biotin to the medium restored growth for all auxotroph mutants. Importantly, malonate was likewise able to restore growth but only in presence of a *matBC* expression vector and with notably diverging strain fitness depending on the anticipated expression level for the bypass proteins. More precisely, pCKmatBC (low copy number, P_{Lac}, GTG start codon for *matB*) exhibited the best growth behavior as compared to pET30matBC and pET30matBC*, which led to intermediate and slow growth, respectively. These results confirmed functionality of the biotin-independent malonyl-CoA bypass and pointed to a preferable low expression level for the bypass proteins, presumably because too high expression levels lead to excessive drainage of the cellular coenzyme A pool, highlighting the significance of expression level optimization for metabolic engineering[39-41].



E. coli strain	plasmid	copy no.	P_{matBC}	$start_{matB}$	colony growth		
					M9	$M9^{BIO+}$	$M9^{MAL+}$
JM83(DE3)	-	-	-	-	++	++	++
JM83(DE3)Δ*bioB*	-	-	-	-	-	++	-
JM83(DE3)Δ*bioB*	pET30matBC*	22-40	P_{T7}	ATG	-	++	+/-
JM83(DE3)Δ*bioB*	pET30matBC	22-40	P_{T7}	GTG	-	++	+
JM83(DE3)Δ*bioB*	pCKmatBC	1-5	P_{Lac}	GTG	-	++	++

Fig. 2 | Engineering of biotin-independent strains of *E. coli* and *C. glutamicum*. (a) Three *E. coli* plasmids for *matBC* expression were constructed differing in plasmid copy number (*ori*'s from pSC101 or pBR322) as well as transcriptional (P_{Lac} vs. P_{T7} promoter) and translational (GTG vs. ATG start codon of *matB*) control. (b) The MatBC bypass restores growth of a biotin auxotrophic mutant (Δ*bioB*) on biotin-free solid medium containing 5 mM malonate ($M9^{MAL+}$) with preference for low expression levels (pCKmatBC). Malonate-free (M9) and biotin-containing ($M9^{BIO+}$) media were included as negative and positive control, respectively. (c) The malonate concentration was optimized to ensure optimal growth of biotin-independent *E. coli*. (**d**) Construction of a *matBC* shuttle vector (pEKEx2matBC) allowed for biotin-independent growth of naturally biotin auxotroph *C. glutamicum*. Bars represent average specific growth rates and/or maximum OD_{600} for three (**c**) or four (**d**) replicate cultures in 96-well format with s.d..

3.2 Optimization of malonate supply

To quantify growth of the biotin-independent strain and investigate a potential influence of the amount of supplemented malonate, we conducted cultivations of JM83(DE3)*ΔbioB* pCKmatBC in microtiter plates (Fig. 2(c)) and shake flasks (see Supplementary Fig. 1) in biotin-free M9 liquid medium. We found growth to depend on the supplied malonate concentration and identified an optimum at which the growth rate was restored roughly to the same level as in presence of biotin which corresponds well with typically observed growth rates for wildtype

strains in mineral media. Concentrations exceeding the apparent optimum negatively affected the strains behavior as far as to complete growth inhibition. Similar observations were made by Lombó and coworkers[35] who observed growth of a normal, biotin prototroph strain at 5 mM but complete inhibition at 40 mM malonate and attributed the inhibitory effect on imbalances in the host's coenzyme A and acyl-CoA metabolism. Moreover, malonate is known to inhibit succinate dehydrogenase, a central enzyme of the Krebs cycle[42]. Consequently, malonate supply needs to be optimized for the desired strain and cultivation vessel in order to exploit the full potential of the malonyl-CoA bypass.

3.3 Grafting of MatBC bypass to Corynebacterium glutamicum

In order to evaluate transferability for the proposed biotin-independent concept, we selected the Gram-positive bacterium *Corynebacterium glutamicum* as a second chassis to demonstrate functionality of the MatBC bypass. *C. glutamicum* is an industrially highly relevant production host[43,44] and naturally biotin auxotrophic therefore requiring supplementation of defined media with the vitamin[9]. This latter practical limitation has been previously addressed by re-introduction of biotin biosynthesis genes into *C. glutamicum* to create biotin-prototrophic phenotypes[8,9]. Besides the essential acetyl-CoA carboxylase, *C. glutamicum* contains a biotin-dependent pyruvate carboxylase, which is responsible for anaplerotic channeling of pyruvate into the tricarboxylic acid cycle[45]. Complementation studies, however, revealed that pyruvate carboxylase is inessential and the corresponding deficient mutants exhibit wild type growth[45]. We therefore hypothesized that the MatBC-bypass would likewise render biotin obsolete in *C. glutamicum* creating mutants proficient to grow in biotin-free medium supplemented with malonate. We constructed the vector pEKEx2matBC with the natural *matBC* cassette under

control of a P_{tac} promoter. Gratifyingly, transformation of *C. glutamicum* wild type (ATCC 13032) with this construct enabled its proliferation in biotin-free media containing malonate whereas in the absence of MatBC (pEKEx2) as well as in medium lacking malonate only marginal growth due to residual biotin transferred from the pre-cultures was observable (Fig. 2(d)). Moreover, we found a strong growth inhibition for high inducer concentrations pointing to a similar preference for low *matBC* expression levels as previously observed for *E. coli* (see Supplementary Fig. 2). Taken together these experiments highlight the feasibility to create biotin-independent organisms using the MatBC bypass and the transferability of the underlying concept to different hosts.

3.4 Utilization of biotin-independence for cytosolic streptavidin production

Next, we sought to demonstrate practical utility of biotin independence and apply a respective *E. coli* strain to address a common problem occurring during expression of high-affinity biotin binders like streptavidin (SAV). Due to the high affinity to biotin (SAV, $K_{D,\ biotin}$ ~10^{-14} M) and its exceptional physicochemical stability, SAV is used for a multitude of biotechnological applications including live cell imaging, immobilization and affinity purification of biotinylated or peptide tagged biomolecules and nanotechnology as well as more recently developed technologies which repurpose the protein for drug targeting and the development of artificial metalloenzymes[46-48]. The secretion of SAV and its homologues by natural hosts such as *Streptomyces avidinii* serves as defense mechanism exploiting the highly efficient sequestration of the vitamin[21,49]. Not coincidentally, the expression of soluble, active SAV in the cytosol has been reported to lead to depletion of the host cell's biotin pool accompanied by impaired growth

and low SAV expression levels[21,22,50], which represents a major limitation for high-yield SAV production.

We presumed that the capability to bypass the critical biotin-sensitive metabolic step should lead to superior behavior in SAV expression without the accompanying negative effects and should therefore be of use for a corresponding SAV production strategy. We therefore introduced a second plasmid (pET30T7SAV) with the SAV gene under the control of a P_{T7} promoter into a strain harboring pCKmatBC (Fig. 3(a)). As production host we selected the strain *E. coli* BL21(DE3) since it is conventionally used for high biomass production and protein expression. Since BL21(DE3) is a biotin prototrophic strain, in the absence of SAV (before induction) the malonyl-CoA bypass is not needed, but its expression from pCKmatBC can be concomitantly activated with SAV induction from pET30T7SAV by addition of IPTG. This "switchable" MatBC bypass allows for normal growth in the off-state without the need to supplement malonate before induction, which is an important practical advantage. At the same time it should facilitate improved growth and production behavior in the on-state after induction of SAV.

To validate this hypothesis we conducted SAV expression studies in shake flasks in M9 medium, both with and without 5 mM malonate supplementation and in the absence (*matBC*$^-$, empty vector control) or presence of pCKmatBC (*matBC*$^+$, Fig. 3(b)). As expected, all four specimens showed very similar growth in the off-state until addition of IPTG (dashed line). Afterwards, the conditionally biotin-independent strain showed a significantly improved growth behavior only in presence of malonate (*matBC*$^+$ MAL$^+$) as compared to the conventional strain (*matBC*$^-$, MAL$^{+/-}$) and the control lacking malonate (*matBC*$^+$ MAL$^-$). More importantly, SAV expression was notably improved in the biotin-independent strain as reflected by a roughly two-fold increased final concentration. We performed similar experiments with strain JM83(DE3) revealing similar

trends and a more than three-fold increase in SAV production (see Supplementary Fig. 3). These results unambiguously demonstrate the functionality of the biotin-independent bypass and its utility for improved expression of SAV.

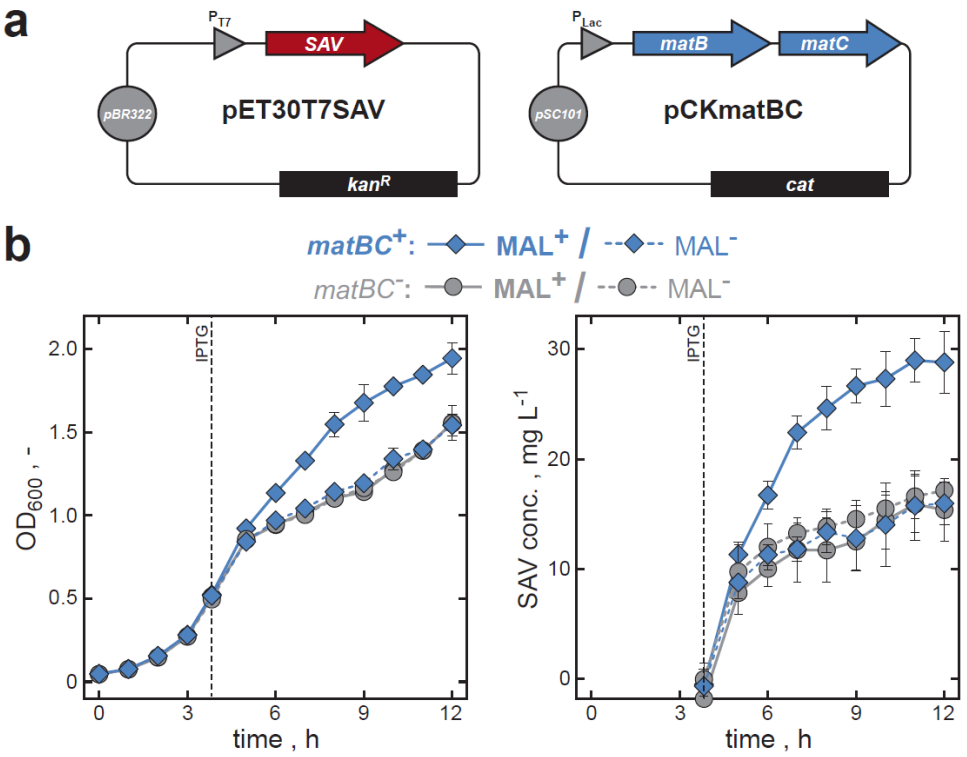


Fig. 3 | Streptavidin (SAV) production in conditionally biotin-independent *E. coli* strains. (a) For production of SAV in *E. coli* the vector pET30T7SAV was constructed. It contains the SAV gene under control of a P_{T7} promoter. (b) Shake flask cultivations with *E. coli* BL21(DE3) revealed significantly improved growth and SAV production behavior after SAV induction in the biotin-independent strain in presence of 5 mM malonate (***matBC*$^+$ MAL$^+$**) compared to the controls lacking either pCKmatBC (*matBC*$^-$ **MAL**$^+$ and *matBC*$^-$ MAL$^-$) or malonate (***matBC***$^+$ MAL$^-$). Data points represent mean OD_{600} and SAV concentrations of three independent cultures with standard deviation.

3.5 Establishment of a biotin-independent streptavidin production process

Next, we transferred the switchable biotin-independent SAV production into a laboratory scale bioreactor process using defined medium. Therefore we developed a cultivation protocol composed of three conceptual stages (Fig. 4(a)): an initial batch phase (I), a fed-batch stage (II)

with exponential glucose-limited feeding (biomass production), and an SAV production stage (III) initiated by induction with IPTG, during which a mixture of malonate and glucose is applied in a constant manner throughout the rest of the process. Since the initial studies had pointed to a critical requirement of a fine-tuned malonate supply depending on the cultivation conditions, we investigated the effect of different malonate-to-glucose ratios in the feed medium during SAV production (III) (Fig. 4(b)). The strain lacking *matBC* reproducibly yielded similar amounts of SAV (~4 g L^{-1}) regardless of the malonate amount fed to the broth. In stark contrast the biotin-independent strain (*matBC*$^{+}$) showed a production behavior depending on the applied malonate amount with a peak concentration of 7.3±0.2 g L^{-1} SAV at 0.56 mmol malonate per gram of glucose. To demonstrate reproducibility we performed replicate bioreactor runs at the identified malonate optimum (Fig. 4(c)). The biotin-independent strain outperformed the conventional strain both with respect to growth and SAV production and allowed for a reproducibly higher maximum product concentration of 7.5±0.7 g L^{-1} of active SAV (compared to 4.7±0.2 g L^{-1}). The best run yielded 8.3±0.2 g L^{-1} (or 126.3±3.0 µM tetramer) of product (compare also Supplementary Fig. 4). This improvement can be attributed both to an improved growth after induction of SAV expression as well as to an increased cell specific product yield of approximately 49±4% of whole cell protein (as compared to 38±1% for the control) indicating that the re-programmed strain's performance was driven close to the reported feasible maximum for recombinant protein expression in *E. coli*[51].

To the best of our knowledge the titers produced with the biotin-independent strain represent the highest SAV concentrations reported to date and constitute a significant increase compared to former benchmark studies[52,53]. Moreover, due to the high specific growth rate of *E. coli*, which allows comparably short overall process times, the volumetric productivity was substantially

increased in comparison to previously used hosts for SAV production such as *S. avidinii*, *P. pastoris*, or *B. subtilis* [52-54].

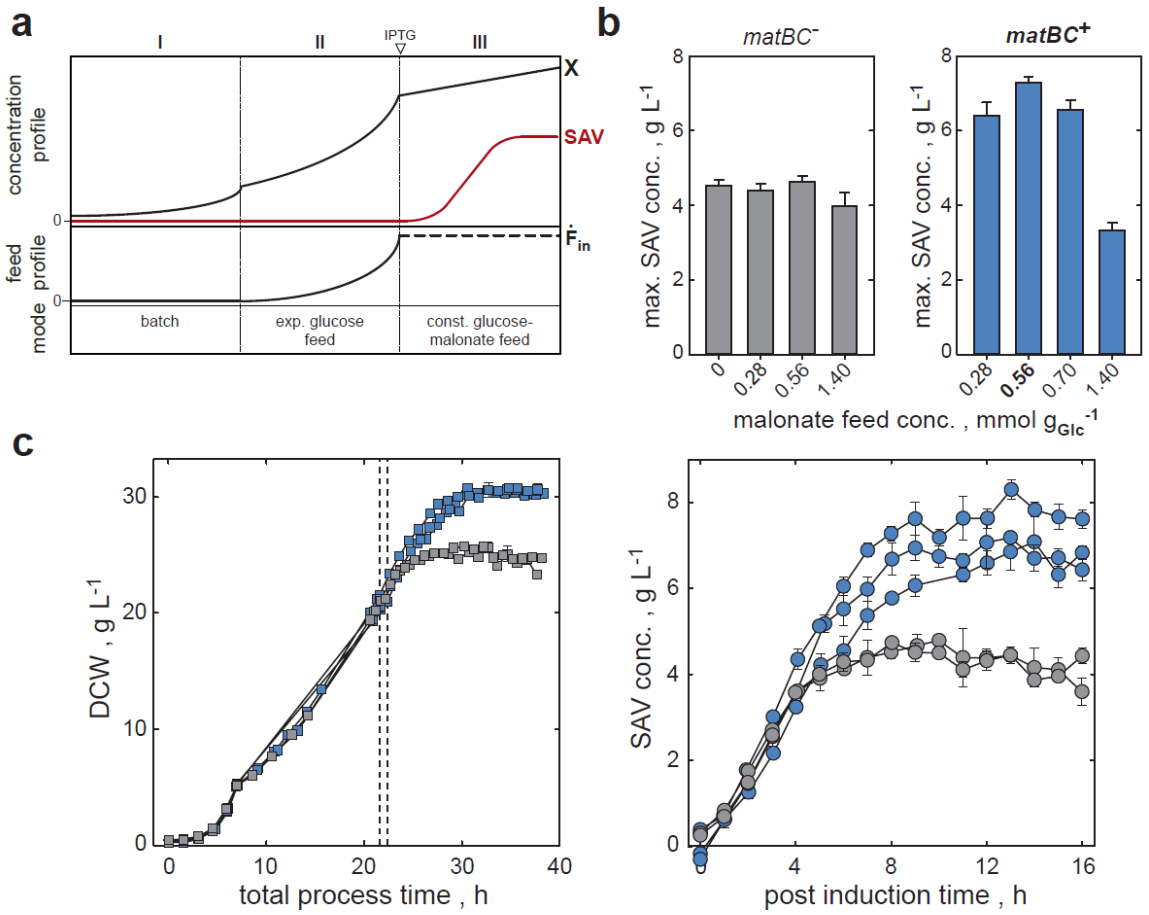


Fig. 4 | Development of a streptavidin (SAV) production process in biotin-independent *E. coli*. (a) A three-stage bioreactor process was developed: I, batch phase; II, exponential glucose-limited fed-batch; III, SAV production phase with constant glucose-malonate feed. The idealized courses for concentration of biomass X and SAV and the volumetric feed flow rate $\dot{F}_{in}$ are conceptually shown. (b) The critical malonate-glucose ratio in the feed (phase III) was optimized. Maximum SAV concentrations eight hours after induction are indicated. (c) The productivity of the optimal setup (0.56 mmol g_{Glc}^{-1}) was verified by three independent bioreactor cultivations of the biotin-independent strain (blue squares/circles) compared to two independent reference cultivations with the conventional strain (*matBC*⁻) without malonate (grey squares/circles). Bars/data points represent averages of triplicate measurements of dry cell weight (DCW) and SAV concentration with standard error. The area between the dashed lines represents the IPTG induction window of all five processes. SDS-PAGE analysis for the biotin-independent process was performed to confirm integrity of the product (see Supplementary Fig. 4)

4. Conclusion

Metabolic engineering is widely used to improve bioprocess performance by directing fluxes into a desired product based on the ever increasing knowledge about cellular metabolic networks[55]. Well established strategies include overexpression or deletion of inherent host enzymes to increase the flux into the target pathway or prevent drainage of intermediates and consequently product loss or side product formation, the integration of enzymes facilitating growth and product formation based on inexpensive substrates, as well as implementation of proteins which simplify downstream processing. These efforts are frequently combined with flux models that help identifying the key bottlenecks within the system [56,57].

A relatively uncharted approach is the fundamental re-organization of central host metabolism in order to enhance bio-production. This strategy seeks to completely re-route central metabolic pathways in order to drive their flux into a desired direction and is based on the notion that natural metabolism, as good as it is to cope with natural challenges, may not be the preferable choice for biotechnological application. Auspicious examples comprise a synthetic non-oxidative glycolysis[58] and a reverse glyoxylate shunt[59], both designed to minimize carbon loss upon utilization of carbohydrates, as well as the engineering of artificial carbon fixation cycles with the goal to increase sequestration of the greenhouse gas carbon dioxide[60-63]. Despite the fact that some of these efforts thus far mainly comprised theoretical considerations and *in vitro* studies and have therefore hardly exceeded the stage of a blueprint, this type of approach could arguably enable to fundamentally change cellular metabolism as we know it today and may allow accessibility to entirely novel processes and bio-products.

In this work we re-route the central pathway of fatty acid biosynthesis by installation of a bypass for malonyl-CoA to liberate the corresponding strains of *E. coli* and *C. glutamicum* from their

dependence on biotin, an essential vitamin evolutionary conserved in all kingdoms of life. The engineered organisms exhibit normal growth in the absence of the cofactor and can be used for biotechnological applications as demonstrated on the test bed of SAV production, which was previously restricted due to toxic biotin depletion in the host cell[21,22,50]. This led to the establishment of an SAV production process with hitherto unmatched maximum titers and productivities.

To extend the biotin-independent concept beyond this proof-of-principle study, the entire cellular machinery associated with biotin could be removed from the host genome. This includes genes involved in its biosynthesis (*bioHFADB*), its loading (*birA*), as well as all acetyl-CoA carboxylase genes (*accABCD*). Furthermore, the presented MatBC bypass could be combined with a module for *in vivo* synthesis of malonate, which has recently been established in an engineered *E. coli* strain that is capable of synthesizing malonate from aspartate via a beta-alanine route[37]. This would close the gap to prevalent metabolites that can be directly derived from central metabolism and render the currently required (yet inexpensive) supplementation of malonate obsolete, leading to a stand-alone biotin-independent organism, which synthesizes fatty acid building-blocks in a completely novel way.

We believe that this work represents a prime example indicating that even fundamental design principles of cellular carbon flux in living cells can be simplified for synthetic purposes. This suggests a hitherto largely unappreciated malleability of core metabolism, which augurs well for future fundamental re-design of bacterial metabolism using abiotic reactions[48,64].

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