

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

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27 **Abstract**

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

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

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52 **1. Introduction**

53 Vitamins play an important role as cofactors in enzymes and fulfill a multitude of other essential
54 biological functions including signaling, regulation, electron transfer, oxidation protection and
55 radical scavenging¹. Correspondingly, vitamin deprivation or inadequate intake cause severe
56 metabolic disorders such as cardiovascular disease, increased risk for cancer and miscarriage,
57 and osteoporosis to name but a few². Biotin (or vitamin B7/H) constitutes an archetypal
58 representative for vitamins since it is inevitably required by most independently living organisms
59 distributed over the three domains of life¹ with some exceptions in archaeal clades³. While only
60 synthesized by some bacteria, yeasts, molds, algae and plants, mammals rely on dietary uptake of
61 the vitamin or its supply from the intestinal microflora^{1,4}. Biotin serves as enzymatic cofactor in
62 carboxylation reactions in fatty acid biosynthesis, amino acid metabolism and gluconeogenesis
63 where it activates CO₂ for the carboxyltransfer domains of the respective enzymes^{4,5}.

64 Several industrially relevant microorganisms lack the ability to independently synthesize biotin
65 including *Saccharomyces cerevisiae*⁶, *Pichia pastoris*⁷ and *Corynebacterium glutamicum*^{8,9}. In
66 the case of *P. pastoris*, for instance, high amounts of the cofactor are added to defined media and
67 process complications are frequently associated with poor quality of the supplemented biotin⁷.
68 Similarly, biotin has to be added in serum-free cell culture medium formulations¹⁰. In order to
69 overcome this limitation, several efforts have been undertaken to genetically engineer
70 prototrophic variants of different organisms for industrial applications⁶⁻⁹. These works comprised
71 introduction of biotin biosynthesis genes from naturally prototrophic hosts like *Escherichia coli*
72 or *Bacillus subtilis*. An alternative approach to the aforementioned efforts could be the metabolic

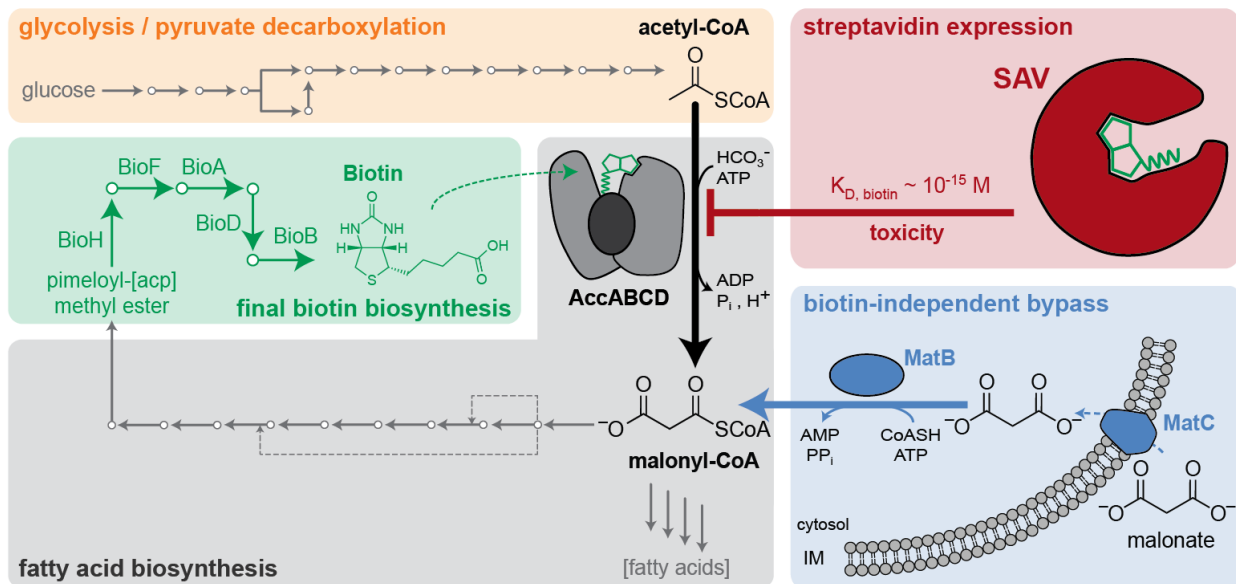
73 engineering of biotin-independent organisms that *a priori* do no longer rely on biotin, which has
74 thus far not been systematically elaborated.

75 Biotin metabolism has been extensively studied in *E. coli*^{4,5,11,12}, where it is used for the
76 carboxylation of acetyl-CoA to yield malonyl-CoA which represents the first committed step in
77 fatty acid synthesis¹³ (Fig. 1). This reaction catalyzed by the acetyl-CoA carboxylase complex
78 (AccABCD) is the only essential utilization of biotin in *E. coli*. Other than that, only propionate
79 metabolism has been reported to rely on biotin for the carboxylation of propionyl-CoA in some
80 strains but the corresponding genes and gene products remain elusive¹⁴. Despite its scarce usage,
81 the biosynthesis of biotin is a metabolically costly procedure involving many enzymatic steps
82 (Fig. 1): starting from glucose, a minimum of twelve enzymatic reaction steps is required to yield
83 acetyl-CoA¹⁵, which is then converted into malonyl-CoA by the aforementioned AccABCD
84 reaction¹³. Subsequently, the fatty acid synthesis machinery is employed to successively couple
85 three malonyl-CoA units in ten enzymatic steps to yield pimeloyl-ACP methyl ester⁴. This
86 precursor is processed into biotin by five final biosynthesis enzymes (BioHFADB) before
87 loading the cofactor onto the biotinyl carboxyl carrier protein (BCCP) by the biotin ligase BirA⁴.
88 Hence, at least 29 steps are required to produce biotin including the comparably inefficient, yet
89 catalytic, final biotin synthase (BioB) reaction^{16,17} and not to mention involved cofactors (SAM,
90 NAD(P)⁺/NAD(P)H, CoA, ATP etc.) and the genetics of biotin biosynthesis including
91 regulation^{18,19}.

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

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

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 104 **Fig. 1 | A biotin-independent bypass for malonyl-CoA synthesis.** Biotin biosynthesis from glucose requires 29
 105 enzymatic steps including synthesis of acetyl-CoA (orange box) and fatty acids (grey box) as well as the final steps
 106 exclusive to biotin production (green box). AccABCD requires biotin as cofactor for the essential conversion of
 107 acetyl-CoA to malonyl-CoA during fatty acid synthesis in most natural organisms²⁰. Bypassing this reaction creates
 108 a biotin-independent phenotype achieved by implementing two heterologous proteins from *R. trifolii* (blue box)
 109 facilitating uptake of malonate (MatC) and its subsequent conversion to malonyl-CoA (MatB). The resulting strain
 110 should be superior to conventional hosts in its capability to produce biotin-binders such as streptavidin (SAV; red
 111 box), which is hitherto restricted due to the sequestration of biotin and inhibition of AccABCD^{21,22}.

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118 **2. Materials and methods**

119 *2.1 Suppliers*

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

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125 *2.2 Growth media*

126 *E. coli* strains were grown in Luria–Bertani (LB) liquid medium or agar²³ for maintenance and
127 genetic engineering supplemented with 50 mg L⁻¹ kanamycin or 34 mg L⁻¹ chloramphenicol
128 where appropriate. The basic M9 medium²³ contained 10 g L⁻¹ glucose and 20 mg L⁻¹ thiamine.
129 For the experiments with JM83 and its derivatives (Fig. 2(b)-(c); Supplementary Fig. 1;
130 Supplementary Fig. 3) basic M9 was additionally supplemented with 1 mM L-proline, 500 μM
131 isopropyl-β-D-thiogalactopyranosid (IPTG) and 50 mg L⁻¹ kanamycin or 34 mg L⁻¹
132 chloramphenicol where appropriate. Additionally 0.2 mg L⁻¹ D-biotin or between 0 and 50 mM
133 malonate were added to yield M9^{BIO+} and M9^{MAL+}, respectively. For SAV expression with
134 BL21(DE3) in shake flasks (Fig. 3(b)) and for the batch phase of bioreactor cultivations (Fig. 4)
135 a defined mineral medium (pH 7.0) was used containing 3.0 g L⁻¹ KH₂PO₄, 4.2 g L⁻¹ Na₂HPO₄,
136 2.3 g L⁻¹ (NH₄)₂SO₄, 1.9 g L⁻¹ NH₄Cl, 1 g L⁻¹ citric acid, 10 g L⁻¹ glucose, 20 mg L⁻¹ thiamine, 55
137 mg L⁻¹ CaCl₂, 240 mg L⁻¹ MgSO₄, 50 mg L⁻¹ kanamycin, 34 mg L⁻¹ chloramphenicol, and

138 1 mL L⁻¹ trace element solution US²⁴. Heterologous gene expression was induced by addition of
139 500 μM IPTG and where appropriate a concomitant malonate pulse to a final concentration of 5
140 mM was added.

141 Glucose feed medium (phase II bioreactor cultivation, Fig. 4(a)) contained 400 g L⁻¹ glucose,
142 13.3 g L⁻¹ MgSO₄ · 7H₂O, 20 mg L⁻¹ thiamine, 50 mg L⁻¹ kanamycin, 34 mg L⁻¹ chloramphenicol
143 and 1 mL L⁻¹ trace element solution US²⁴. Glucose-malonate feed medium (phase III bioreactor
144 cultivation, Fig. 4(a)) was prepared likewise and additionally contained between 0 and 560 mM
145 malonate (pH adjusted to 7.0 by addition of sodium hydroxide).

146 For strain maintenance *C. glutamicum* was grown in LB liquid medium or agar containing
147 10 g L⁻¹ glucose and where appropriate 25 mg L⁻¹ kanamycin. Transformation was performed by
148 electroporation as described elsewhere²⁵. For biotin complementation experiments with
149 *C. glutamicum* the minimal medium CGXII²⁶ with 2% glucose (w/v) was used either with
150 (20 mg L⁻¹) or without biotin or supplemented with varying concentrations of malonate. Unless
151 stated otherwise 10 μM IPTG were added for induction of *matBC* genes in this strain.

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153 2.3 Cultivation conditions

154 *E. coli* growth experiments in microtiter plates were carried out in an Infinite M200 plate reader
155 (Tecan, Männedorf, Switzerland) at 37°C and under agitation (orbital shaking, 2 mm amplitude)
156 and bacterial growth was monitored by measuring the optical density of the cultures (200 μL
157 total volume) at 600 nm (OD₆₀₀). Shake flask cultivations were carried out using 1 L Erlenmeyer
158 flasks and a culture volume of 100 mL in a shaking incubator (37°C, 220 r.p.m.) and growth was
159 monitored by OD₆₀₀ determination in a cuvette photometer. SAV concentrations were
160 determined by a fluorescence quenching assay (see section 2.7).

161 *C. glutamicum* was cultivated in 96-deepwell plates in 500 μ L 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 OD₆₀₀
164 was determined in an Infinite M1000 plate reader (Tecan, Männedorf, Switzerland).

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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 OD₆₀₀
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 μ L 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 ($\mu_{\text{set}} =$
179 0.09 h⁻¹) until an approximate OD₆₀₀ 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 μ M 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 OD₆₀₀ determination and DCW

184 measurement and SAV concentrations were determined by a fluorescence quenching assay (see
185 section 2.7).

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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 elsewhere²⁷.
194 Subsequently the kanamycin resistance gene was removed using FRT recombination with
195 plasmid pCP20, which was cured from the resulting strain JM83(DE3)Δ*bioB* by incubation at
196 43°C²⁸. 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).

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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 pCK01²⁹ 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

207 amplified (primers 7 and 4) and the resulting PCR product was digested and ligated into the
208 backbone of pET-30b(+) treated with the same restriction enzymes (*NdeI* and *EcoRV*).
209 pET30matBC*, in which the natural GTG start codon of the *matB* gene is replaced by ATG, was
210 constructed by digestion of the PCR product of the *matBC* cassette (Supplementary Tab. 3) and
211 primers 8 and 9 with *NdeI* and *BamHI* and subsequent ligation into the backbone of pET-30b(+)
212 treated with the same restriction enzymes (*NdeI* and *BamHI*).
213 pEKEx2matBC was constructed by PCR amplification of the natural *matBC* cassette
214 (Supplementary Tab. 3) with primers 10 and 11 followed by restriction digest with *BamHI* and
215 *KpnI* and subsequent ligation into the backbone of pEKEx2³⁰ treated with the same restriction
216 enzymes.

217

218 2.7 Quantification of active SAV

219 Cell lysates of *E. coli* were produced by spinning down (20'000 rcf, 5 min, 4° C) 1 mL of broth
220 and re-suspending the cell pellet in lysis buffer (10 mM Tris buffer at pH 7.4 containing 1.0 g L⁻¹
221 lysozyme, 1 mM MgSO₄ and 10 mg L⁻¹ DNase). Afterwards, three consecutive freeze-thaw
222 cycles were performed and the SAV-containing supernatant was cleared from cell debris by
223 centrifugation (20'000 rcf, 10 min, 4° C). Free biotin binding sites in SAV were then quantified
224 using a fluorescent quenching assay derived from a previously described protocol³¹. For this
225 purpose, a binding site buffer containing 1 μM Atto-565-biotin (Atto-Tec, Siegen, Germany) and
226 0.1 g L⁻¹ bovine serum albumin in phosphate buffered saline²³ was freshly prepared for each
227 measurement. Aliquots of 10 μL of samples (diluted into the linear range of the assay; 0-0.95
228 μM biotin-binding sites) were mixed with 190 μL of binding site buffer and incubated for 30
229 minutes at ambient temperature to ensure binding of the dye to SAV. Afterwards a fluorescent

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

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255 **3. Results and discussion**

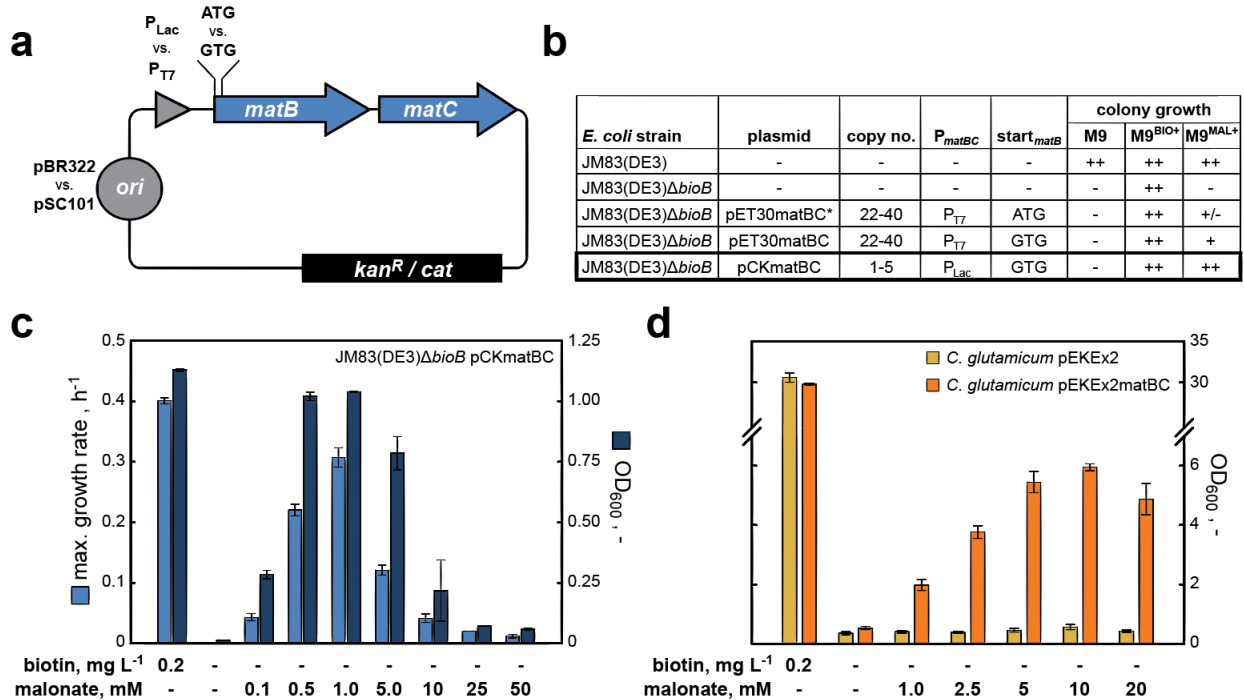
256 *3.1 Construction of biotin-independent Escherichia coli strains*

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 carboxylase³⁶.
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

276 (wild type GTG vs. ATG) of *matB* (Fig. 2(a)). To test whether the only biotin-dependent reaction
277 can be bypassed by MatBC, we constructed a strain with disrupted chromosomal biotin synthase
278 gene *bioB* which reportedly prevents biotin production and thus growth in biotin-free medium³⁸.
279 The resulting strain JM83(DE3) Δ *bioB* was transformed with the different *matBC* expression
280 plasmids and plated on selective M9-agar containing either 0.2 mg L⁻¹ biotin or 5 mM malonate
281 as well as neither of the two supplements (Fig. 2(b)). Whereas parent strain JM83(DE3)
282 exhibited normal growth even in biotin absence, the *bioB* mutants failed to proliferate on biotin-
283 free agar. As expected, supplementation of biotin to the medium restored growth for all
284 auxotroph mutants. Importantly, malonate was likewise able to restore growth but only in
285 presence of a *matBC* expression vector and with notably diverging strain fitness depending on
286 the anticipated expression level for the bypass proteins. More precisely, pCK*matBC* (low copy
287 number, P_{Lac}, GTG start codon for *matB*) exhibited the best growth behavior as compared to
288 pET30*matBC* and pET30*matBC**, which led to intermediate and slow growth, respectively.
289 These results confirmed functionality of the biotin-independent malonyl-CoA bypass and pointed
290 to a preferable low expression level for the bypass proteins, presumably because too high
291 expression levels lead to excessive drainage of the cellular coenzyme A pool, highlighting the
292 significance of expression level optimization for metabolic engineering³⁹⁻⁴¹.



293
294 **Fig. 2 | Engineering of biotin-independent strains of *E. coli* and *C. glutamicum*.** (a) Three *E. coli* plasmids for
295 *matBC* expression were constructed differing in plasmid copy number (*ori*'s from pSC101 or pBR322) as well as
296 transcriptional (P_{Lac} vs. P_{T7} promoter) and translational (GTG vs. ATG start codon of *matB*) control. (b) The MatBC
297 bypass restores growth of a biotin auxotrophic mutant (Δ *bioB*) on biotin-free solid medium containing 5 mM
298 malonate (M9^{MAL+}) with preference for low expression levels (pCKmatBC). Malonate-free (M9) and biotin-
299 containing (M9^{BIO+}) media were included as negative and positive control, respectively. (c) The malonate
300 concentration was optimized to ensure optimal growth of biotin-independent *E. coli*. (d) Construction of a *matBC*
301 shuttle vector (pEKEx2matBC) allowed for biotin-independent growth of naturally biotin auxotroph *C. glutamicum*.
302 Bars represent average specific growth rates and/or maximum OD₆₀₀ for three (c) or four (d) replicate cultures in 96-
303 well format with s.d..

305 3.2 Optimization of malonate supply

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

312 strains in mineral media. Concentrations exceeding the apparent optimum negatively affected the
313 strains behavior as far as to complete growth inhibition. Similar observations were made by
314 Lombó and coworkers³⁵ who observed growth of a normal, biotin prototroph strain at 5 mM but
315 complete inhibition at 40 mM malonate and attributed the inhibitory effect on imbalances in the
316 host's coenzyme A and acyl-CoA metabolism. Moreover, malonate is known to inhibit succinate
317 dehydrogenase, a central enzyme of the Krebs cycle⁴². Consequently, malonate supply needs to
318 be optimized for the desired strain and cultivation vessel in order to exploit the full potential of
319 the malonyl-CoA bypass.

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321 *3.3 Grafting of MatBC bypass to Corynebacterium glutamicum*

322 In order to evaluate transferability for the proposed biotin-independent concept, we selected the
323 Gram-positive bacterium *Corynebacterium glutamicum* as a second chassis to demonstrate
324 functionality of the MatBC bypass. *C. glutamicum* is an industrially highly relevant production
325 host^{43,44} and naturally biotin auxotrophic therefore requiring supplementation of defined media
326 with the vitamin⁹. This latter practical limitation has been previously addressed by re-
327 introduction of biotin biosynthesis genes into *C. glutamicum* to create biotin-prototrophic
328 phenotypes^{8,9}. Besides the essential acetyl-CoA carboxylase, *C. glutamicum* contains a biotin-
329 dependent pyruvate carboxylase, which is responsible for anaplerotic channeling of pyruvate into
330 the tricarboxylic acid cycle⁴⁵. Complementation studies, however, revealed that pyruvate
331 carboxylase is inessential and the corresponding deficient mutants exhibit wild type growth⁴⁵.
332 We therefore hypothesized that the MatBC-bypass would likewise render biotin obsolete in
333 *C. glutamicum* creating mutants proficient to grow in biotin-free medium supplemented with
334 malonate. We constructed the vector pEKEx2matBC with the natural *matBC* cassette under

335 control of a P_{tac} promoter. Gratifyingly, transformation of *C. glutamicum* wild type (ATCC
336 13032) with this construct enabled its proliferation in biotin-free media containing malonate
337 whereas in the absence of MatBC (pEKEx2) as well as in medium lacking malonate only
338 marginal growth due to residual biotin transferred from the pre-cultures was observable
339 (Fig. 2(d)). Moreover, we found a strong growth inhibition for high inducer concentrations
340 pointing to a similar preference for low *matBC* expression levels as previously observed for
341 *E. coli* (see Supplementary Fig. 2). Taken together these experiments highlight the feasibility to
342 create biotin-independent organisms using the MatBC bypass and the transferability of the
343 underlying concept to different hosts.

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345 *3.4 Utilization of biotin-independence for cytosolic streptavidin production*

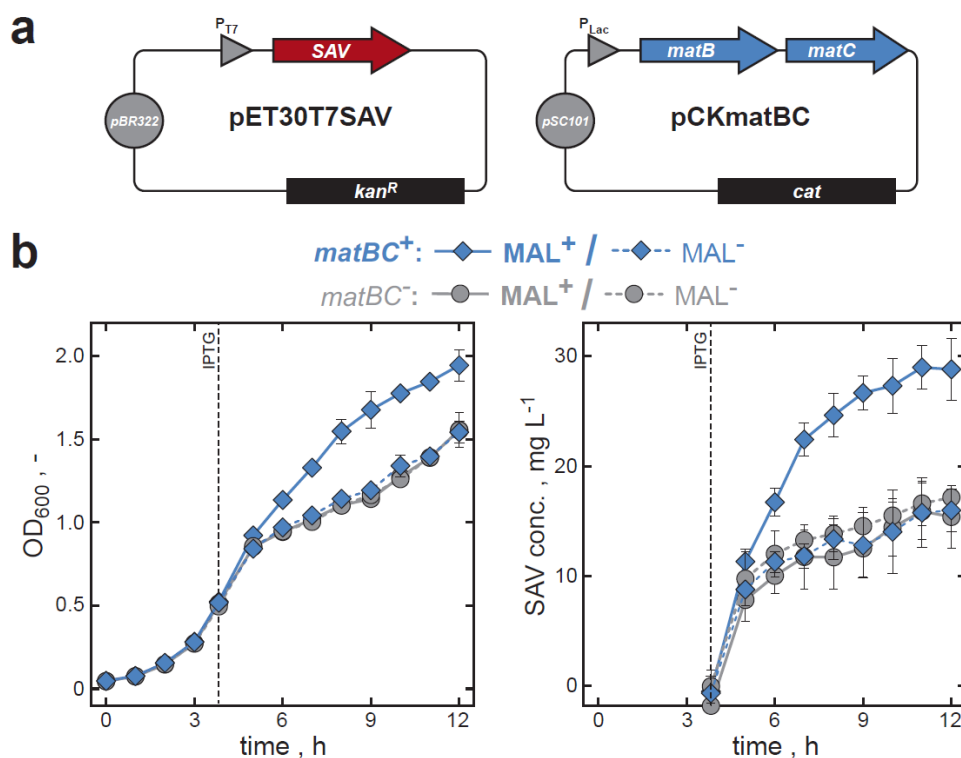
346 Next, we sought to demonstrate practical utility of biotin independence and apply a respective
347 *E. coli* strain to address a common problem occurring during expression of high-affinity biotin
348 binders like streptavidin (SAV). Due to the high affinity to biotin (SAV, $K_{D, \text{biotin}} \sim 10^{-14}$ M) and
349 its exceptional physicochemical stability, SAV is used for a multitude of biotechnological
350 applications including live cell imaging, immobilization and affinity purification of biotinylated
351 or peptide tagged biomolecules and nanotechnology as well as more recently developed
352 technologies which repurpose the protein for drug targeting and the development of artificial
353 metalloenzymes⁴⁶⁻⁴⁸. The secretion of SAV and its homologues by natural hosts such as
354 *Streptomyces avidinii* serves as defense mechanism exploiting the highly efficient sequestration
355 of the vitamin^{21,49}. Not coincidentally, the expression of soluble, active SAV in the cytosol has
356 been reported to lead to depletion of the host cell's biotin pool accompanied by impaired growth

357 and low SAV expression levels^{21,22,50}, which represents a major limitation for high-yield SAV
358 production.

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

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

380 trends and a more than three-fold increase in SAV production (see Supplementary Fig. 3). These
 381 results unambiguously demonstrate the functionality of the biotin-independent bypass and its
 382 utility for improved expression of SAV.
 383



384
 385 **Fig. 3 | Streptavidin (SAV) production in conditionally biotin-independent *E. coli* strains.** (a) For production of
 386 SAV in *E. coli* the vector pET30T7SAV was constructed. It contains the SAV gene under control of a P_{T7} promoter.
 387 (b) Shake flask cultivations with *E. coli* BL21(DE3) revealed significantly improved growth and SAV production
 388 behavior after SAV induction in the biotin-independent strain in presence of 5 mM malonate (*matBC*⁺ MAL⁺)
 389 compared to the controls lacking either pCKmatBC (*matBC*⁻ MAL⁺ and *matBC*⁻ MAL⁻) or malonate (*matBC*⁺
 390 MAL⁻). Data points represent mean OD₆₀₀ and SAV concentrations of three independent cultures with standard
 391 deviation.

392

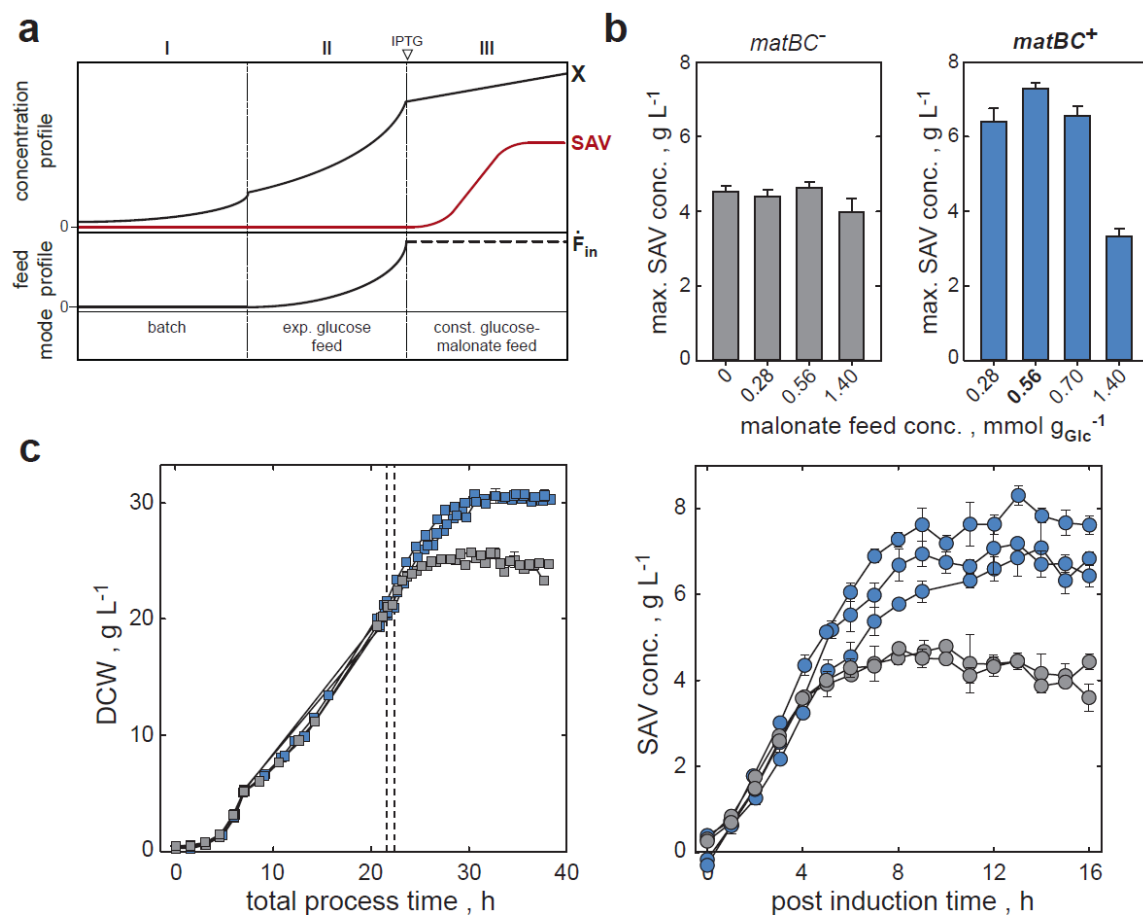
393 3.5 Establishment of a biotin-independent streptavidin production process

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

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

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

420 increased in comparison to previously used hosts for SAV production such as *S. avidinii*, *P.*
 421 *pastoris*, or *B. subtilis*⁵²⁻⁵⁴.
 422



423 **Fig. 4 | Development of a streptavidin (SAV) production process in biotin-independent *E. coli*.** (a) A three-
 424 stage bioreactor process was developed: I, batch phase; II, exponential glucose-limited fed-batch; III, SAV
 425 production phase with constant glucose-malonate feed. The idealized courses for concentration of biomass X and
 426 SAV and the volumetric feed flow rate \dot{F}_{in} are conceptually shown. (b) The critical malonate-glucose ratio in the
 427 feed (phase III) was optimized. Maximum SAV concentrations eight hours after induction are indicated. (c) The
 428 productivity of the optimal setup (0.56 mmol g_{Glc}⁻¹) was verified by three independent bioreactor cultivations of the
 429 biotin-independent strain (blue squares/circles) compared to two independent reference cultivations with the
 430 conventional strain (*matBC*⁻) without malonate (grey squares/circles). Bars/data points represent averages of
 431 triplicate measurements of dry cell weight (DCW) and SAV concentration with standard error. The area between the
 432 dashed lines represents the IPTG induction window of all five processes. SDS-PAGE analysis for the biotin-
 433 independent process was performed to confirm integrity of the product (see Supplementary Fig. 4)
 434
 435

436 **4. Conclusion**

437 Metabolic engineering is widely used to improve bioprocess performance by directing fluxes into
438 a desired product based on the ever increasing knowledge about cellular metabolic networks⁵⁵.

439 Well established strategies include overexpression or deletion of inherent host enzymes to
440 increase the flux into the target pathway or prevent drainage of intermediates and consequently
441 product loss or side product formation, the integration of enzymes facilitating growth and
442 product formation based on inexpensive substrates, as well as implementation of proteins which
443 simplify downstream processing. These efforts are frequently combined with flux models that
444 help identifying the key bottlenecks within the system^{56,57}.

445 A relatively uncharted approach is the fundamental re-organization of central host metabolism in
446 order to enhance bio-production. This strategy seeks to completely re-route central metabolic
447 pathways in order to drive their flux into a desired direction and is based on the notion that
448 natural metabolism, as good as it is to cope with natural challenges, may not be the preferable
449 choice for biotechnological application. Auspicious examples comprise a synthetic non-oxidative
450 glycolysis⁵⁸ and a reverse glyoxylate shunt⁵⁹, both designed to minimize carbon loss upon
451 utilization of carbohydrates, as well as the engineering of artificial carbon fixation cycles with
452 the goal to increase sequestration of the greenhouse gas carbon dioxide⁶⁰⁻⁶³. Despite the fact that
453 some of these efforts thus far mainly comprised theoretical considerations and *in vitro* studies
454 and have therefore hardly exceeded the stage of a blueprint, this type of approach could arguably
455 enable to fundamentally change cellular metabolism as we know it today and may allow
456 accessibility to entirely novel processes and bio-products.

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

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

465 To extend the biotin-independent concept beyond this proof-of-principle study, the entire cellular
466 machinery associated with biotin could be removed from the host genome. This includes genes
467 involved in its biosynthesis (*bioHFADB*), its loading (*birA*), as well as all acetyl-CoA
468 carboxylase genes (*accABCD*). Furthermore, the presented MatBC bypass could be combined
469 with a module for *in vivo* synthesis of malonate, which has recently been established in an
470 engineered *E. coli* strain that is capable of synthesizing malonate from aspartate via a beta-
471 alanine route³⁷. This would close the gap to prevalent metabolites that can be directly derived
472 from central metabolism and render the currently required (yet inexpensive) supplementation of
473 malonate obsolete, leading to a stand-alone biotin-independent organism, which synthesizes fatty
474 acid building-blocks in a completely novel way.

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

479

480

481

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488
489 **Author contributions:** M.J. conceived the project and carried out the experimental work. M.J.
490 and M.O.B. developed biotin-independent SAV production. V.S. carried out bioreactor
491 cultivations. P.M., T. R.W. and S.P. supervised the study. M.J., T. R. W. and S.P. wrote the
492 manuscript.

493

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