

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 resulting
31 in the requirement to supplement defined media. Here we describe the creation of biotin-
32 independent strains of *Escherichia coli* and *Corynebacterium glutamicum* through installation of
33 an optimized malonyl-CoA bypass, which re-routes natural fatty acid synthesis, rendering the
34 previously essential vitamin completely obsolete. We utilize biotin-independent *E. coli* for the
35 production of the high-value protein streptavidin which was hitherto restricted because of toxic
36 effects due to biotin depletion. The engineered strain revealed significantly improved streptavidin
37 production resulting in the highest titers and productivities reported for this protein to date.

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

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51 **1. Introduction**

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

63 Several industrially relevant microorganisms lack the ability to independently synthesize biotin
64 including *Saccharomyces cerevisiae*⁶, *Pichia pastoris*⁷ and *Corynebacterium glutamicum*^{8,9}. In the
65 case of *P. pastoris*, for instance, high amounts of the cofactor are added to defined media and
66 process complications are frequently associated with poor quality of the supplemented biotin⁷.
67 Similarly, biotin has to be added in serum-free cell culture medium formulations¹⁰. In order to
68 overcome this limitation, several efforts have been undertaken to genetically engineer prototrophic
69 variants of different organisms for industrial applications⁶⁻⁹. These works comprised introduction
70 of biotin biosynthesis genes from naturally prototrophic hosts like *Escherichia coli* or *Bacillus*
71 *subtilis*. An alternative approach to the aforementioned efforts could be the metabolic engineering
72 of biotin-independent organisms that *a priori* do no longer rely on biotin, which has thus far not
73 been systematically elaborated.

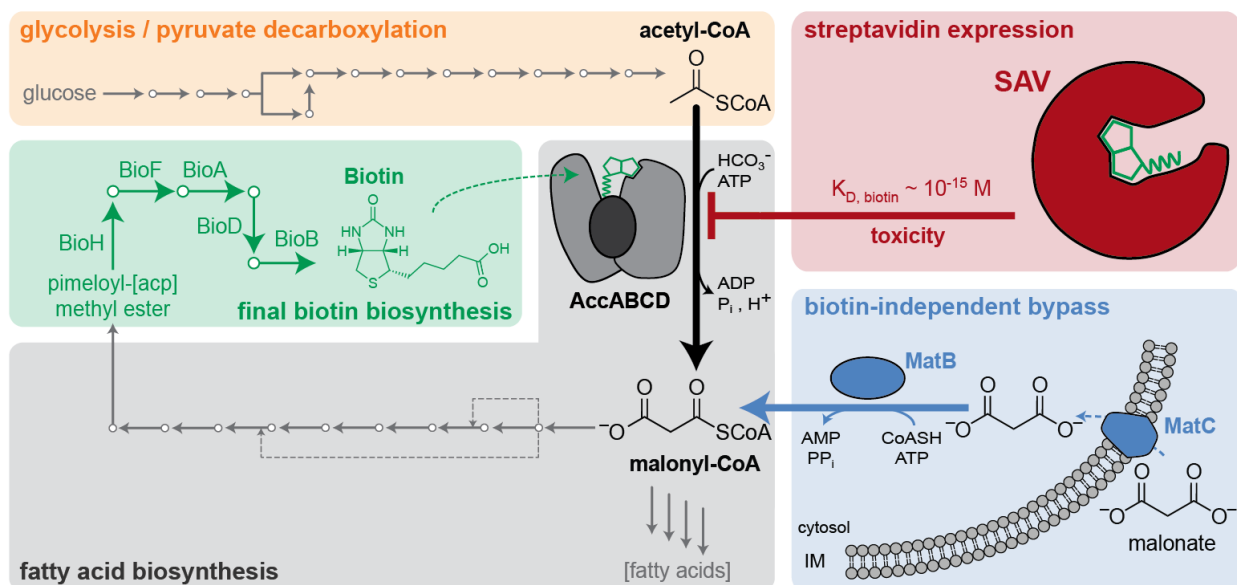
74 Biotin metabolism has been extensively studied in *E. coli*^{4,5,11,12}, where it is used for the
75 carboxylation of acetyl-CoA to yield malonyl-CoA which represents the first committed step in
76 fatty acid synthesis¹³ (Fig. 1). This reaction catalyzed by the acetyl-CoA carboxylase complex
77 (AccABCD)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

90 In this work we describe the creation of biotin-independent phenotypes by re-wiring initial fatty
91 acid biosynthesis using a malonyl-CoA bypass. The resulting engineered strains of *E. coli* and
92 *C. glutamicum* are able to survive and proliferate in the complete absence of biotin. We apply this
93 concept of biotin-independence to improve the production of the high-affinity biotin binder
94 streptavidin (SAV hereafter), which was thus far restricted due to toxic biotin depletion in the host
95 cell. The biotin-independent *E. coli* strain revealed a significantly enhanced SAV production as
96 well as improved growth behavior as compared to a conventional strain. Transferring this strategy

97 to an SAV production process in lab-scale bioreactors and fine-tuning of the bypass led to the
 98 highest streptavidin titers reported to date (up to $8.3 \pm 0.2 \text{ g L}^{-1}$).

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102 **Fig. 1 | A biotin-independent bypass for malonyl-CoA synthesis.** Biotin biosynthesis from glucose requires 29

103 enzymatic steps including synthesis of acetyl-CoA (orange box) and fatty acids (grey box) as well as the final steps

104 exclusive to biotin production (green box). AccABCD requires biotin as cofactor for the essential conversion of acetyl-

105 CoA to malonyl-CoA during fatty acid synthesis in most natural organisms²⁰. Bypassing this reaction creates a biotin-

106 independent phenotype achieved by implementing two heterologous proteins from *R. trifolii* (blue box) facilitating

107 uptake of malonate (MatC) and its subsequent conversion to malonyl-CoA (MatB). The resulting strain should be

108 superior to conventional hosts in its capability to produce biotin-binders such as streptavidin (SAV; red box), which

109 is hitherto restricted due to the sequestration of biotin and inhibition of AccABCD^{21,22}.

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116 **2. Materials and methods**

117 *2.1 Suppliers*

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

122 *2.2 Growth media*

124 *E. coli* strains were grown in Luria–Bertani (LB) liquid medium or agar²³ for maintenance and
125 genetic engineering supplemented with 50 mg L⁻¹ kanamycin or 34 mg L⁻¹ chloramphenicol where
126 appropriate. The basic M9 medium²³ contained 10 g L⁻¹ glucose and 20 mg L⁻¹ thiamine. For the
127 experiments with JM83 and its derivatives (Fig. 2(b)-(c); Supplementary Fig. 1; Supplementary
128 Fig. 3) basic M9 was additionally supplemented with 1 mM L-proline, 500 μM isopropyl-β-D-
129 thiogalactopyranosid (IPTG) and 50 mg L⁻¹ kanamycin or 34 mg L⁻¹ chloramphenicol where
130 appropriate. Additionally 0.2 mg L⁻¹ D-biotin or between 0 and 50 mM malonate were added to
131 yield M9^{BIO+} and M9^{MAL+}, respectively. For SAV expression with BL21(DE3) in shake flasks (Fig.
132 3(b)) and for the batch phase of bioreactor cultivations (Fig. 4) a defined mineral medium (pH 7.0)
133 was used containing 3.0 g L⁻¹ KH₂PO₄, 4.2 g L⁻¹ Na₂HPO₄, 2.3 g L⁻¹ (NH₄)₂SO₄, 1.9 g L⁻¹ NH₄Cl,
134 1 g L⁻¹ citric acid, 10 g L⁻¹ glucose, 20 mg L⁻¹ thiamine, 55 mg L⁻¹ CaCl₂, 240 mg L⁻¹ MgSO₄,
135 50 mg L⁻¹ kanamycin, 34 mg L⁻¹ chloramphenicol, and 1 mL L⁻¹ trace element solution US ²⁴.
136 Heterologous gene expression was induced by addition of 500 μM IPTG and where appropriate a
137 concomitant malonate pulse to a final concentration of 5 mM was added.

138 Glucose feed medium (phase II bioreactor cultivation, Fig. 4(a)) contained 400 g L⁻¹ glucose,
139 13.3 g L⁻¹ MgSO₄ · 7H₂O, 20 mg L⁻¹ thiamine, 50 mg L⁻¹ kanamycin, 34 mg L⁻¹ chloramphenicol
140 and 1 mL L⁻¹ trace element solution US²⁴. Glucose-malonate feed medium (phase III bioreactor
141 cultivation, Fig. 4(a)) was prepared likewise and additionally contained between 0 and 560 mM
142 malonate (pH adjusted to 7.0 by addition of sodium hydroxide).
143 For strain maintenance *C. glutamicum* was grown in LB liquid medium or agar containing 10 g L⁻¹
144 ¹ glucose and where appropriate 25 mg L⁻¹ kanamycin. Transformation was performed by
145 electroporation as described elsewhere²⁵. For biotin complementation experiments with
146 *C. glutamicum* the minimal medium CGXII²⁶ with 2% glucose (w/v) was used either with (20 mg
147 L⁻¹) or without biotin or supplemented with varying concentrations of malonate. Unless stated
148 otherwise 10 μM IPTG were added for induction of *matBC* genes in this strain.

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150 2.3 Cultivation conditions

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

158 *C. glutamicum* was cultivated in 96-deepwell plates in 500 μL culture volume. Wells were
159 inoculated from single colonies from biotin-containing CGXII plates (1.6% agarose) and

160 cultivation was performed in a shaking incubator (30°C, 250 r.p.m.) for 48 h. The final OD₆₀₀ was
161 determined in an Infinite M1000 plate reader (Tecan, Männedorf, Switzerland).

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163 *2.4 Bioreactor cultivation*

164 Bioreactor cultivations with *E. coli* were carried out with a Labfors-5 benchtop fermenter system
165 (Infors HT, Bottmingen, Switzerland) with 7.5 L vessel volume at 37°C and a pH of 7.0 which
166 was maintained by titration with 10% (v/v) sulfuric acid and 5 M ammonium hydroxide. The
167 dissolved oxygen concentration was maintained above 30% of the saturation level by firstly
168 adjusting the stirrer speed (300 to 1250 rpm, 50 rpm increments; two six-blade Rushton impellers)
169 and secondly the air flow (1 to 5 vvm).

170 The batch phase was started by inoculation of 2 L of defined mineral medium to an initial OD₆₀₀
171 of 0.15 from an overnight shake flask pre-culture in the same medium. In order to prevent foaming
172 1 mL of 20% (v/v) of polypropylene glycol was added to the culture and additionally as necessary
173 in 500 µL increments throughout the cultivation. After the consumption of the initial amount of
174 glucose (10 g L⁻¹), as indicated by a sudden rise in the dissolved oxygen signal, glucose feed
175 medium was gradually applied to the culture in an exponential manner ($\mu_{\text{set}} = 0.09 \text{ h}^{-1}$) until an
176 approximate OD₆₀₀ of 55 was reached (corresponding to a dry cell weight concentration (DCW)
177 of roughly 21 g L⁻¹). Subsequently, heterologous gene expression was induced by addition of 500
178 µM IPTG and the administered medium was switched to glucose-malonate feed medium which
179 was applied at a constant rate of 0.53 mL min⁻¹ until the end of the process. Cell growth was
180 monitored throughout the process by OD₆₀₀ determination and DCW measurement and SAV
181 concentrations were determined by a fluorescence quenching assay (see section 2.7).

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183 2.5 Strain engineering

184 All strains used in this study are listed in Supplementary Table 1. In order to facilitate transcription
185 using P_{T7} promoters, the T7 RNA polymerase was integrated into the chromosome of strain JM83
186 using the λ DE3 Lysogenization Kit (Merck-Millipore, Darmstadt, Germany) and the resulting
187 strain was designated JM83(DE3). A biotin auxotroph derivative of JM83(DE3) was created by
188 P1 transduction from the Keio collection strain BW25113, which carries an insertional knockout
189 of the biotin synthase gene (*bioB:kan*), as described elsewhere²⁷. Subsequently the kanamycin
190 resistance gene was removed using FRT recombination with plasmid pCP20, which was cured
191 from the resulting strain JM83(DE3) Δ *bioB* by incubation at 43°C²⁸. Colony PCR was performed
192 to verify both successful transduction and removal of the resistance gene using primers 1 and 2
193 flanking the *bioB* gene (Supplementary Tab. 2).

194

195 2.6 Cloning procedures

196 All plasmids used in this study are listed in Supplementary 1. The natural *matBC* cassette from *R.*
197 *trifolii* was obtained as a synthetic DNA fragment (Life Technologies, Regensburg, Germany;
198 Supplementary Tab. 3) and PCR-amplified using oligonucleotides 3 and 4 in order to introduce
199 flanking restriction sites for *Bam*HI and *Eco*RV. Accordingly, the plasmid pCK01²⁹ was PCR-
200 amplified with primers 5 and 6 introducing sites for *Bam*HI and *Eco*RV. Both PCR products were
201 digested (*Bam*HI and *Eco*RV) and joined by ligation resulting in plasmid pCKmatBC. To generate
202 pET30matBC the synthetic DNA construct of *matBC* (Supplementary Tab. 3) was PCR amplified
203 (primers 7 and 4) and the resulting PCR product was digested and ligated into the backbone of
204 pET-30b(+) treated with the same restriction enzymes (*Nde*I and *Eco*RV).

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

209 pEKEx2matBC was constructed by PCR amplification of the natural *matBC* cassette
210 (Supplementary Tab. 3) with primers 10 and 11 followed by restriction digest with *BamHI* and
211 *KpnI* and subsequent ligation into the backbone of pEKEx2³⁰ treated with the same restriction
212 enzymes.

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214 2.7 Quantification of active SAV

215 Cell lysates of *E. coli* were produced by spinning down (20'000 rcf, 5 min, 4° C) 1 mL of broth
216 and re-suspending the cell pellet in lysis buffer (10 mM Tris buffer at pH 7.4 containing 1.0 g L⁻¹
217 lysozyme, 1 mM MgSO₄ and 10 mg L⁻¹ DNase). Afterwards, three consecutive freeze-thaw cycles
218 were performed and the SAV-containing supernatant was cleared from cell debris by
219 centrifugation (20'000 rcf, 10 min, 4° C). Free biotin binding sites in SAV were then quantified
220 using a fluorescent quenching assay derived from a previously described protocol³¹. For this
221 purpose, a binding site buffer containing 1 μM Atto-565-biotin (Atto-Tec, Siegen, Germany) and
222 0.1 g L⁻¹ bovine serum albumin in phosphate buffered saline²³ was freshly prepared for each
223 measurement. Aliquots of 10 μL of samples (diluted into the linear range of the assay; 0-0.95 μM
224 biotin-binding sites) were mixed with 190 μL of binding site buffer and incubated for 30 minutes
225 at ambient temperature to ensure binding of the dye to SAV. Afterwards a fluorescent
226 measurement was performed ($\lambda_{Ex} = 563$ nm, $\lambda_{Em} = 620$ nm) in black 96-well microtiter plates using
227 an Infinite M1000Pro microtiter plate reader (Tecan, Männedorf, Switzerland) and SAV

228 concentrations were calculated by correlation with an SAV standard curve (prepared from purified,
229 lyophilized SAV) similarly prepared as the samples and recorded in the same plate. The cell
230 specific SAV yields were calculated from the measured concentrations of SAV and DCW of the
231 samples assuming a whole cell protein content of 0.5 g per gram DCW. In order to verify integrity
232 of the product and confirm the validity of the optical quantification by a second method SDS-
233 PAGE analysis was performed (Supplementary Fig. 4). Therefore cell lysates from one of the
234 biotin-independent bioreactor processes (Fig. 4(c)) were run in comparison to a purified,
235 lyophilized SAV standard corresponding to a concentration of 4 g L⁻¹.

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251 **3. Results and discussion**

252 *3.1 Construction of biotin-independent Escherichia coli strains*

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

257 In order to create this biotin-independent bypass (Fig. 1) we selected two heterologous genes
258 (*matBC*) from the *Rhizobium trifolii* malonate utilization operon that are responsible for the uptake
259 of malonate (*matC*, malonate transporter) and its subsequent conversion to malonyl-CoA (*matB*,
260 malonyl-CoA synthetase)³². Malonic acid is a cheap bulk chemical readily synthesized by plants
261 but its bulk production mainly relies on chemical synthesis from chloroacetic acid^{33,34}. The
262 *matABC* gene cluster has been expressed in *E. coli* previously to improve polyketide synthesis^{32,35}.
263 Importantly, James and Cronan elegantly demonstrated that this system can be used to create
264 deletion mutants for various subunits of the essential acetyl-CoA carboxylase³⁶. Building up on
265 these previous studies, we anticipated that it should be possible to create a biotin-independent
266 phenotype using *matBC* and supplementation of growth media with malonate and thereby
267 insulating fatty acid synthesis from central carbon metabolism. Alternatively, malonate could also
268 be directly synthesized from glucose as very recently demonstrated in genetically engineered *E.*
269 *coli*³⁷.

270 We constructed three different *matBC* expression vectors for *E. coli* differing in the anticipated
271 expression levels by using different copy numbers and promoters and varying the start codon (wild
272 type GTG vs. ATG) of *matB* (Fig. 2(a)). To test whether the only biotin-dependent reaction can be
273 bypassed by MatBC, we constructed a strain with disrupted chromosomal biotin synthase gene

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

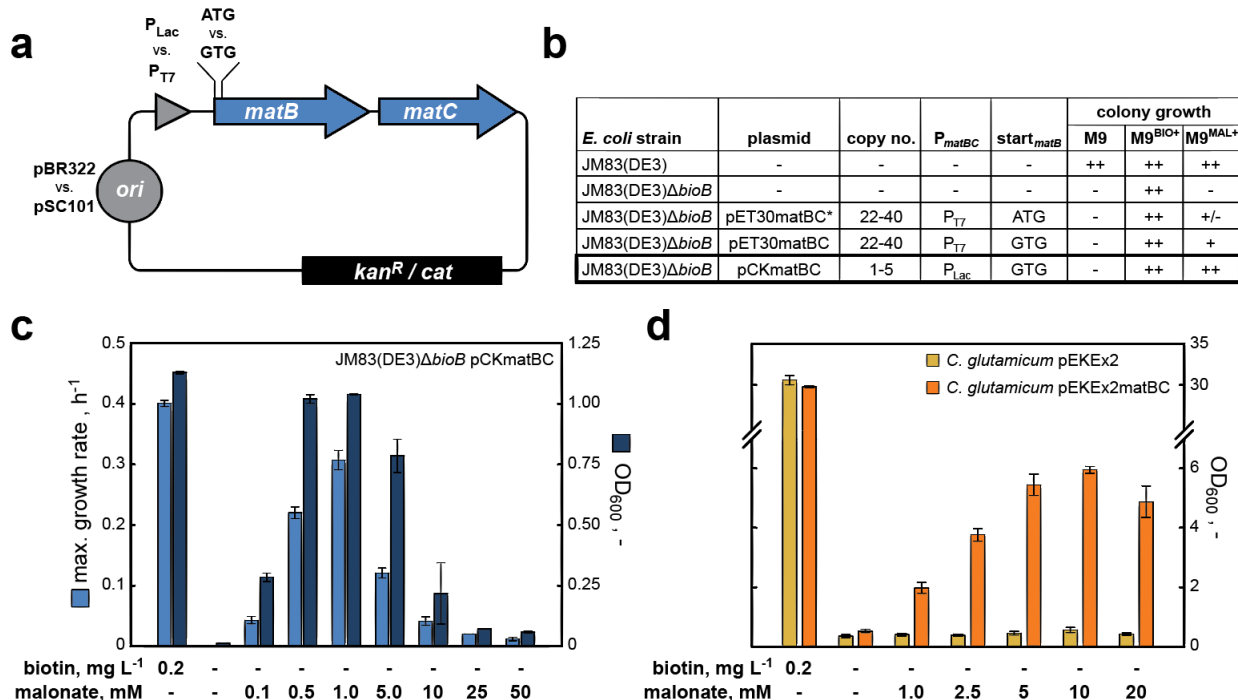


Fig. 2 | Engineering of biotin-independent strains of *E. coli* and *C. glutamicum*. (a) Three *E. coli* plasmids for *matBC* expression were constructed differing in plasmid copy number (*ori*'s from pSC101 or pBR322) as well as transcriptional (P_{Lac} vs. P_{T7} promoter) and translational (GTG vs. 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 OD₆₀₀ 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) Δ *bioB* 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.

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

314

315 *3.3 Grafting of MatBC bypass to Corynebacterium glutamicum*

316 In order to evaluate transferability for the proposed biotin-independent concept, we selected the
317 Gram-positive bacterium *Corynebacterium glutamicum* as a second chassis to demonstrate
318 functionality of the MatBC bypass. *C. glutamicum* is an industrially highly relevant production
319 host^{43,44} and naturally biotin auxotrophic therefore requiring supplementation of defined media
320 with the vitamin⁹. This latter practical limitation has been previously addressed by re-introduction
321 of biotin biosynthesis genes into *C. glutamicum* to create biotin-prototrophic phenotypes^{8,9}.
322 Besides the essential acetyl-CoA carboxylase, *C. glutamicum* contains a biotin-dependent pyruvate
323 carboxylase, which is responsible for anaplerotic channeling of pyruvate into the tricarboxylic acid
324 cycle⁴⁵. Complementation studies, however, revealed that pyruvate carboxylase is inessential and
325 the corresponding deficient mutants exhibit wild type growth⁴⁵. We therefore hypothesized that
326 the MatBC-bypass would likewise render biotin obsolete in *C. glutamicum* creating mutants
327 proficient to grow in biotin-free medium supplemented with malonate. We constructed the vector
328 pEKEx2matBC with the natural *matBC* cassette under control of a P_{tac} promoter. Gratifyingly,
329 transformation of *C. glutamicum* wild type (ATCC 13032) with this construct enabled its

330 proliferation in biotin-free media containing malonate whereas in the absence of MatBC (pEKEx2)
331 as well as in medium lacking malonate only marginal growth due to residual biotin transferred
332 from the pre-cultures was observable (Fig. 2(d)). Moreover, we found a strong growth inhibition
333 for high inducer concentrations pointing to a similar preference for low *matBC* expression levels
334 as previously observed for *E. coli* (see Supplementary Fig. 2). Taken together these experiments
335 highlight the feasibility to create biotin-independent organisms using the MatBC bypass and the
336 transferability of the underlying concept to different hosts.

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338 *3.4 Utilization of biotin-independence for cytosolic streptavidin production*

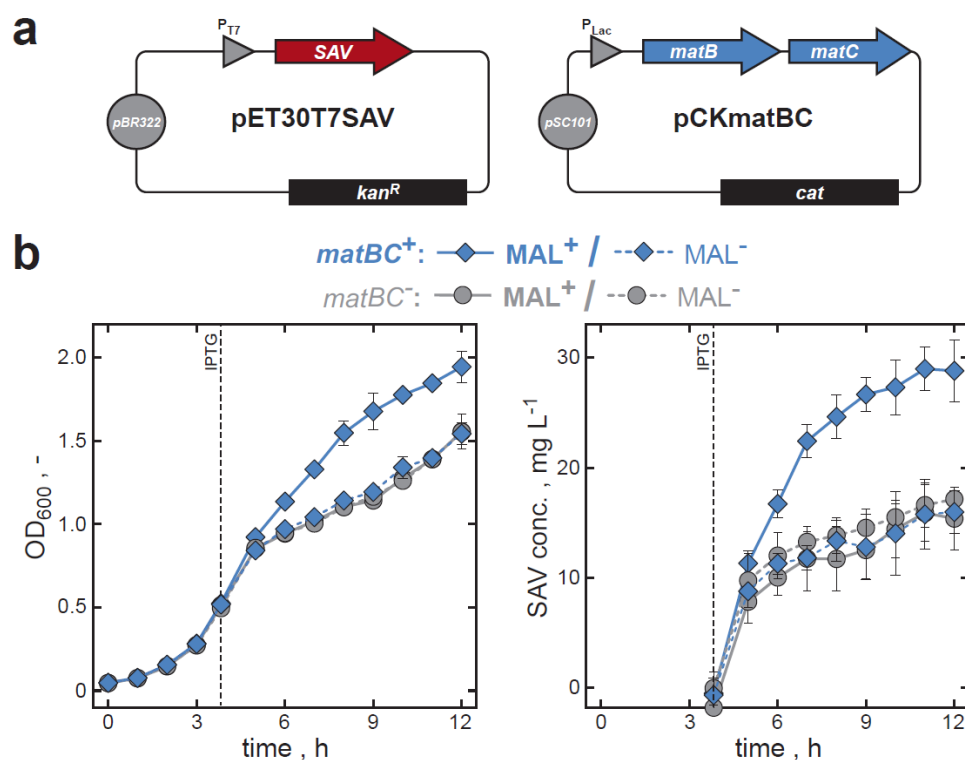
339 Next, we sought to demonstrate practical utility of biotin independence and apply a respective
340 *E. coli* strain to address a common problem occurring during expression of high-affinity biotin
341 binders like streptavidin (SAV). Due to the high affinity to biotin (SAV, $K_{D, \text{biotin}} \sim 10^{-14}$ M) and its
342 exceptional physicochemical stability, SAV is used for a multitude of biotechnological
343 applications including live cell imaging, immobilization and affinity purification of biotinylated
344 or peptide tagged biomolecules and nanotechnology as well as more recently developed
345 technologies which repurpose the protein for drug targeting and the development of artificial
346 metalloenzymes⁴⁶⁻⁴⁸. The secretion of SAV and its homologues by natural hosts such as
347 *Streptomyces avidinii* serves as defense mechanism exploiting the highly efficient sequestration of
348 the vitamin^{21,49}. Not coincidentally, the expression of soluble, active SAV in the cytosol has been
349 reported to lead to depletion of the host cell's biotin pool accompanied by impaired growth and
350 low SAV expression levels^{21,22,50}, which represents a major limitation for high-yield SAV
351 production.

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

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

374 unambiguously demonstrate the functionality of the biotin-independent bypass and its utility for
 375 improved expression of SAV.

376



377 **Fig. 3 | Streptavidin (SAV) production in conditionally biotin-independent *E. coli* strains.** (a) For production of
 378 SAV in *E. coli* the vector pET30T7SAV was constructed. It contains the SAV gene under control of a P_{T7} promoter.
 379 (b) Shake flask cultivations with *E. coli* BL21(DE3) revealed significantly improved growth and SAV production
 380 behavior after SAV induction in the biotin-independent strain in presence of 5 mM malonate ($matBC^+ MAL^+$)
 381 compared to the controls lacking either pCKmatBC ($matBC^- MAL^+$ and $matBC^- MAL^-$) or malonate ($matBC^+ MAL^-$).
 382 Data points represent mean OD₆₀₀ and SAV concentrations of three independent cultures with standard deviation.
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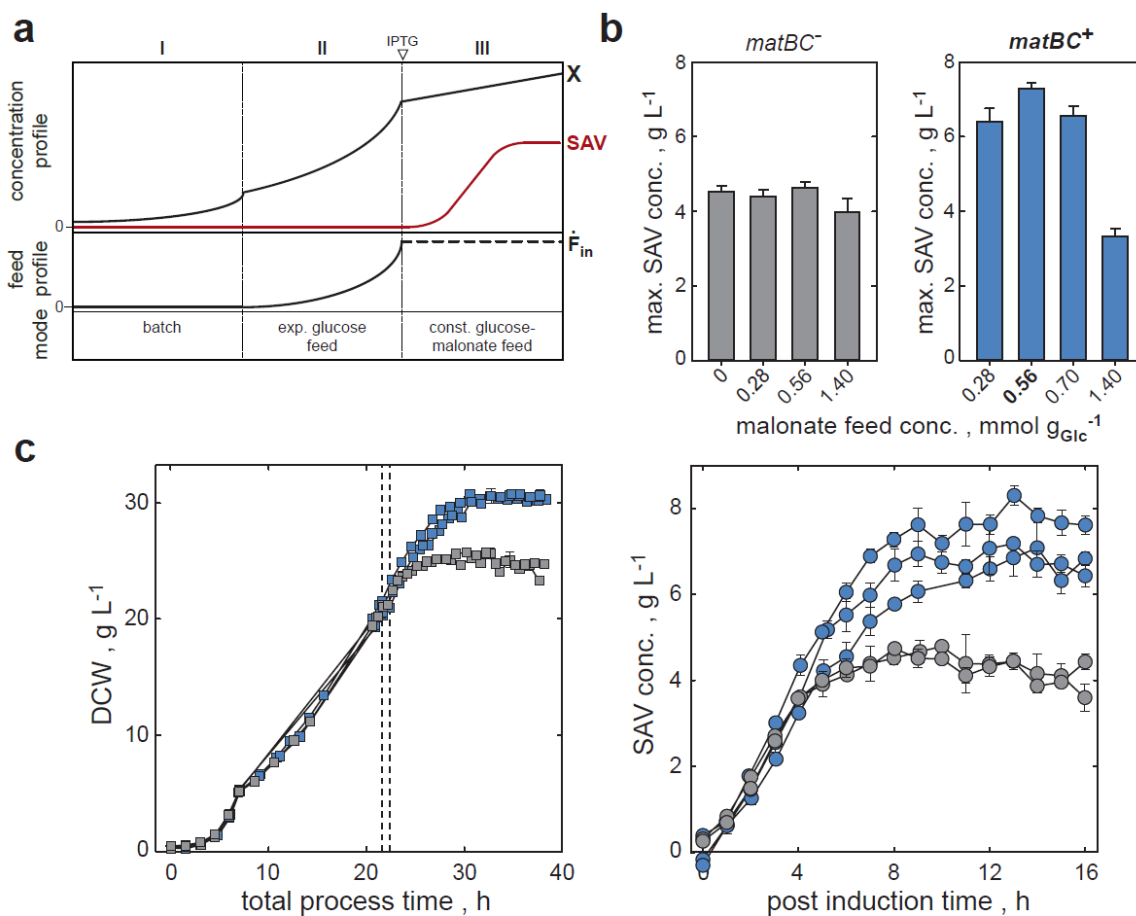
385 3.5 Establishment of a biotin-independent streptavidin production process

386 Next, we transferred the switchable biotin-independent SAV production into a laboratory scale
 387 bioreactor process using defined medium. Therefore we developed a cultivation protocol
 388 composed of three conceptual stages (Fig. 4(a)): an initial batch phase (I), a fed-batch stage (II)
 389 with exponential glucose-limited feeding (biomass production), and an SAV production stage (III)

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

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

412 increased in comparison to previously used hosts for SAV production such as *S. avidinii*, *P.*
 413 *pastoris*, or *B. subtilis*⁵²⁻⁵⁴.
 414



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

426
 427

428 **4. Conclusion**

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

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

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

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

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

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

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

471

472

473

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480
481 **Author contributions:** M.J. conceived the project and carried out the experimental work. M.J.
482 and M.O.B. developed biotin-independent SAV production. V.S. carried out bioreactor
483 cultivations. P.M., T. R.W. and S.P. supervised the study. M.J., T. R. W. and S.P. wrote the
484 manuscript.

485

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