

1 Emergence of NADP⁺-reducing enzymes in *Escherichia coli* central metabolism via
2 adaptive evolution

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17

18 **Abstract**

19 The nicotinamide cofactor specificity of enzymes plays a key role in regulating metabolic processes and
20 attaining cellular homeostasis. Multiple studies have used enzyme engineering tools or a directed evolution
21 approach to switch the cofactor preference of specific oxidoreductases. However, whole-cell adaptation
22 towards the emergence of novel cofactor regeneration routes was not previously explored. To address
23 this challenge, we used an *Escherichia coli* NADPH-auxotroph strain. We continuously cultivated this strain
24 under selective conditions. After 500-1100 generations of adaptive evolution using different carbon
25 sources, we isolated several strains capable of growing without an external NADPH source. Most isolated
26 strains were found to harbor a mutated NAD-dependent malic enzyme (MaeA). A single mutation in MaeA
27 was found to switch cofactor specificity while lowering enzyme activity. Most mutated MaeA variants also
28 harbored a second mutation that restored the catalytic efficiency of the enzyme. Remarkably, the best
29 MaeA variants identified this way displayed overall superior kinetics relative to the wildtype variant with
30 NAD⁺. In other evolved strains, the dihydrolipoamide dehydrogenase (Lpd) was mutated to accept NADP⁺
31 thus enabling the pyruvate dehydrogenase and 2-ketoglutarate dehydrogenase complexes to regenerate
32 NADPH. Interestingly, no other central metabolism oxidoreductase seems to evolve towards reducing
33 NADP⁺, which we attribute to several biochemical constraints such as unfavorable thermodynamics. This
34 study demonstrates the potential and biochemical limits of evolving oxidoreductases within the cellular
35 context towards changing cofactor specificity, further showing that long-term adaptive evolution can
36 optimize enzyme activity beyond what is achievable via rational design or directed evolution using small
37 libraries.

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39 **Importance**

40 In the cell, NAD(H) and NADP(H) cofactors have different functions. The former mainly accepts electrons
41 from catabolic reactions and carries them to respiration, while the latter provides reducing power for
42 anabolism. Correspondingly, the ratio of the reduced to the oxidized form differs for NAD (low) and NADP
43 (high), reflecting their distinct roles. We challenged the flexibility of *E. coli*'s central metabolism in multiple
44 adaptive evolution experiments using an NADPH-auxotroph strain. We found several mutations in two
45 enzymes, changing the cofactor preference of malic enzyme and dihydrolipoamide dehydrogenase. Upon
46 deletion of their corresponding genes we performed additional evolution experiments which did not lead
47 to the emergence of any additional mutants. We attribute this restricted number of mutational targets to
48 intrinsic thermodynamic barriers: The high ratio of NADPH to NADP⁺ limits metabolic redox reactions which
49 can regenerate NADPH, mainly by mass action constraints.

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52 **Introduction**

53 The cofactor preference of enzymes is crucial for ensuring balanced production and consumption of
54 resources, proper regulation of metabolic processes, and general cellular homeostasis. The two primary
55 electron carriers, NAD and NADP, demonstrate this quite well, as the former is mainly involved in catabolic
56 and respiratory processes while the latter mostly participates in biosynthetic pathways. The physiological
57 reduction levels of NAD and NADP pools reflect this distinction: the NAD pool is highly oxidized, providing
58 a thermodynamic push for catabolic processes, which are mostly oxidative and use NAD⁺ as an electron
59 acceptor; in contrast, the NADP pool is relatively reduced, thermodynamically supporting anabolic
60 processes, which are mostly reductive, using NADPH as an electron donor.

61 Multiple previous studies have demonstrated how the replacement of only a few residues in the active site
62 of an oxidoreductase enzyme can switch its cofactor preference from NAD to NADP or *vice versa* [1-4].
63 Several studies also constructed mutant libraries of specific oxidoreductases and harnessed natural
64 selection to identify variants with altered cofactor specificity [5-7]. Yet, until now, no study has attempted
65 to systematically explore the overall evolvability of central metabolism oxidoreductases towards the use of
66 a different cofactor. This could help shed light on the flexibility of the metabolic network and identify
67 emergent regeneration processes, thus adding to our understanding on the plasticity of cellular
68 metabolism.

69 Here, we applied a whole cell evolution approach towards the emergence of new NADPH regeneration
70 routes. We used an NADPH-auxotroph strain, deleted in all enzymes capable of regenerating NADPH
71 ($\Delta zwf \Delta maeB \Delta icd \Delta pntAB \Delta sthA$), with the exception of 6-phosphogluconate dehydrogenase [8]. This
72 strain could grow on a minimal medium only when gluconate is added as an NADPH source. We conducted
73 multiple parallel evolution experiments in continuous culture with limiting supply of gluconate, thus
74 selecting for the emergence of mutated oxidoreductases that could reduce NADP⁺. After long cultivation
75 periods under selective conditions (500-1100 generations), we were able to isolate evolved strains, from
76 10 independent evolution experiments, capable of growing without gluconate. Despite conducting the
77 experiments with different carbon sources, each enforcing a different distribution of central metabolism
78 fluxes, we found that all the evolved strains harbor mutations in one of only two enzymes: NAD-dependent
79 malic enzyme (MaeA) or dihydrolipoamide dehydrogenase (Lpd). We show that while the mutated MaeAs
80 strongly prefer the reduction of NADP⁺ over NAD⁺, the catalytic efficiencies of the Lpd variants are in
81 similar ranges with both cofactors. Several mutated MaeA variants also displayed higher overall activity
82 than their wildtype counterpart, demonstrating the strength of selective adaptation for identifying superior
83 mutants. Interestingly, no other central metabolism oxidoreductase evolved towards the use of NADP⁺,
84 which could be attributed to structural or thermodynamic constraints.

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88 **Results**

89 **The NADPH-auxotroph strain and oxidoreductase candidates for NADPH regeneration**

90 Five enzymes are known to support NADPH regeneration in *E. coli*: glucose 6-phosphate dehydrogenase
91 (Zwf), 6-phosphogluconate dehydrogenase (Gnd), the NADP-dependent malic enzyme (MaeB), isocitrate
92 dehydrogenase (Icd), and the membrane-bound transhydrogenase (PntAB) [9]. In a previous study we
93 constructed a strain in which the genes coding for these NADP-dependent oxidoreductases were deleted,
94 with the exception of *gnd* ($\Delta zwf \Delta maeB \Delta icd \Delta pntAB \Delta sthA$; the gene *sthA*, which encodes the soluble
95 transhydrogenase, was also deleted to remove a major NADPH sink) [8]. For this NADPH-auxotroph strain
96 to grow on a minimal medium, gluconate must be added as a precursor of 6-phosphogluconate, the
97 substrate of Gnd. Due to the deletion of *icd*, the supply of 2-ketoglutarate as a precursor for glutamate and
98 the downstream C₅ amino acids glutamine, proline, and arginine is also mandatory. We demonstrated that,
99 when gluconate is omitted, the NADPH-auxotroph strain can serve as an effective *in vivo* platform to test
100 and optimize different enzymatic systems for NADPH regeneration [8].

101 We speculated that cultivating the NADPH-auxotroph strain under limiting amounts of gluconate would
102 lead to the emergence of mutated oxidoreductase enzymes capable of regenerating NADPH. Such an
103 enzyme would need to sustain very high flux to support a NADPH regeneration rate sufficiently high to
104 enable cell growth. We therefore regarded oxidoreductase enzymes that participate in central metabolism
105 as the major candidates for evolution towards NADP⁺ reduction. Central metabolism employs multiple
106 oxidoreductases (Fig. 1), including glyceraldehyde 3-phosphate dehydrogenase (GapA), glycerol
107 dehydrogenase (GldA), glycerol 3-phosphate dehydrogenase (GpsA), pyruvate/2-ketoglutarate
108 dehydrogenases (or, more precisely, their lipoamide dehydrogenase subunit, Lpd), lactate dehydrogenase
109 (LdhA), the NAD-dependent malic enzyme (MaeA), and malate dehydrogenase (Mdh).

110 Since the entry point of carbon into central metabolism dictates the carbon flux distribution, we
111 hypothesized that the choice of the carbon source could predispose different NAD⁺-dependent
112 oxidoreductases as targets for mutations. For example, mutations in GapA that enable it to accept NADP⁺
113 would be useful to regenerate NADPH only if the cell is fed with a carbon source that enters upper
114 glycolysis and induces glycolytic flux (rather than gluconeogenesis). Similarly, mutagenesis of GpsA or
115 LdhA towards accepting NADP⁺ could effectively produce NADPH only when glycerol or lactate
116 (respectively) serves as the carbon source.

117 **Adaptive evolution of the NADPH-auxotroph strain led to mutations in *maeA* and *lpd***

118 We conducted twelve evolution experiments using six carbon sources: fructose, glycerol, pyruvate, lactate,
119 2-ketoglutarate and succinate (two parallel cultures for each carbon source). Fructose and glycerol are
120 expected to force glycolytic and anaplerotic fluxes, pyruvate and lactate are expected to force
121 gluconeogenic and anaplerotic fluxes, while 2-ketoglutarate and succinate are expected to force
122 gluconeogenic and cataplerotic fluxes (cataplerosis being the reverse of anaplerosis, that is,

123 decarboxylation of a C₄ intermediate of the TCA cycle to generate a C₃ glycolytic intermediate). Hence,
124 the six carbon sources nicely cover a large variation in flux distribution across central metabolism (Fig. 1).

125 We used GM3 cultivation devices to apply a medium-swap continuous culture regime [10, 11] in order to
126 evolve the NADPH-auxotroph strain towards novel NADPH regeneration routes. Cultures of growing cells
127 subjected to this regime are diluted, at fixed time intervals, by one of two growth media, permissive or
128 stressing, the choice depending on the turbidity of the culture measured in real time. Specifically, if the
129 turbidity is below a predefined value, a dilution pulse comes from the permissive medium; otherwise, the
130 stressing medium is used to dilute the culture [10, 11]. This approach enables gradual genetic adaptation
131 of a bacterial population to grow on the stressing medium. Here, the permissive medium contained one of
132 the six canonical carbon sources listed above, gluconate as NADPH source, and 2-ketoglutarate as
133 glutamate source. The stressing medium had the same composition except for gluconate which was
134 omitted. Continuous cultivation under these conditions is expected to select for the emergence of novel
135 NADPH regenerating enzymes, adapting the cells to grow with less and less gluconate, until growth on
136 the stressing medium alone is reached.

137 Of the 12 parallel adaptive evolution experiments, 8 evolved to rely completely on the stressing medium
138 (100% stressing medium pulses), including at least one culture for all six carbon sources used (Fig. 2A).
139 The adaptation kinetics and the number of generations required to attain growth without gluconate were
140 comparable for all the eight cultures (Table 1). In most cases, the stressing/relaxing dilution ratio only
141 slightly increased during a prolonged period of the adaptation until a sharp rise occurred and growth on
142 the stressing medium was attained, pointing to the appearance of a key adaptive mutation in the
143 population.

144 We isolated strains from all the cultures that were evolved to grow on the stressing medium and cultivated
145 each of these isolated strains on a minimal medium supplemented with different carbon sources (Fig. 3).
146 Each of these strains could grow well on the carbon source used in the evolution experiment in which it
147 has emerged. The isolated strains could also grow on (almost) all other carbon sources tested, indicating
148 that the metabolic adaptation was not restricted to a particular flux distribution in central metabolism.

149 Genomic sequencing of the adapted strains and comparison with the non-evolved parent strain revealed
150 5 to 10 point mutations as well as small insertions and deletions in all genomes sequenced (Table 1).
151 Importantly, we sequenced two isolates from each successful adaptive evolution experiment; for each
152 experiment, these isolates displayed an almost identical mutation profile (Supplementary Data),
153 suggesting that the bacterial populations in the cultures were rather homogeneous. The strains isolated
154 from the two glycerol cultures were exceptional as each contained more than 20 mutations, including a
155 missense mutation in gene *mutL* coding for a DNA mismatch repair protein. Notably, in 7 of the 8 cultures,
156 the gene *maeA*, which codes for the NAD-dependent malic enzyme, carried one or two non-silent
157 mutations. Furthermore, the isolates from the fructose and succinate cultures carried an amplified
158 chromosomal region containing the *maeA* gene, which points to overexpression of the mutated gene as
159 an additional adaptive trait. The only divergent isolate was from one of the cultures cultivated on 2-

160 ketoglutarate, in which *maeA* was not mutated. Instead, *lpd*, coding for lipoamide dehydrogenase, was
161 mutated in this strain.

162 **A mutation in a single residue in MaeA changed cofactor specificity but other mutations were** 163 **essential to recover catalytic efficiency**

164 Three of the isolated strains harbored a single mutation in *maeA*: D336N. In four strains, *maeA* had two
165 mutations, of which one was either D336N or D336A (Table 1). We chose to focus on three mutated
166 variants: D336N, D336N L176V, and D336A I283N. We introduced each of these mutation sets into the
167 non-evolved, parental strain using Multiplex Automated Genomic Engineering (MAGE [12]) and
168 characterized the growth of the resulting strains (Fig. 4).

169 The strain harboring *maeA* D336N was able to grow only with succinate and 2-ketoglutarate but not with
170 the other carbon sources (Fig. 4A). This is in line with the fact that two of the three evolved strains
171 displaying this mutation were cultivated on either succinate or 2-ketoglutarate, while the third one –
172 cultivated on fructose – also showed an amplification of the chromosomal region containing the *maeA*
173 gene (Table 1). It therefore seems that the D336N mutation enhanced NADP⁺ reduction by MaeA, but only
174 to a limited extent. Therefore, only carbon sources that enter the TCA cycle (i.e., succinate and 2-
175 ketoglutarate) and thus force high cataplerotic flux via MaeA, support sufficiently high NADPH regeneration
176 rate. When another carbon source is used (e.g., fructose) overexpression of *maeA* D336N seems
177 necessary to enable sufficient NADPH regeneration.

178 On the other hand, the strains harboring either *maeA* D336N L176V or *maeA* D336A I283N were able to
179 grow on (almost) all carbon sources (Fig. 4B,C). This suggests that these mutation sets increased the
180 activity of MaeA with NADP⁺ to a sufficiently high level such that even carbon sources that do not induce
181 cataplerotic flux could sustain high NADPH regeneration rate without further overexpression of *maeA*.

182 To test whether these interpretations are correct, we purified the mutated MaeA variants and performed
183 steady state analysis with NAD⁺ and NADP⁺ (Table 2). We found that while wildtype (WT) MaeA can accept
184 NADP⁺, it uses this cofactor with a low $k_{cat}/K_M = 11 \text{ s}^{-1} \text{ mM}^{-1}$, more than two orders of magnitude lower than
185 the $k_{cat}/K_M > 1800 \text{ s}^{-1} \text{ mM}^{-1}$ measured with NAD⁺. The D336N mutation lowered k_{cat}/K_M with NAD⁺ to 49 s^{-1}
186 mM^{-1} , while increasing k_{cat}/K_M with NADP⁺ ≈ 80 -fold to $\approx 870 \text{ s}^{-1} \text{ mM}^{-1}$. This mutation thus increased MaeA
187 preference toward NADP – as indicated by the ratio $(k_{cat}/K_M)_{\text{NADP}}/(k_{cat}/K_M)_{\text{NAD}}$ – by a factor of 3,000, from
188 0.006 to ≈ 18 .

189 The combined D336N L176V and D336A I283N mutations increased the activity with NADP⁺ even more,
190 resulting in k_{cat}/K_M of $\approx 6500 \text{ s}^{-1} \text{ mM}^{-1}$ and $\approx 8600 \text{ s}^{-1} \text{ mM}^{-1}$ (respectively), a 600- to 800-fold increase relative
191 to MaeA WT. Interestingly, for these two mutation sets, the catalytic efficiency with NAD⁺ increased to the
192 same extent as with NADP⁺, relative to that observed in MaeA D336N. Hence, the preference of MaeA
193 D336N L176V and MaeA D336A I283N toward NADP⁺ was effectively identical to that of MaeA D336N. It
194 therefore seems that the main role of the L176V and I283N mutations is the recovery of the catalytic
195 efficiency lost upon cofactor switching by the mutation of D336 [1, 13]. Notably, the k_{cat}/K_M values of MaeA

196 D336N L176V and MaeA D336A I283N with NADP⁺ are ≈4-fold higher than the k_{cat}/K_M of MaeA WT with
197 NAD⁺, presenting one of rare cases in which overall relative catalytic efficiency was improved upon
198 switching the cofactor specificity.

199 **Upon deletion of *maeA*, adaptive evolution led to mutations in *lpd***

200 As 7 of the 8 evolved strains contained a mutation in *maeA*, we decided to delete this gene in the NADPH-
201 auxotroph strain and repeat the evolution experiment in the hope to prompt the emergence of other
202 mutations enabling NADPH regeneration. Four cultures of the $\Delta zwf \Delta maeB \Delta icd \Delta pntAB \Delta sthA \Delta maeA$
203 strain, two supplemented with pyruvate and two with glycerol, were cultivated under the medium swap
204 continuous culture regime described above. For both carbon sources, growth on gluconate-free stressing
205 medium was attained for one of the two parallel cultures (Fig. 5A). The culture supplemented with pyruvate
206 showed a rather rapid adaptation, characterized by a steady increase in the stressing/relaxing dilution
207 pulse ratio. On the other hand, the culture fed with glycerol showed a two-phase plateau-acceleration
208 development. We isolated strains from the two cultures on the stressing medium and cultivated them on a
209 minimal medium supplemented with different carbon sources (Fig. 5B). Both strains were able to grow on
210 all carbon sources, with the exception of those entering the TCA cycle – that is succinate and 2-
211 ketoglutarate.

212 Genomic sequencing of isolates from the two cultures revealed missense mutations in *lpd* (Table 1 and
213 Supplementary Data). In all isolates, residue E205 was mutated either to glycine or to alanine; in one
214 isolate, the E205A mutation was further accompanied by an E366K mutation. We note that the E205A
215 mutation was also identified following the adaptive evolution of the NADPH-auxotroph strain cultivated on
216 2-ketoglutarate in which *maeA* did not mutate (see above, Table 1). It therefore seems that Lpd, which
217 participates as a subunit in the pyruvate dehydrogenase and 2-ketoglutarate dehydrogenase complexes
218 [14], was mutated to accept NADP⁺.

219 We used MAGE to introduce the three observed mutation sets – E205G, E205A, and E205A E366K – to
220 the *lpd* gene in the non-evolved, parental strain (NADPH auxotroph deleted in *maeA*). All resulting strains
221 were found to grow without gluconate on (almost) all carbon sources, where the E205G mutation seems
222 to enable the best growth (Fig. 6). Interestingly, while the isolated strains from the evolved culture could
223 not grow on 2-ketoglutarate and succinate (Fig. 5B), the MAGE-constructed strains could grow on these
224 carbon sources (Fig. 6, with the exception of Lpd E205A cultivated on succinate). This could potentially be
225 attributed to the adaptation of the evolved strains for growth on carbon sources that enter glycolysis and
226 hence force anaplerotic flux (glycerol, pyruvate) rather than enter the TCA cycle.

227 We further characterized the kinetics of the purified Lpd WT, Lpd E205G, Lpd E205A, and Lpd E205G
228 E366K. We found that while Lpd WT displayed no detectable activity with NADP⁺, the mutated Lpd
229 versions catalyzed NADP⁺ reduction with k_{cat}/K_M of 7.8, 6.5, and 10.6 s⁻¹ mM⁻¹ for Lpd E205G, Lpd E205A,
230 and Lpd E205G E366K, respectively (Table 3). Interestingly, the preference of Lpd E205G, Lpd E205A,
231 and Lpd E205G E366K towards NADP⁺, $(k_{cat}/K_M)_{NADP}/(k_{cat}/K_M)_{NAD}$ between 0.6 and 1.9, is considerably
232 lower than that observed with the mutated variants of MaeA. And yet, such slight preference seems to

233 ensure effective regeneration of NADPH by the mutated Lpds, thus enabling growth in a medium lacking
234 gluconate. This might be explained by the fact that the combined flux via the pyruvate dehydrogenase and
235 2-ketoglutarate dehydrogenase complexes is substantially higher than that via the malic enzyme,
236 especially since 2-ketoglutarate, as a necessary supplement (due to the *icd* deletion), was present in all
237 experiments.

238 Finally, to assess the possibility that other central metabolism oxidoreductases could mutate to enable
239 NADPH regeneration, we deleted both *maeA* and *lpd* genes in the NADPH-auxotroph strain. We subjected
240 the resulting strain ($\Delta zwf \Delta maeB \Delta icd \Delta pntAB \Delta sthA \Delta maeA \Delta lpd$) to medium swap adaptation as
241 described above, using either glycerol or succinate as a carbon source (both relaxing and stressing media
242 were further supplemented with acetate to cope with *lpd* deletion). However, even after prolonged
243 cultivation (>1000 generations), none of the four cultures evolved towards growth without gluconate.

244 Discussion

245 In this study we demonstrated whole-cell adaptive evolution towards the emergence of novel routes for
246 NADPH regeneration. In ten successful such experiments, using different carbon sources, we found two
247 NAD-dependent oxidoreductases within central metabolism that have evolved to accept NADP⁺: the malic
248 enzyme MaeA and lipoamide dehydrogenase Lpd.

249 Notably, the key residue that was mutated in all identified MaeA variants – aspartate 336 (Table 2) – was
250 also found to mutate in a previous study, enabling reductive, CO₂-assimilating flux using NADPH as an
251 electron donor [15]. The preference towards NADP⁺ of the MaeA D336G variant described before was
252 ≈12, somewhat lower than that observed for our mutants, i.e., 17-18. Moreover, the affinity of MaeA D336G
253 towards NADP⁺, $K_{M,app} = 0.23$ mM, was an order of magnitude lower than that observed with our mutants
254 MaeA D336N L176V and MaeA D336A I283N, i.e., $K_{M,app} < 0.02$ mM. This emphasizes the supportive role
255 of the L176V and I283N mutations in restoring high catalytic efficiency upon cofactor switching. As the
256 physiological concentration of NADP⁺ is exceedingly low, characteristically ≈ 1 μM in *E. coli* WT [16],
257 sustaining high affinity towards this cofactor is key for its effective reduction. This explains why in most
258 mutated MaeA variants identified, D336 was not mutated alone, but was rather accompanied by another
259 mutation. As mentioned above, the relative catalytic efficiency of MaeA D336N L176V and MaeA D336A
260 I283N with NADP⁺ was found to be 4-fold higher than that of the MaeA WT with NAD⁺, thus representing
261 one of the few cases in which cofactor switching is coupled to improved overall kinetics. An *a priori*
262 identification of the L176 or I283 residues as targets for recovery and increase of catalytic activity would
263 be very difficult, thus demonstrating the power of natural selection in leading to superior, yet non-trivial
264 solutions.

265 The key residue that was mutated in all three Lpd variants – glutamate 205 (Table 3) – was also previously
266 mutated to switch Lpd cofactor specificity [17]. In this study, the catalytic efficiency with NADP⁺ of the best
267 engineered variant of Lpd, which harbored the E205V mutation and four additional point mutations, was
268 100-fold higher than that with NAD⁺; and 4-fold superior to the catalytic efficiency of the Lpd WT with NAD⁺.

269 The consequences of the sole mutation at position 205 (E205V) on the kinetic parameters of the enzyme
270 were not investigated. The selection of the same five combined mutations in an evolutionary experiment
271 in continuous culture would require several events of point mutation to arise and be fixed in the same
272 gene, which makes the occurrence of such a variant highly improbable. Comparatively, the variant Lpd
273 enzymes selected in our experiments exhibited similar catalytic efficiencies with both nicotinamide
274 cofactors (ratio $(k_{cat}/K_M)_{NADP}/(k_{cat}/K_M)_{NAD}$ between 0.6 and 1.9) and far lower catalytic efficiencies with NAD⁺
275 than Lpd WT. The mutations selected by the process of natural selection enlarged the cofactor specificity
276 of Lpd with a concomitant decrease of activity, which apparently constitutes a good compromise for
277 maintaining balanced pools of NADH and NADPH and for ensuring sufficient carbon flux to sustain growth.

278 Many of the evolved strains harbor a mutation in the gene coding for citrate synthase (*gltA*, Supplementary
279 Data). Interestingly, we found that even a short-term cultivation of the NADPH-auxotroph strain using a
280 'permissive medium' – having gluconate in the minimal medium – frequently led to mutations in this gene.
281 These mutations included a missense mutation changing residue 150 from leucine to arginine which
282 resulted in a 30-fold decrease in specific activity (Supplementary Table S1) and a base pair deletion
283 causing a frameshift which shortened the polypeptide from 427 AA to 317 AA (Supplementary Data).
284 Citrate synthase activity, while not being required for the NADPH-auxotroph strain in the presence of 2-
285 ketoglutarate, is probably deleterious as it leads to overproduction and accumulation of citrate and
286 isocitrate, which cannot be further metabolized easily due to the deletion of *Icd* (considering limited flux
287 via the glyoxylate shunt). The downregulation or elimination of *GltA* activity avoids this overproduction and
288 hence might be beneficial for cell growth.

289 It is worth noticing that out of all possible oxidoreductase enzymes in central metabolism only *MaeA* and
290 *Lpd* were found to mutate during the adaptive evolution. This might be explained by the fact that shifting
291 the cofactor specificity of these two enzymes was attainable thanks to a unique point mutation. Changing
292 the cofactor preference of the other central metabolism oxidoreductases might require the accumulation
293 of multiple mutations, for both cofactor switching and recovery of activity [1, 13]. However, a more plausible
294 option is that the evolution of other oxidoreductases towards NADPH regeneration is biochemically
295 constrained due to unfavorable thermodynamics. For example, the reactions catalyzed by glycerol-3-
296 phosphate dehydrogenase (*GpsA*) or lactate dehydrogenase (*LdhA*) strongly favor NADH consumption
297 ($\Delta_rG'^m < -20$ kJ/mol [18]). Hence, even if these enzymes were mutated to accept NADP⁺, their ability to
298 regenerate NADPH with a sufficiently high rate – when cells are fed with the respective carbon source –
299 would be highly limited. Indeed, glycerol and lactate catabolism involve oxidoreductases using a quinone
300 as electron acceptor rather than NAD(P)⁺ (Fig. 1).

301 Similarly, if glyceraldehyde 3-phosphate dehydrogenase (*GapA*) would evolve to accept NADP⁺, a severe
302 thermodynamic barrier might arise. Even with NAD⁺, substrate-level phosphorylation (involving *GapA*,
303 $\Delta_rG'^m = 24,9$ kJ/mol) represents the major thermodynamic barrier in glycolysis [19]. As the cellular NADP
304 pool is substantially more reduced than the NAD pool, switching the cofactor preference of *GapA* from
305 NAD⁺ to NADP⁺ would further reduce the reaction driving force, rendering glycolysis practically inoperative.
306 Finally, the oxidation of malate to oxaloacetate is the most thermodynamically challenging reaction in

307 central metabolism ($\Delta_rG'^m = 30,3$ kJ/mol), which can operate only if the concentration of oxaloacetate is
308 kept very low (~ 1 μ M) [19]. Hence, replacing NAD⁺ with NADP⁺, thus further decreasing the driving force
309 for malate oxidation, would certainly render the TCA cycle thermodynamically infeasible. Taken together,
310 it seems that only those central metabolism oxidoreductases which thermodynamically prefer the NAD⁺
311 reduction direction (MaeA, $\Delta_rG'^m = -4.1$ kJ/mol; Lpd as part of pyruvate dehydrogenase, $\Delta_rG'^m = -35.3$
312 kJ/mol and as part of 2-ketoglutarate dehydrogenase -27.2 kJ/mol) could evolve to accept NADP⁺, as only
313 these enzymes could sustain a high rate of NADPH regeneration.

314 Interestingly, instead of evolving a central metabolism oxidoreductase to accept NADP⁺, the adaptive
315 evolution could have increased metabolic flux towards routes that natively produce NADPH but usually
316 carry only low fluxes. For example, increasing flux towards serine and glycine biosynthesis and one carbon
317 metabolism could boost NADPH regeneration via the NADP-dependent bifunctional 5,10-methylene-
318 tetrahydrofolate dehydrogenase/5,10-methenyl-tetrahydrofolate cyclohydrolase (FolD). The fact that we
319 did not observe such adaptation indicates that it is easier to change the cofactor preference of an enzyme
320 via few mutations rather than redistribute fluxes within the endogenous metabolic network.

321 Overall, the presented work shows the power of evolution and the flexibility, but also the limits of
322 metabolism to adapt to metabolic challenges. Finding a solution counteracting the increased metabolic
323 constraint by the additional deletion of both *maeA* and *lpd* in the NADPH-auxotroph was not within reach
324 in our setup, most likely because of the thermodynamic constraints of the remaining oxidoreductases,
325 which additionally might not provide easy starting points for a cofactor switch to NADP⁺. However, the
326 failure of the evolution attempt of the NADPH-auxotroph Δ *maeA* Δ *lpd* indicates that this strain provides a
327 stringent host for the *in vivo* testing of heterologous NADP⁺ specific oxidoreductases and their evolution.

328 **Methods**

329 **Reagents and chemicals.** Primers were synthesized by Eurofins (Ebersberg, Germany) (Supplementary
330 Table S2). Screening PCRs were performed using DreamTaq polymerase (Thermo Fisher Scientific,
331 Dreieich, Germany). PCR reactions for amplifying deletion cassettes were done using PrimeSTAR MAX
332 DNA Polymerase (Takara). NAD⁺ and NADP⁺(Na)₂ were purchased from Carl Roth GmbH, malic acid and
333 chicken egg lysozyme from Sigma Aldrich AG and DNase I from Roche Diagnostics. Dihydrolipoamide
334 was synthesized by borohydride reduction of lipoamide (Sigma Aldrich SA) as described by Reed et al.
335 [20]. Purity (> 95 %) was checked by NMR and infusion mass-spectrometry analysis.

336 **Media.** LB medium (1% NaCl, 0.5% yeast extract, 1% tryptone) was used for strain maintenance. When
337 appropriate, kanamycin (25 μ g/mL), ampicillin (100 μ g/mL) or chloramphenicol (30 μ g/mL) was used.
338 Minimal MA medium (31 mM Na₂HPO₄, 25 mM KH₂PO₄, 18 mM NH₄Cl, 1 mM MgSO₄, 40 μ M trisodic
339 nitrilotriacetic acid, 3 μ M CaCl₂, 3 μ M FeCl₃·6H₂O, 0.3 μ M ZnCl₂, 0.3 μ M CuCl₂·2H₂O, 0.3 μ M CoCl₂·2H₂O,
340 0.3 μ M H₃BO₃, 1 μ M MnCl₂, 0.3 μ M CrCl₃, 6 H₂O, 0.3 μ M Ni₂Cl, 6 H₂O, 0.3 μ M Na₂MoO₄, 2 H₂O, 0.3 μ M
341 Na₂SeO₃, 5 H₂O) was used for long-term continuous cultures. M9 minimal medium (50 mM Na₂HPO₄, 20
342 mM KH₂PO₄, 1 mM NaCl, 20 mM NH₄Cl, 2 mM MgSO₄ and 100 μ M CaCl₂, 134 μ M EDTA, 13 μ M

343 FeCl₃·6H₂O, 6.2 μM ZnCl₂, 0.76 μM CuCl₂·2H₂O, 0.42 μM CoCl₂·2H₂O, 1.62 μM H₃BO₃, 0.081 μM
344 MnCl₂·4H₂O) was used for cell growth analysis. The mineral media were supplemented with various
345 carbon sources as indicated in the main text and hereafter.

346 **Strains and plasmids.** *E. coli* K12 strains used in this study are derivatives of strain SIJ488, which was
347 used as wildtype reference (Table 4). The deletion of the *maeA* gene was carried out by λ-Red
348 recombination using a kanamycin resistance cassette generated via PCR using the FRT-PGK-gb2-neo-
349 FRT (Km) cassette (Gene Bridges, Germany) and the primer pair *maeA_KO_fw* and *maeA_KO_rv*.
350 Primers *maeA_KO_Ver-F* and *maeA_KO_Ver-R* were used to verify the deletion of *maeA* (Supplementary
351 Table S2). Cell preparation and transformation for gene deletion was carried out as described [21, 22].
352 The coding sequences of the WT sequences of *maeA*, *lpd* and *gltA*, as well as the respective mutated
353 genes were amplified by PCR using the primer pairs *maeA_Nter_histag_fw* and *maeA_rv*,
354 *lpd_Nter_histag_fw* and *lpd_rv*, *gltA_Nter_histag_fw* and *gltA-R*, respectively (Supplementary Table S2).
355 The amplified fragments were inserted into a modified Novagen pET22b(+) expression vector
356 (Supplementary Table S3) by using a ligation independent directional cloning method [23]. The sequence
357 of the inserts of the resulting plasmids was verified by Sanger sequencing.

358 **Evolution in GM3-driven long-term continuous culture.** Pre-cultures of the auxotrophic strains
359 NADPH-auxotroph and NADPH-auxotroph Δ*maeA* were obtained in permissive growth media consisting
360 in minimal MA medium supplemented with 5 mM D-gluconate, 5 mM 2-ketoglutarate and one of the
361 following carbon sources: D-fructose (10 mM), succinate (17 mM), pyruvate (25 mM), lactate (25 mM),
362 glycerol (20 mM), 2-ketoglutarate (20 mM final). Each pre-culture was then used to inoculate the growth
363 chambers (16 ml per chamber) of two parallel independent GM3 devices [10]. A continuous gas flow of
364 sterile air through the culture vessel ensured constant aeration and growth in suspension by counteracting
365 cell sedimentation. The cultures were grown in the corresponding medium under turbidostat mode (dilution
366 threshold set to 80 % transmittance (OD ≈ 0.4, 37°C) until stable growth of the bacterial population. The
367 cultures were then submitted to conditional medium swap regime. This regime enabled gradual adaptation
368 of the bacterial populations to grow in a non-permissive or stressing medium of composition equivalent to
369 the permissive medium but lacking D-gluconate. Dilutions of the growing cultures were triggered every 10
370 minutes with a fixed volume of medium calculated to impose a generation time of 3h10 on the cell
371 population, if not otherwise stated. The growing cultures were fed by permissive or stressing medium
372 depending on the turbidity of the culture with respect to a set OD threshold (OD₆₀₀ value of 0.4). When the
373 OD exceeded the threshold, a pulse of stressing medium was injected; otherwise a pulse of permissive
374 medium. The cultures were maintained under medium swap regime until the bacterial cell populations
375 grew in 100 % stressing medium. Cultures which did not evolve towards growth in stressing medium were
376 aborted after culturing for 1000 generations. Four isolates were obtained on agar-solidified stressing
377 medium for each successful evolution experiment and further analyzed.

378 **Genomic analysis of evolved strains.** Pair-end libraries (2x150 bp) were prepared with 1 μg genomic
379 DNA from the evolved strains and sequenced using a MiSeq sequencer (Illumina). The PALOMA pipeline,
380 integrated in the platform Microscope (<http://www.genoscope.cns.fr/agc/microscope>) was used to map the

381 reads against *E. coli* K12 wildtype strain MG1655 reference sequence (NC_000913.3) for detecting single
382 nucleotide variations, short insertions or deletions (in/dels) as well as read coverage variations [24].

383 **Growth experiments.** Overnight cultures were obtained in 4 mL M9 medium supplemented with 12 mM
384 gluconate and 3 mM 2-ketoglutarate (permissive growth condition). Strains were harvested (6,000g, 3 min)
385 and washed thrice in M9 medium to remove residual carbon sources. Cells were then inoculated into the
386 various test media to OD₆₀₀ of 0.01 and distributed into 96-well microtiter plates (Nunclon Delta Surface,
387 Thermo Scientific). Each well contained 150 µL of culture and 50 µL mineral oil (Sigma-Aldrich) to avoid
388 evaporation. Growth monitoring and incubation at 37 °C was carried out in a microplate reader (EPOCH
389 2, BioTek). The program (controlled by Gen5 3.04) consisted in 4 shaking phases, 60 seconds each: linear
390 shaking 567 cpm (3 mm), orbital shaking 282 cpm (3 mm), linear shaking 731 cpm (2 mm), orbital shaking
391 365 cpm (2 mm). After 3 shaking cycles absorbance OD₆₀₀ was measured. Raw data were calibrated to 1
392 cm-wide cuvette measured OD₆₀₀ values according to $OD_{cuvette} = OD_{plate} / 0.23$. Matlab was used to
393 calculate growth parameters, repeatedly based on at least three technical replicates. Average values were
394 used to generate the growth curves. Variability between triplicate measurements was less than 5% in all
395 cases displayed.

396 **Reverse engineering.** The pORTMAGE system which allows an efficient directed genome editing in *E.*
397 *coli* [25] was used to introduce into the naïve ancestor strains the mutations fixed in the genes *maeA* and
398 *lpd* during the evolution experiments. MAGE oligos were designed using <http://modest.biosustain.dtu.dk/>
399 (Supplementary Table S2); they contained thioester bonds at 5' and 3' ends and the wanted mutation.
400 Cells carrying the pORTMAGE-2 plasmid were incubated at 30°C. When cultures reached an OD₆₀₀ of 0.5,
401 the system was induced by incubation at 42°C for 15 min. Afterwards cells were immediately chilled on ice
402 until they were prepared for electroporation by 3 consecutive cycles of washing and centrifugation (11,000
403 rpm, 30 sec, 2°C) with ice-cold 10 % glycerol solution. MAGE oligos were introduced into the strains by
404 electroporation (1 mm cuvette, 1.8 kV, 25 µF, 200 Ω). Strains were directly transferred to LB, 10 mM
405 gluconate, 3 mM 2-ketoglutarate and incubated for 1 hour. After three rounds of MAGE cells were plated
406 on LB-plates containing 10 mM gluconate and 3 mM 2-ketoglutarate. The respective loci were amplified
407 by PCR using respective primer pairs Ver-F and Ver-R (Supplementary Table S2), and sequenced by
408 Sanger sequencing, to identify strains with the wanted mutations.

409
410 **Protein Expression and purification.** The His-tagged WT and mutated MaeA proteins were expressed
411 in *E. coli* BL21 DE3. Cells in Terrific Broth containing 100 µg/mL ampicillin were grown at 37 °C until they
412 reached an OD₆₀₀ of 0.8-1 upon which expression for 16 h at 23 °C was induced by addition of 250 µM
413 IPTG (Isopropyl-D-β-thiogalactopyranoside). Cells were harvested for 15 min at 6'000 g at 4°C then
414 resuspended in 2 mL of Buffer A (50 mM Tris, 500 mM NaCl, pH 7.5) per gram of pellet. The suspension
415 was treated with 10 mg/mL of DNase I, 5 mM MgCl₂ and 6 µg/mL lysozyme on ice for 20 min upon which
416 cells were lysed by sonication. The lysate was clarified at 45'000 g at 4°C for 45 min and the supernatant
417 was filtered through a 0.4 µm syringe tip filter (Sarstedt, Nümbrecht, Germany). Lysate was loaded onto a
418 pre-equilibrated 1 mL HisTrap FF column and washed with 12 % Buffer B (50 mM Tris, 500 mM NaCl, 500

419 mM imidazole, pH 7.5) for 20-30 column volumes until the UV 280 nm reached the baseline level. The
420 protein was eluted by applying 100% buffer B, collected then pooled and desalted into 12.5 mM Tris, 125
421 mM NaCl, pH 7.5, 10 % glycerol. The protein was frozen in N₂ (l) and stored at -80°C if not immediately
422 used for assays.

423 The His-tagged WT and mutated Lpd proteins were expressed in *E. coli* BL21 DE3 Codon+ (Invitrogen).
424 Cells in 400 ml Terrific broth containing 100 µg/mL carbenicillin were grown at 37 °C until they reached an
425 OD₆₀₀ = of 0.8-1 upon which expression for 16 h at 20 °C was induced by addition 500 µM IPTG. Cells
426 were harvested by centrifugation for 30 min at 10000 g at 4°C. Cell pellets were frozen at -80°C for one
427 night. Thawed cells were then suspended in 32 ml of Buffer A (50 mM phosphate (Na/K), 500 mM NaCl,
428 30 mM imidazole, 15% glycerol, pH 8.0) and lysed for 30 min at room temperature after addition of 3.6 ml
429 of Bug Buster (Novagen) 32 µl DTT (dithiothreitol) 1M, 320 µl Pefabloc 0.1 M (Millipore) and 23 µl Lysonase
430 (Novagen). Lysate was clarified at 9000g for 45 min at 4°C then loaded onto a 5 ml HisTrap FF column
431 pre-equilibrated in Buffer A. The protein was eluted in Buffer B (50 mM phosphate (Na/K), 500 mM NaCl,
432 250 mM imidazole, 1 mM DTT 15% glycerol, pH 8.0) and desalted on a gel-filtration column Hi Load 16/60
433 Superdex 200 pg in Buffer C (50mM Tris, 50 mM NaCl, glycerol 15%, 1 mM DTT, pH8.0). The protein was
434 frozen and stored at -80°C if not immediately used for assays.

435 **Biochemical assays.** *Characterization of MaeA kinetic parameters.* Assays were performed on a Cary-
436 60 UV/Vis spectrophotometer (Agilent) at 30°C using quartz cuvettes (10 mm path length; Hellma).
437 Reactions were performed in 50 mM Tris HCl pH 7.5 10 mM MgCl₂. Kinetic parameters for one substrate
438 were determined by varying its concentration while the others were kept constant at 6-10 times their K_M
439 value. Reaction procedure was monitored by following the reduction of NAD(P)⁺ at 340 nm ($\epsilon_{\text{NADPH},340\text{nm}} =$
440 $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$). Each concentration was measured in triplicates and the obtained curves were fit using
441 GraphPad Prism 8. Hyperbolic curves were fit to the Michaelis-Menten equation to obtain apparent k_{cat}
442 and K_M values.

443 *Characterization of Lpd kinetic parameters.* Assays were performed using a Safas UV mc2 double beam
444 spectrophotometer at room temperature using quartz cuvettes (10mm path length). The concentration of
445 purified Lpd enzyme was determined spectrophotometrically using an extinction coefficient of 34.0 mM⁻¹.cm⁻¹
446 at 280 nm. The concentration of FAD was determined using an extinction coefficient of 15.4 mM⁻¹.cm⁻¹
447 at 446 nm [26]. The absorbances at 446 nm and at 280 nm were measured and the ratio calculated
448 to determine the fraction of active FAD-containing catalysts within each batch of purified enzyme and to
449 normalize the results between the different enzyme forms. Assays of Lpd-catalyzed oxidation of
450 dihydrolipoamide were conducted in 100 mM Na phosphate, 100 mM KCl, 8 mM TCEP pH 7.6. Kinetic
451 parameters for NAD(P)⁺ were determined by varying its concentration in the presence of a saturating
452 concentration of dihydrolipoamide (4 mM). The reactions were monitored by recording the accumulation
453 of NAD(P)H at 340 nm. Kinetic constants were determined by non-linear analysis of initial rates from
454 duplicate experiments using SigmaPlot 9.0 (Systat Software, Inc.).

455 **Data availability**

456 The data supporting the findings of this work are available within the paper and its Supplementary
457 Information files. Strains used here are available on request from the corresponding author. For ΔG
458 calculations data from eQuilibrator (<http://equilibrator.weizmann.ac.il/>) was used.

459 **References**

- 460 1. Cahn, J. K., Werlang, C. A., Baumschlager, A., Brinkmann-Chen, S., Mayo, S. L. & Arnold, F. H. (2017)
461 A General Tool for Engineering the NAD/NADP Cofactor Preference of Oxidoreductases, *ACS synthetic*
462 *biology*. **6**, 326-333.
- 463 2. Chanique, A. M. & Parra, L. P. (2018) Protein Engineering for Nicotinamide Coenzyme Specificity in
464 Oxidoreductases: Attempts and Challenges, *Frontiers in microbiology*. **9**, 194.
- 465 3. Selles Vidal, L., Kelly, C. L., Mordaka, P. M. & Heap, J. T. (2018) Review of NAD(P)H-dependent
466 oxidoreductases: Properties, engineering and application, *Biochimica et biophysica acta Proteins and*
467 *proteomics*. **1866**, 327-347.
- 468 4. Wang, M., Chen, B., Fang, Y. & Tan, T. (2017) Cofactor engineering for more efficient production of
469 chemicals and biofuels, *Biotechnology advances*. **35**, 1032-1039.
- 470 5. Zhang, L., King, E., Luo, R. & Li, H. (2018) Development of a High-Throughput, In Vivo Selection
471 Platform for NADPH-Dependent Reactions Based on Redox Balance Principles, *ACS synthetic biology*. **7**,
472 1715-1721.
- 473 6. Kramer, L., Le, X., Rodriguez, M., Wilson, M. A., Guo, J. & Niu, W. (2020) Engineering Carboxylic Acid
474 Reductase (CAR) through a Whole-Cell Growth-Coupled NADPH Recycling Strategy, *ACS synthetic*
475 *biology*. **9**, 1632-1637.
- 476 7. Calzadiaz Ramirez, L., Calvo Tusell, C., Stoffel, G. M., Lindner, S. N., Osuna, S., Erb, T. J., Garcia-
477 Borràs, M., Bar-Even, A. & Acevedo-Rocha, C. G. (2020) In vivo selection for formate dehydrogenases
478 with high efficiency and specificity towards NADP+, *ACS Catal*. **10**, 7512–7525.
- 479 8. Lindner, S. N., Ramirez, L. C., Krusemann, J. L., Yishai, O., Belkhef, S., He, H., Bouzon, M., Doring,
480 V. & Bar-Even, A. (2018) NADPH-Auxotrophic E. coli: A Sensor Strain for Testing in Vivo Regeneration of
481 NADPH, *ACS synthetic biology*. **7**, 2742-2749.
- 482 9. Sauer, U., Canonaco, F., Heri, S., Perrenoud, A. & Fischer, E. (2004) The soluble and membrane-
483 bound transhydrogenases UdhA and PntAB have divergent functions in NADPH metabolism of
484 *Escherichia coli*, *J Biol Chem*. **279**, 6613-9.
- 485 10. Marliere, P., Patrouix, J., Doring, V., Herdewijn, P., Tricot, S., Cruveiller, S., Bouzon, M. & Mutzel, R.
486 (2011) Chemical evolution of a bacterium's genome, *Angew Chem Int Ed Engl*. **50**, 7109-14.
- 487 11. Bouzon, M., Perret, A., Loreau, O., Delmas, V., Perchat, N., Weissenbach, J., Taran, F. & Marliere,
488 P. (2017) A Synthetic Alternative to Canonical One-Carbon Metabolism, *ACS synthetic biology*. **6**, 1520-
489 1533.
- 490 12. Wang, H. H., Isaacs, F. J., Carr, P. A., Sun, Z. Z., Xu, G., Forest, C. R. & Church, G. M. (2009)
491 Programming cells by multiplex genome engineering and accelerated evolution, *Nature*. **460**, 894-8.
- 492 13. Tawfik, D. S. (2014) Accuracy-rate tradeoffs: how do enzymes meet demands of selectivity and
493 catalytic efficiency?, *Curr Opin Chem Biol*. **21**, 73-80.
- 494 14. Carothers, D. J., Pons, G. & Patel, M. S. (1989) Dihydrolipoamide dehydrogenase: functional
495 similarities and divergent evolution of the pyridine nucleotide-disulfide oxidoreductases, *Arch Biochem*
496 *Biophys*. **268**, 409-25.
- 497 15. Zelle, R. M., Harrison, J. C., Pronk, J. T. & van Maris, A. J. (2011) Anaplerotic role for cytosolic malic
498 enzyme in engineered *Saccharomyces cerevisiae* strains, *Appl Environ Microbiol*. **77**, 732-8.
- 499 16. Bennett, B. D., Kimball, E. H., Gao, M., Osterhout, R., Van Dien, S. J. & Rabinowitz, J. D. (2009)
500 Absolute metabolite concentrations and implied enzyme active site occupancy in *Escherichia coli*, *Nat*
501 *Chem Biol*. **5**, 593-9.

- 502 17. Bocanegra, J. A., Scrutton, N. S. & Perham, R. N. (1993) Creation of an NADP-dependent pyruvate
503 dehydrogenase multienzyme complex by protein engineering, *Biochemistry*. **32**, 2737-40.
- 504 18. Flamholz, A., Noor, E., Bar-Even, A. & Milo, R. (2012) eQuilibrator--the biochemical thermodynamics
505 calculator, *Nucleic Acids Res.* **40**, D770-5.
- 506 19. Noor, E., Bar-Even, A., Flamholz, A., Reznik, E., Liebermeister, W. & Milo, R. (2014) Pathway
507 thermodynamics highlights kinetic obstacles in central metabolism, *PLoS Comput Biol.* **10**, e1003483.
- 508 20. Reed, L. J., Koike, M., Levitch, M. E. & Leach, F. R. (1958) Studies on the nature and reactions of
509 protein-bound lipoic acid, *J Biol Chem.* **232**, 143-58.
- 510 21. Wenk, S., Yishai, O., Lindner, S. N. & Bar-Even, A. (2018) An Engineering Approach for Rewiring
511 Microbial Metabolism, *Methods Enzymol.* **608**, 329-367.
- 512 22. Jensen, S. I., Lennen, R. M., Herrgard, M. J. & Nielsen, A. T. (2015) Seven gene deletions in seven
513 days: Fast generation of *Escherichia coli* strains tolerant to acetate and osmotic stress, *Scientific reports.*
514 **5**, 17874.
- 515 23. Aslanidis, C. & de Jong, P. J. (1990) Ligation-independent cloning of PCR products (LIC-PCR), *Nucleic*
516 *Acids Res.* **18**, 6069-74.
- 517 24. Vallenet, D., Calteau, A., Dubois, M., Amours, P., Bazin, A., Beuvin, M., Burlot, L., Bussell, X.,
518 Fouteau, S., Gautreau, G., Lajus, A., Langlois, J., Planel, R., Roche, D., Rollin, J., Rouy, Z., Sabatet, V. &
519 Medigue, C. (2020) MicroScope: an integrated platform for the annotation and exploration of microbial
520 gene functions through genomic, pangenomic and metabolic comparative analysis, *Nucleic Acids Res.* **48**,
521 D579-D589.
- 522 25. Nyerges, Á., Csörgő, B., Nagy, I., Bálint, B., Bihari, P., Lázár, V., Apjok, G., Umenhoffer, K., Bogos,
523 B., Pósfai, G. & Pál, C. (2016) A highly precise and portable genome engineering method allows
524 comparison of mutational effects across bacterial species, *Proceedings of the National Academy of*
525 *Sciences.* **113**, 2502-2507.
- 526 26. Thorpe, C., Matthews, R. G. & Williams, C. H., Jr. (1979) Acyl-coenzyme A dehydrogenase from pig
527 kidney. Purification and properties, *Biochemistry.* **18**, 331-7.
528
529

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533 **Author contributions**

534 S.N.L. and A.B.-E. conceived the study. M.B., V.D., A.P., T.E., S.N.L., and A.B.-E. designed the
535 experiments. M.B. and V.D. supervised the evolution experiments and analyzed the genome sequencing
536 data. I.D. and A.B. ran the continuous cultures, isolated and characterized evolved strains. D.R. performed
537 comparative genomic analysis. S.N.L., S.M., L.C.R. performed reverse engineering and growth
538 experiments. G.S., L.C.R., M.F., A.B., A.P. and T.E. performed the *in vitro* experiments. S.N.L., A.B.-E,
539 V.D., and M.B. analyzed the results and wrote the manuscript with contributions from all authors.

540 **Competing Interests**

541 The authors declare no competing interests.

542

543

544 **Tables**

545 Table 1. Outcome of the successful adaptive evolutions of NADPH-auxotroph strains performed in the
546 GM3 cultivation device. See Supplementary Data, for all identified mutations.

Ancestor strain	Carbon source	N° generations (until growth w/o gluconate)	N° mutations in evolved isolates	Mutated dehydrogenase	Genomic amplification
NADPH-aux ($\Delta zwf \Delta maeB \Delta icd \Delta pntAB \Delta sthA$)	Fructose	700	5	MaeA D336N	+ (MaeA)
	Glycerol	750	24	MaeA D336N L176V	
		580	24	MaeA D336N L166V	
	Pyruvate	690	10	MaeA D336A I283N	
	Lactate	1105	10	MaeA D336N S30C	
	2-ketoglutarate	609	8	Lpd E205A	
640		10	MaeA D336N		
Succinate	850	9	MaeA D336N	+ (MaeA)	
NADPH-aux $\Delta maeA$ ($\Delta zwf \Delta maeB \Delta icd \Delta pntAB \Delta sthA \Delta maeA$)	Glycerol	740	9	Lpd E205A E366K ^a	
	Pyruvate	460	8	Lpd E205G	

547 ^a The E366K mutation was found in only one isolate from this culture. Other isolates from the same culture harbored only the
548 E205A mutation

549

550 Table 2. Apparent steady state parameters of MaeA variants. Parameters are indicated as mean value \pm
 551 standard error. Underlying Michaelis-Menten kinetics can be found in Supplementary Figure S1.

Enzyme	Substrate	K_M (mM)	k_{cat} (s ⁻¹)	k_{cat} / K_M (s ⁻¹ mM ⁻¹)	$(k_{cat} / K_M)_{NADP} /$ $(k_{cat} / K_M)_{NAD}$
MaeA WT	L-malate	0.63 \pm 0.04			0.0059
	NAD ⁺	0.10 \pm 0.02	188 \pm 9	1843	
	NADP ⁺	3.6 \pm 0.4	39 \pm 2	11	
MaeA D336N	L-malate	1.8 \pm 0.2			17.6
	NAD ⁺	1.5 \pm 0.2	74 \pm 3	49	
	NADP ⁺	0.091 \pm 0.01	79 \pm 2	868	
MaeA D336N L176V	L-malate	1.4 \pm 0.3			16.7
	NAD ⁺	0.27 \pm 0.03	105 \pm 2	389	
	NADP ⁺	0.016 \pm 0.001	102 \pm 1	6497	
MaeA D336A I283N	L-malate	0.48 \pm 0.04			18.0
	NAD ⁺	0.27 \pm 0.03	130 \pm 3	480	
	NADP ⁺	0.013 \pm 0.00	112 \pm 2	8615	

552

553

554 Table 3. Apparent steady state parameters of Lpd variants with NAD⁺ and NADP⁺. Underlying Michaelis-
555 Menten kinetics can be found in Supplementary Figure S2.

Enzyme	Substrate	K_M (mM)	K_{cat} (s ⁻¹)	K_{cat} / K_M (s ⁻¹ mM ⁻¹)	$(K_{cat} / K_M)_{NADP} /$ $(K_{cat} / K_M)_{NAD}$
Lpd WT	dihydrolipoamide	0.18 ± 0.02			
	NAD ⁺	0.44 ± 0.06	363 ± 60	830	
	NADP ⁺	no activity			
Lpd E205G	dihydrolipoamide	0.24 ± 0.09			0.6
	NAD ⁺	9.49 ± 1.82	121.2 ± 0.9	12.8	
	NADP ⁺	3.48 ± 1.03	27.2 ± 3.4	7.8	
Lpd E205A	dihydrolipoamide	0.02 ± 0.00			1.2
	NAD ⁺	6.35 ± 0.38	34.3 ± 0.9	5.4	
	NADP ⁺	2.48 ± 0.69	16.2 ± 2.5	6.5	
Lpd E205A E366K	dihydrolipoamide	0.03 ± 0.01			1.9
	NAD ⁺	8.07 ± 0.45	45.2 ± 1.3	5.6	
	NADP ⁺	3.26 ± 0.81	34.4 ± 2.9	10.6	

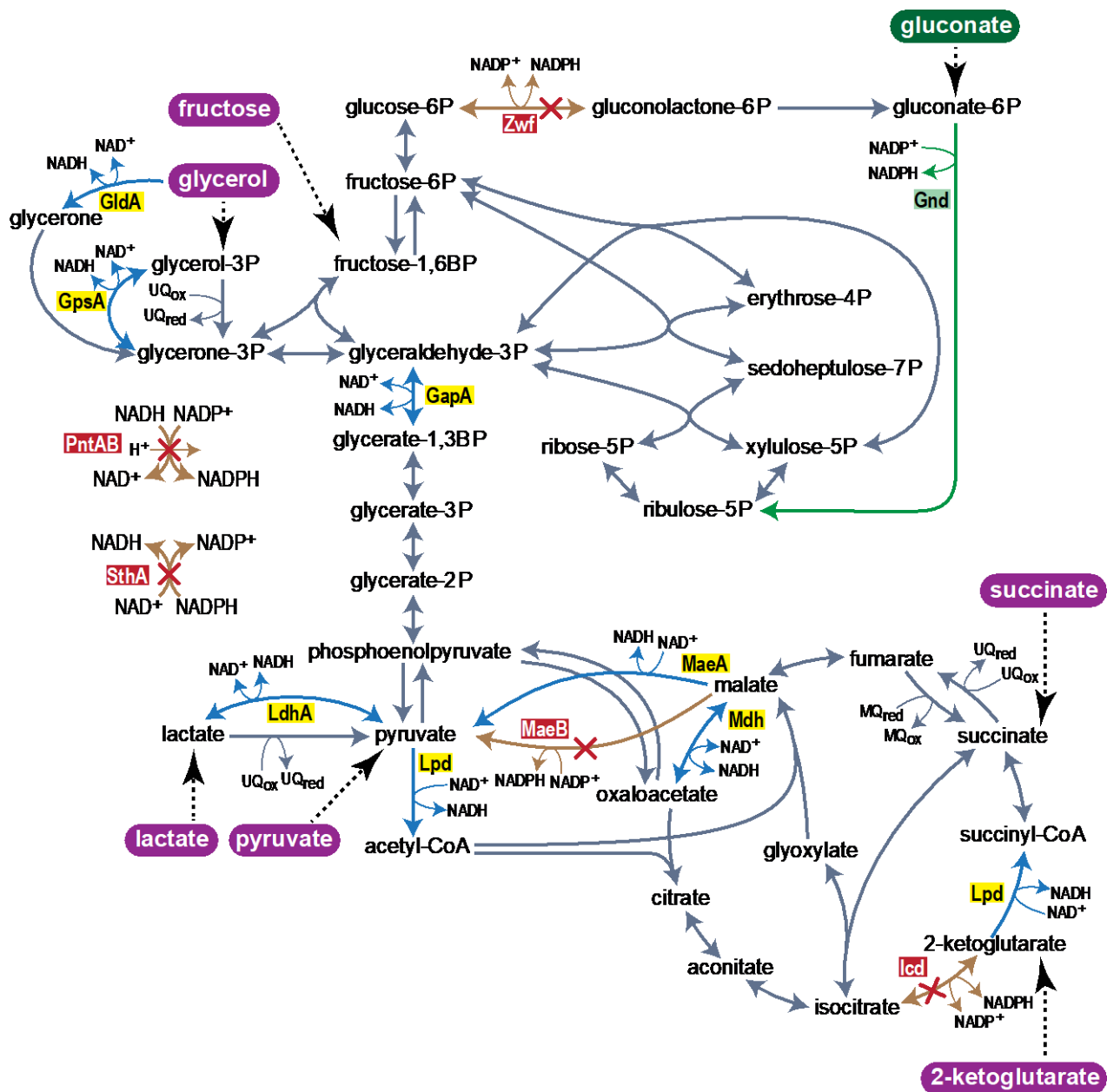
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557 Table 4: *E. coli* strains and their genetic modifications used in this study.

Strain	Genotype	References
SIJ488	WT	[22]
NADPH-auxotroph	$\Delta zwf \Delta maeB \Delta icd \Delta pntAB \Delta sthA$	[8]
NADPH-auxotroph $\Delta maeA$	$\Delta zwf \Delta maeB \Delta icd \Delta pntAB \Delta sthA \Delta maeA$	This work
NADPH-auxotroph $\Delta maeA \Delta lpd$	$\Delta zwf \Delta maeB \Delta icd \Delta pntAB \Delta sthA \Delta maeA \Delta lpd$	This work
NADPH-auxotroph $maeA^{D336N}$	$maeA^{D336N}$ introduced by MAGE in the NADPH-auxotroph	This work
NADPH-auxotroph $maeA^{D336N L176V}$	$maeA^{D336N L176V}$ introduced by MAGE in the NADPH-auxotroph	This work
NADPH-auxotroph $maeA^{D336A I283N}$	$maeA^{D336A I283N}$ introduced by MAGE in the NADPH-auxotroph	This work
NADPH-auxotroph $\Delta maeA lpd^{P05G}$	lpd^{E205G} introduced by MAGE in the NADPH-auxotroph $\Delta maeA$	This work
NADPH-auxotroph $\Delta maeA lpd^{E205A}$	lpd^{E205A} introduced by MAGE in the NADPH-auxotroph $\Delta maeA$	This work
NADPH-auxotroph $\Delta maeA lpd^{E205A E366K}$	$lpd^{E205A E366K}$ introduced by MAGE in the NADPH-auxotroph $\Delta maeA$	This work

558

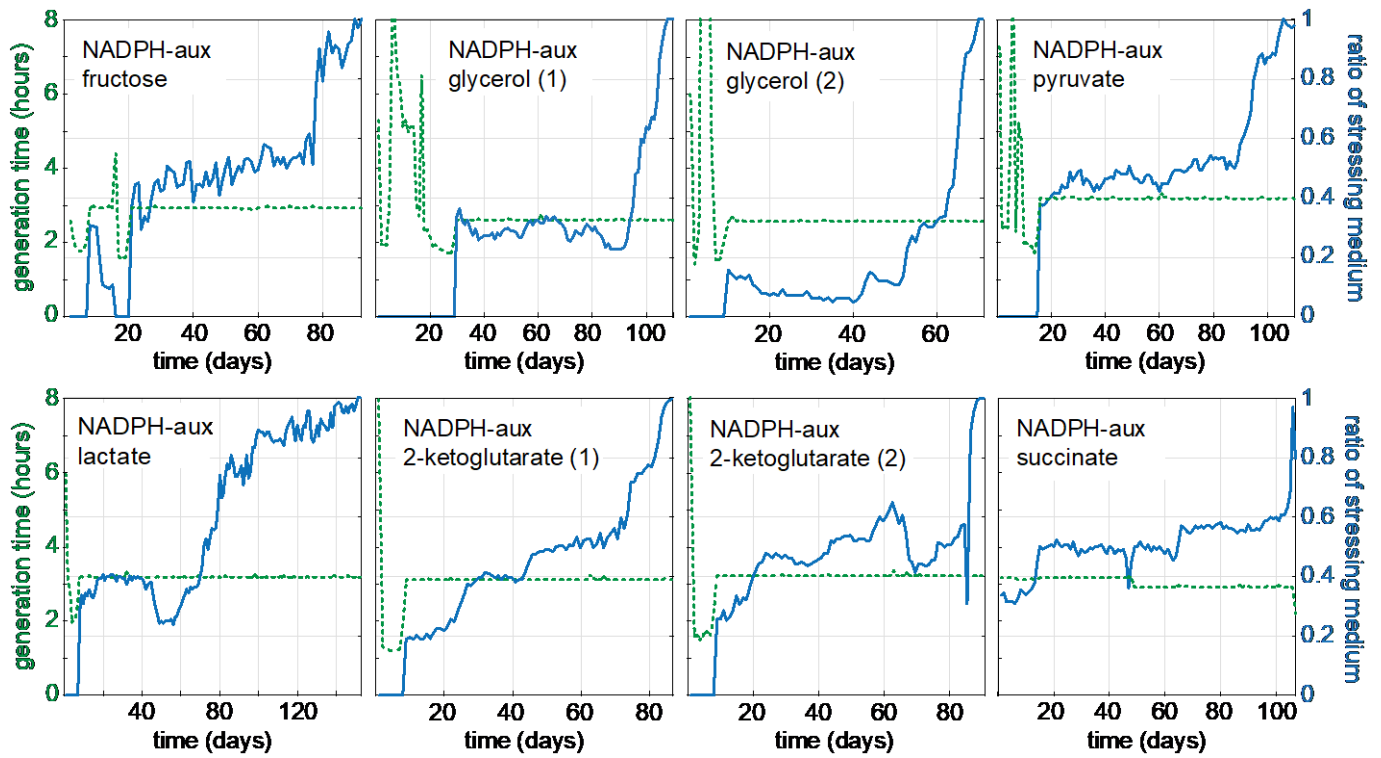
559 **Figures**



560

561 **Figure 1:**

562 Central carbon metabolism of *E. coli*. NADP⁺ reducing reactions, which were deleted to construct the
 563 NADPH-auxotroph strain are shown by red crossed orange arrows and red underlain enzyme name. The
 564 green arrow indicates gluconate dependent NADPH generation. Blue arrows combined with yellow
 565 underlain enzyme names highlight NAD⁺-dependent oxidoreductases and potential candidates for a
 566 cofactor change from NAD⁺ to NADP⁺ in evolution experiments. Highlighted in purple are carbon sources
 567 used in evolution experiments. Abbreviations of enzymes: Zwf, glucose 6-phosphate dehydrogenase; Gnd,
 568 gluconate 6-phosphate dehydrogenase; GldA, glycerol dehydrogenase; GpsA, glycerol 3-phosphate
 569 dehydrogenase; GapA, glyceraldehyde 3-phosphate dehydrogenase; PntAB, membrane bound
 570 transhydrogenase; SthA, soluble transhydrogenase; LdhA, lactate dehydrogenase; Lpd, dihydrolipoamide
 571 dehydrogenase; MaeB, NADP⁺-dependent malic enzyme; MaeA, NAD⁺-dependent malic enzyme; Mdh,
 572 malate dehydrogenase; Icd, isocitrate dehydrogenase.



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Figure 2:

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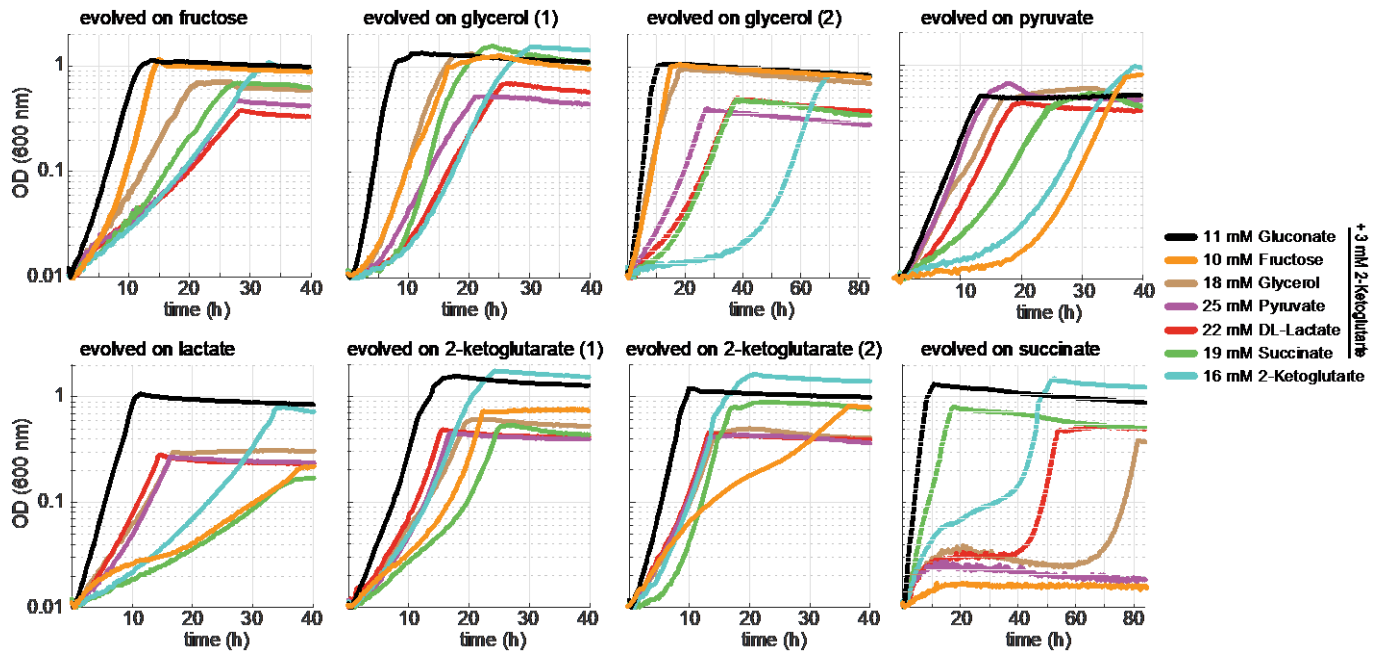
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Evolution of the NADPH-auxotroph strain for growth with either fructose, glycerol, pyruvate, lactate, 2-ketoglutarate, or succinate in the absence of gluconate. For each carbon source 2 independent cultures were subjected to a medium swap regime in GM3 devices (see methods section). Shown are the eight cultures which evolved to growth in stressing medium. Blue lines show the ratio of stressing medium over relaxing medium. Stressing media contained 5 mM 2-ketoglutarate plus one of the following carbon sources: D-fructose (10mM), succinate (17 mM), pyruvate (25 mM), lactate (25 mM), glycerol (20mM), 2-ketoglutarate (20mM final)) (right axes). Relaxing media were composed as stressing medium supplemented with 5 mM D-gluconate. Generation times are indicated by the green dashed lines (left axes).



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Figure 3:

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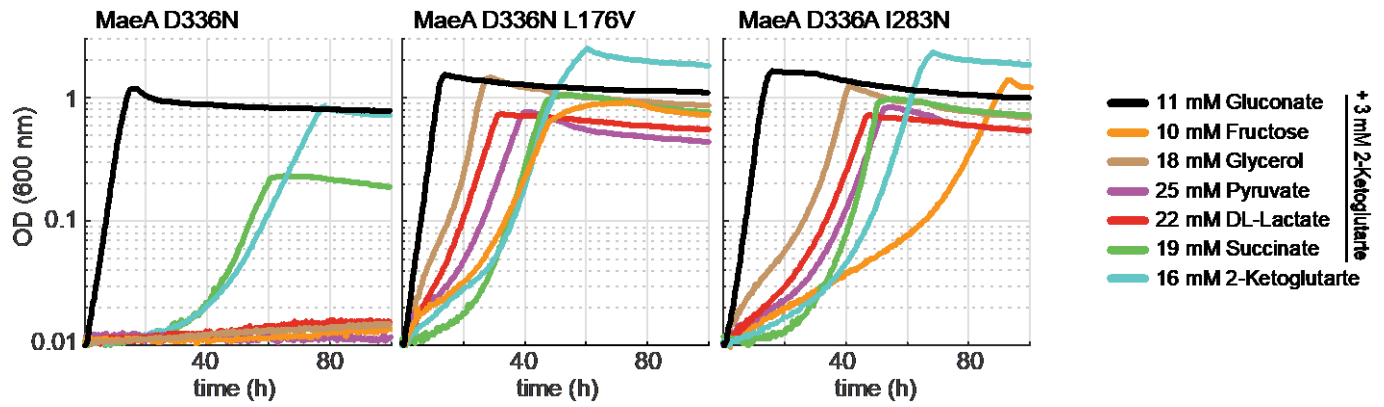
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Growth curves of isolates obtained from cultures adapted in GM3 to proliferate without gluconate on either fructose, glycerol, pyruvate, lactate, 2-ketoglutarate, or succinate, each supplemented with 5 mM 2-ketoglutarate. Growth of the isolates was determined on 11 mM gluconate, 10 mM fructose, 18 mM glycerol, 25 mM pyruvate, 22 mM D/L-lactate, 19 mM succinate (all supplemented with 3 mM 2-ketoglutarate), and 16 mM 2-ketoglutarate. Growth curves were recorded in triplicates, showing similar growth ($\pm 5\%$).

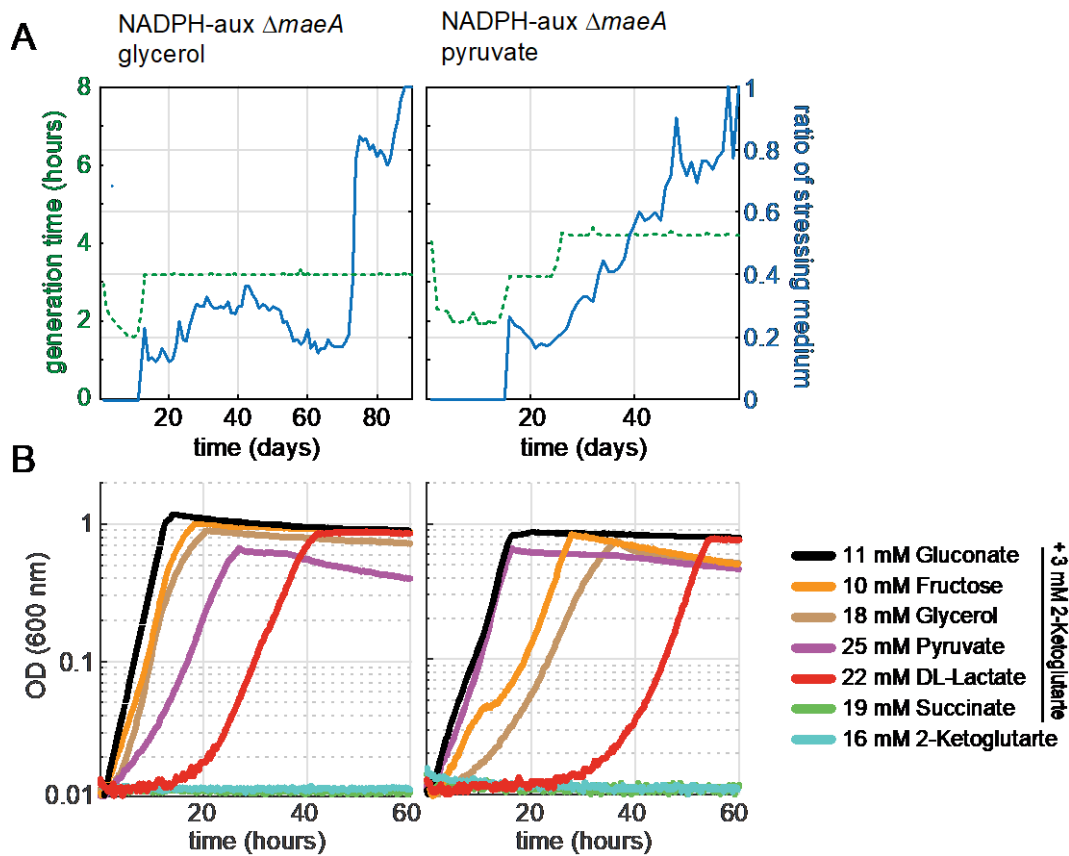


596
597 Figure 4:

598 Growth of NADPH-auxotroph strain derivatives carrying mutations in *maeA* (D336N, D336N + L176V,
599 D336A + I283N) introduced by MAGE. Growth was determined on 11 mM gluconate, 10 mM fructose, 18
600 mM glycerol, 25 mM pyruvate, 22 mM D/L-lactate, 19 mM succinate (all supplemented with 3 mM 2-
601 ketoglutarate), and 16 mM 2-ketoglutarate. Growth curves were recorded in triplicates, showing similar
602 growth ($\pm 5\%$).

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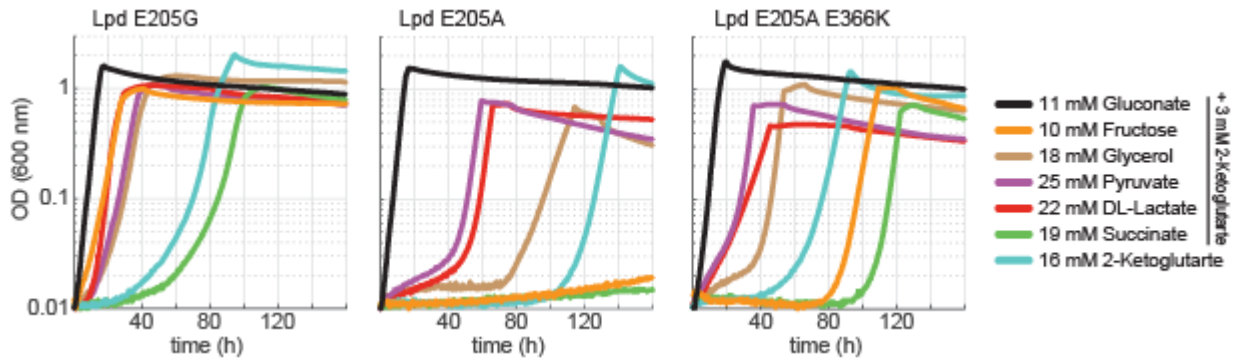


605
606 **Figure 5:**

607 Evolution of the NADPH-auxotroph $\Delta maeA$ strain for growth on glycerol or pyruvate in the absence of
608 gluconate. For each carbon source 2 independent cultures were subjected to a medium swap regime in
609 GM3 devices (see methods section). For both carbon sources, one of the two cultures evolved to growth
610 in stressing medium. **A** Evolutionary kinetics of the cultures in the GM3 device. The ratio of stressing
611 medium (5 mM 2-ketoglutarate plus glycerol (20mM) or pyruvate (25 mM)) over relaxing medium (same
612 composition as stressing medium plus 5 mM D-gluconate) is shown by the blue line (right axes).
613 Generation times are indicated by the green dashed lines (left axes). **B** Growth of isolated mutants was
614 determined on 11 mM gluconate, 10 mM fructose, 18 mM glycerol, 25 mM pyruvate, 22 mM D/L-lactate,
615 19 mM succinate (all supplemented with 3 mM 2-ketoglutarate), and 16 mM 2-ketoglutarate. Growth
616 curves were recorded in triplicates, showing similar growth ($\pm 5\%$).

617

618



620 **Figure 6:**

621 Growth of the NADPH-auxotroph Δ *maeA* strain derivatives mutated in *lpd* (E205G, E205A, E205A +
622 E366K) using the MAGE protocol (see methods section). Growth was determined on 11 mM gluconate,
623 10 mM fructose, 18 mM glycerol, 25 mM pyruvate, 22 mM D/L-lactate, 19 mM succinate (all supplemented
624 with 3 mM 2-ketoglutarate), and 16 mM 2-ketoglutarate. Curves were recorded in triplicates, showing
625 similar growth (\pm 5%).

626

627 **Supplementary material**

628 Figure S1: Michaelis-Menten kinetics of MaeA variants. Data represents mean values +/- SD from three
629 independent experiments (n = 3).

630

631 Figure S2: Michaelis-Menten kinetics of LPD variants. Data represents mean values +/- SD from three
632 independent experiments (n = 3).

633

634 Table S1: Citrate synthase activity.

635

636 Table S2: List of DNA oligo primers used in this study. *thioester bound

637

638 Table S3. Plasmids constructed for protein purification

639

640 **Supplementary Methods**

641 **GltA expression and purification.** The His-tagged WT and L150R-mutated GltA proteins were expressed
642 in *E. coli* BL21 DE3 Codon+ (Invitrogen). Cells in Terrific broth containing 100 µg/mL carbenicillin were
643 grown at 37 °C until they reached an OD₆₀₀ = of 0.8-1 upon which expression for 16 h at 20 °C was induced
644 by addition of 500 µM IPTG. Cells were harvested by centrifugation for 30 min at 10000g at 4°C. Cell
645 pellets were frozen at -80°C for one night. Thawed cells were then suspended in 3 ml of Buffer A (50 mM
646 HEPES, 50 mM NaCl, 30 mM imidazole, 10% glycérol, pH 8.0) and incubated with Pefabloc and Lysonase
647 for 20 min then lysed by sonication. The lysate was clarified at 12000 g for 30 min at 4 °C. The supernatant
648 was loaded onto a pre-equilibrated Ni-NTA minicolumn (QIAGEN) and washed thrice with Buffer A. The
649 protein was eluted in elution buffer B (50 mM HEPES, 50 mM NaCl 250 mM imidazole, 10 % glycerol),
650 collected, pooled and desalted on a Amicon Ultra-4 10kD column in buffer C (50 mM HEPES 50 mM NaCl
651 10 % glycerol). The protein was frozen and stored at -80°C if not immediately used for assays.

652

653 **Measurement of GltA specific activity.** Citrate synthase activity was determined by measuring the initial
654 rate of reaction at 412 nm by means of the DTNB method [1]. Reactions were conducted in 100 mM Tris
655 pH 8, 200 µM DTNB, sub-saturating concentrations of acetyl-CoA (200 µM) and oxaloacetate (100 µM)
656 and with or without the addition of NADH (200 µM) and KCl (100 mM). Specific activity (µmole min⁻¹ mg⁻¹)
657 was determined.

658 **Supplementary reference**

659 1. Moriyama, T. & Srere, P. A. (1971) Purification of rat heart and rat liver citrate synthases. Physical,
660 kinetic, and immunological studies, *J Biol Chem.* **246**, 3217-23.

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