1 Review

2 **Evolutionary engineering of *Corynebacterium glutamicum***

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Abstract

A unique feature of biotechnology is that we can harness the power of evolution to improve process performance. Rational engineering of microbial strains has led to the establishment of a variety of successful bioprocesses, but is hampered by the overwhelming complexity of biological systems. Evolutionary engineering represents a straightforward approach for fitness-linked phenotypes (e.g. growth or stress tolerance) and has been successfully applied to select for strains with improved properties for the particular industrial application. In recent years, synthetic evolution strategies enabled selection for increased small molecule production by linking metabolic productivity to growth as a selectable trait. In this review, we will summarize evolutionary engineering strategies performed with the industrial platform organism *Corynebacterium glutamicum*. An increasing number of recent studies highlight the potential of adaptive laboratory evolution (ALE) to improve growth or stress resistance, implement the utilization of alternative carbon sources or improve small molecule production. Advances in next-generation sequencing and automation technologies will foster the application of ALE strategies to streamline microbial strains for bioproduction and enhance our understanding of biological systems.

1 Introduction

The Gram-positive actinomycete *Corynebacterium glutamicum* has a long-standing history in microbial biotechnology, following its isolation in 1957 by Kinoshita et al. due to its potential to secrete large amounts of L-glutamate.[1, 2] In the last decades, *C. glutamicum* has been engineered for the production of a variety of value-added products, including amino acids, organic acids, polymer precursors, aromatic chemicals and proteins. These achievements are mainly the result of classical metabolic engineering and have been summarized in a number of recent reviews.[3–6]

Systems biology and metabolic engineering take a rational approach at strain development and are aided by the high level of information available for *C. glutamicum*, including a comprehensive overview on the transcriptomic map and detailed information on metabolic pathways and their regulation.[5, 7, 8] Strain construction is accelerated by an ever increasing amount of synthetic biology tools, such as CRISPR/Cpf1[9] and CRISPRi[10], which is covered by several reviews.[5, 11, 12] Transcription factor-based biosensors, which enable the visualization of cellular productivity at the single cell level, have proven a powerful tool for the high-throughput screening of strain or enzyme libraries and for single cell analysis of producer strains.[13–16]

While rational approaches are indispensable for the development of industrial platform strains in general, their application is often hampered by the overwhelming complexity of biological systems. Even for well-established model systems, like *C. glutamicum* or *Escherichia coli*, we are still not able to quantitatively predict responses to environmental changes or genetic perturbations. However, nature itself provides us with the most powerful approach for the optimization of biological systems: evolution. Evolutionary engineering, also known as adaptive laboratory evolution (ALE), has a long-standing history in the development of microbial production strains and has been heavily applied to improve growth rates, stress or product tolerance.[17, 18] ALE requires microbial growth, which is

63   usually facilitated by repetitive batch cultivations or continuous cultivations, which can be

64   simple (e.g. shake flasks) or more advanced (e.g. pH auxostats).[17, 19] When combined with

65   next-generation sequencing and comprehensive omics analysis, ALE strategies can be used

66   to obtain better production strains, but also to identify non-intuitive targets for strain

67   engineering and ultimately to gain a comprehensive understanding of biological pathway

68   regulation. Furthermore, advances in automation of laboratory workflows and microbial

69   phenotyping are fostering a tremendous increase in throughput and efficiency.

70   This review gives an overview of evolutionary engineering of *C. glutamicum*, including

71   approaches to improve growth on glucose or alternative carbon sources, stress tolerance,

72   or small molecule production by growth coupling strategies and biosensor guided evolution

73   (Figure 1, examples summarized in Table 1). We conclude by highlighting the potential of a

74   smart combination of synthetic biology and workflow automation for the next-generation

75   evolutionary engineering approaches.

76

77   **2 Accelerating growth on glucose**

78   The time needed for biomass production represents a key factor in the economic success of

79   biotechnological processes. While ALE approaches have been intensively explored to

80   accelerate growth of *E. coli*[20, 21], so far only two recent studies reported on the evolution of

81   *C. glutamicum* towards higher growth rates on glucose minimal medium (Table 1).[22, 23]

82   Interestingly, these studies revealed similar key targets for enhancing metabolic flux and

83   increasing substrate uptake rates for both species indicating that microorganisms follow

84   similar principles to adapt to fast growth.

85   Among the key targets improving growth on glucose, we recently described mutations in

86   the *pyk* gene, encoding the pyruvate kinase (PK). Re-introduction of identified SNPs into the

87   parental strain caused a significant decrease in PK activity (T12A, and A20V).[22]

88   Furthermore, a mutation of alanine 271 to threonine (A271T) was enriched in one cell line.

89   Intriguingly, a PK enzyme carrying this mutation was previously described as being

90   desensitized towards the allosteric activator fructose 1,6-bisphosphate (FBP), resulting in

91   a reduced PK activity upon accumulation of the glycolytic intermediate FBP.[24] Mutations

92   in the *pyk* gene were also identified in the prominent *E. coli* long-term evolution experiment

93   that lasted for more than 25 years, and in a recent study by LaCroix et al. where cells were

94   evolved towards fast growth on glucose.[25, 26] It is interesting to note that fast proliferating

95   eukaryotic cells, like embryonic stem cells and cancer cells, also harbour a less active PKM2

96   isoform of the PK enzyme.[27, 28] From these findings, we can conclude that lowering the PK

97   activity appears to be a conserved strategy of fast growing cells that need to exploit large

98   amounts of glucose for anabolic pathways.

99   Further key targets identified by Pfeifer et al. were mutations in *fruK*, which encodes for 1-

100   phosphofructokinase (Pfk1). In a previous study, deletion of the *fruK* gene was actually

101   reported to enhance glucose uptake of *C. glutamicum*. The authors speculated that this was

102   the result of an accumulation of F1P relieving *ptsG* repression via the SugR regulator.[29]

103   However, further analyses are required to understand the impact of Pfk1 on the sugar

104   phosphate pool and its impact on regulatory networks in these strains.

105   The *corA* gene, coding for a putative Mg^{2+}/Co^{2+} transport protein[30], represented an

106   unexpected mutational hotspot in the *C. glutamicum* long-term evolution and was also

107   identified, but not discussed, in the *E. coli* experiment of LaCroix et al..[22, 25] Mg^{2+} limitation

108   was previously described to increase glucose consumption rates and metabolic flux of *E. coli*

109   in the stationary phase. Here, the authors assumed that reduced Mg^{2+} levels might cause a

110   block in pyruvate dehydrogenase (PDH) activity leading to an accumulation of upstream

111   intermediates like PEP, which stimulate glucose uptake.[31] However, the availability of

112   magnesium ions is known to affect a variety of different enzymes and to have an impact on

113   the stability of polyphosphates.[30] For example, the biologically active form of ATP is a

114   complex with Mg^{2+}. Based on these findings, we can conclude that mutation of *corA* linked

to magnesium limitation appears to be a prime target to enhance glucose uptake, but the underlying mechanism is not fully understood and requires further theoretical and experimental attention.

In another recent ALE experiment, *C. glutamicum* cultures were evolved for about 1500 generations in defined media with glucose.[23] The evolved cell line was found to lack large parts of the CGP3 element.[32] Deletion of the CGP3 prophage was also revealed as a competitive fitness advantage in the other *C. glutamicum* ALE study, where a competitive growth experiment showed that a prophage-free strain (MB001) outcompetes the wild type.[22] Besides the lack of the prophage element, the authors found mutations in the *gntR1* gene, encoding a repressor of gluconate catabolism and an activator of *ptsG*[33], and in *ramA*, encoding a LuxR-type regulator, which inherits a global role in the coordination of central metabolism.[34] Re-introduction into the wild type background revealed that both mutations significantly increased the growth rate and glucose consumption rate (Table 1).[23] Metabolomic flux and transcriptome analysis showed an increased flux through the pentose phosphate pathway but did not indicate significant rewiring of the central metabolic network, which is in agreement with similar studies in *E. coli*.[35]

3 Improving performance under industrial conditions

Evolutionary engineering provides an efficient means to improve the performance of microbial strains under harsh industrial conditions and/or to realize the utilization of second and third generation feedstock.[36, 37] Recent studies have employed ALE strategies for both aspects, and while the division is not always clear (e.g. in the case of methanol tolerance and utilization), they will be discussed separately in this section (Table1).

3.1 Improving stress tolerance

In an economically optimized bioprocess, microorganisms should be able to grow at low-purity feedstock (lower substrate costs) and at favorable process conditions (e.g. at a higher

141 temperature, to reduce cooling costs). An improved heat tolerance was reported for *C.*

142 *glutamicum* after a repetitive batch ALE (rbALE) of 65 days, in which the temperature was

143 gradually increased from 38 °C to 41.5 °C.[38] Sequencing of three evolved isolates revealed

144 a surprisingly high amount of 295 point mutations and 2 genomic deletions in total. This

145 was caused by an acquired mutator phenotype, which resulted in a mutation rate 40-fold to

146 80-fold higher than that of the parental strain. A genetic basis for this phenotype was not

147 found. However, the combined reengineering of the mutations g*lmU*(E295K) and

148 *otsA*(R328H) resulted in an improved heat tolerance and increased the maximum specific

149 growth rate at 40.7 °C from 0.27 ± 0.02 h^{-1} to 0.33 ± 0.03 h^{-1}.

150 Two different studies applied rbALE to increase tolerance for inhibitors present in corn

151 stover hydrolysate[39] and for methanol, which is an impurity found in certain glycerol waste

152 streams.[40] A fixed amount of inhibitor was added, and cells were selected for growth. The

153 corn stover evolved strain showed a higher degradation rate of the inhibitors furfural, HMF,

154 vanillin, syringaldehyde, 4-hydroxybenzaldehyde and acetic acid.[39] When grown on corn

155 stover hydrolysate, the evolved strain also showed a 68% higher glutamic acid titer and

156 35% higher yield than the parental strain. Increased glucose consumption rate probably

157 resulted from a higher *ptsI* expression, which codes for a component of the

158 phosphotransferase system (PTS). In the methanol study, tolerance was increased by using

159 media with 50 mM methanol.[40] The evolved strain showed a higher growth rate under

160 methanol concentrations between 500 and 2000 mM. Sequencing identified 29 mutations

161 and combined engineering of two of them, *metY*(A165T) and *cat*(Q342*), increased the

162 methanol tolerance of the parental strain to that of the evolved strain. Interestingly, both

163 the reverse engineered strain and the evolved strain lost the ability to grow on ethanol. This

164 showed that, in contrast to an observed beneficial cross-tolerance for isobutanol after ALE

165 for increased temperatures[38], a single-target ALE approach is typically associated with

166 significant negative trade-off effects.

167

3.2 Increasing feedstock flexibility

The utilization of alternative, non-native carbon sources is key to the implementation of non-glucose based second or third generation processes in modern biotechnology, which provides a strong incentive to engineer industrial strains that utilize most of the components present in these substrate feeds. Attractive targets to realize a flexible feedstock concept are, for example, carbon sources derived from complex polymers (e.g. starch, cellulose or xylan), di- and monosaccharides (e.g. arabinose or xylose) and C1 compounds, like methanol.[36, 37] If the microbe has no native capacity to consume the particular substrate of interest, targeted engineering can provide an initial, non-optimal strain which can subsequently be improved by ALE. This combinatorial strategy was applied in three recent publications that describe improved growth on xylose, cellobiose and methanol (Table 1).[41–43]

Two different rbALE approaches were described for co-utilization of xylose and methanol[41] or xylose and cellobiose[42]. In the xylose and methanol study, targeted engineering was first applied to obtain a strain that required both xylose and ribose for growth. After the first ALE, this strain showed an increased maximum specific growth rate, from 0.03 h^{-1} to 0.15 h^{-1}. Then, the evolved strain was rationally engineered for the co-utilization of xylose and methanol and submitted to another round of ALE. After 206 days, the evolved strain was able to grow, but the growth rate of 0.03 h^{-1} is still far away from industrial applications. Interestingly, the authors describe the *metY*(G1256A) mutation; another mutation in the same gene (*metY*(A165T)) was also found in the methanol tolerance study.[40] An elegant approach to realize methanol-essential growth was recently reported for *E. coli*.[44] The assimilation of methanol and gluconate was stoichiometrically coupled in a strain that had the ribulose monophosphate cycle. This study highlights the

192   potential of metabolic re-routing for the successful implementation of synthetic

193   methylotrophy in established industrial platform organisms.

194   In the xylose and cellobiose study, targeted engineering was first applied to obtain two

195   different strains that could grow on cellobiose as sole carbon source.[42] ALE was performed

196   until growth and cellobiose consumption did not improve anymore. Sequencing analysis

197   revealed a high number of mutations (36 and 300), which could not be explained. After the

198   ALE for cellobiose, the xylose consumption pathway was introduced and co-utilization of

199   xylose and cellobiose was shown.

200   An improved growth rate on only D-xylose was shown by Radek and co-workers, using a

201   rationally engineered strain that was subsequently evolved by high throughput rbALE for

202   35 generations in only 13 days.[43] A best performer showed more than a doubling of the

203   maximum specific growth rate, compared to the parental strain (0.26 ± 0.02 h^{-1} and $0.10 \pm$

204   0.02 h^{-1}, respectively). Sequencing analysis revealed putative loss-of-function mutations in

205   the transcriptional regulator IolR, involved in myo-inositol uptake and degradation, and

206   cg3388, encoding for a putative transcriptional regulator. In a subsequent study, the

207   functional loss of IolR was shown to result in an upregulation of the gene *iolT1* (encoding

208   for the myo-inositol/proton symporter IolT1), and additional reengineering could prove

209   the importance of IolT1 for xylose uptake.[45] Interestingly, the identified mutations are

210   different from those identified by the xylose-methanol ALE study.[41] Altogether, these

211   examples once more highlight the importance of subsequent re-engineering of key

212   mutations to ultimately expand our knowledge on biological systems.

213

214   **4 Metabolic engineering to guide evolution**

215   Combining evolutionary engineering with rationally engineered system perturbations is a

216   promising approach for the identification of metabolic traits that can be beneficial for target

217   molecule production.[46] Gene knockouts are frequently used to investigate the metabolic

218 and regulatory function of gene products[47, 48], but also for pre-defining the cell´s metabolic

219 network with regard to precursor supply for biotechnological applications (Figure 2).[49]

220 Growth defects that result from systems perturbations by introduction of gene knockouts

221 provide a guided selection pressure towards fitness recovery. This can lead to alternative

222 re-optimized metabolic states, for example by harnessing the underground metabolism by

223 building on promiscuous enzyme activity of the particular species.[47, 50]

224 Schwentner et al. used the inactivation of anaplerotic reactions to evolve an alternative

225 producer platform for the amino acid L-valine.[51] This effort was motivated by the fact that

226 metabolic engineering of *C. glutamicum* for L-valine production is typically based on

227 complete inactivation or downregulation of the pyruvate dehydrogenase complex

228 (PDHC).[52, 53] Schwentner et al. evolutionary engineered an alternative route for increased

229 pyruvate supply by inactivating the genes encoding the PEP and pyruvate carboxylase

230 (PEPCx, *ppc* and PCx, *pyc*). The resulting strain was unable to grow on glucose as sole carbon

231 source since the replenishment of the oxaloacetate pool was impaired.[54]. Whole genome

232 sequencing of the evolved strains revealed *icd*, encoding isocitrate dehydrogenase (ICD), as

233 a consistent mutation target in all evolved strains. Re-engineering of identified key

234 mutations in *icd* revealed an upregulation of the glyoxylate shunt[55] as an alternative route

235 to replenish the oxaloacetate pool in the Δ*pyc* Δ*ppc* background.[51] However, further studies

236 are necessary to identify the molecular mechanisms underlying the activation of the

237 glyoxylate shunt as a secondary effect of reduced ICD activity. In accordance with

238 assumptions of Baumgart et al., the authors postulated that the reduced ICD activity and the

239 resulting accumulation of isocitrate promote activation of the glyoxylate shunt.[51, 56] Based

240 on a 1.9-fold increased intracellular L-valine level and a predicted increased flux through

241 the PDHC, Schwentner et al. suggested that the re-engineered strains represent promising

242 candidates for L-valine producers. Indeed, plasmid-based overexpression of L-valine

243 biosynthesis genes (pJC4*ilvBNCE*) led to a fourfold-enhanced L-valine product yield in

244 comparison to the wild type.[51]

245 The study of Schwentner and co-workers demonstrated the potential of guided evolution

246 strategies to improve glucose conversion to value-added compounds like L-valine. In

247 contrast, the previously discussed guided evolution approaches of Lee et al. and Tuyishime

248 and colleagues with pre-engineered *C. glutamicum* cells resulted in strains showing efficient

249 co-utilization of alternative substrates such as xylose and methanol[41] or xylose and

250 cellobiose[42] demonstrating the broad application spectrum of guided evolution strategies

251 (Table 1).

252 McCloskey and colleagues intensively studied growth recovery of different *E. coli* knockout

253 strains during ALE at systems level. The combination of ALE and multi-omics technologies

254 provided deep insights into the versatile pathways allowing re-balancing of the metabolite

255 and redox state.[47, 48] Further, growth coupling approaches with *E. coli* used the strong

256 evolutionary driving force towards redox homeostasis for increasing fermentative

257 production of lactic acid[49], 1-butanol[57] and linear higher alcohols[58] by inactivating

258 competing NADH oxidizing enzymes. These examples highlight, that not only the

259 experimental conditions but also the genetic strain background need to be streamlined for

260 the particular aim of the ALE.

261

262 **5 The benefit of automation**

263 A number of different ALE approaches are already established that can roughly be classified

264 according to the cultivation conditions (i.e., chemostat vs. repetitive batch culture),

265 experimental throughput (i.e., lab-scale vs. micro-scale bioreactor systems) and degree of

266 automation (i.e., manual vs. autonomous operation). See[17, 59] for a detailed discussion of

267 the advantages and disadvantages of the chemostat and repetitive batch approach.

In recent years, the rbALE approach has become more popular due to its lower costs for operation, simpler experimental implementation and easier expandability. In short, individual cultivation batches are sequentially inoculated until a termination point (e.g., no further improvement in fitness criterion) is reached. Adaptation and diversification of cells during rbALE depends on the applied selection pressure, which can greatly vary between conditions of feast (i.e., exponential growth) and famine (i.e., stationary phase).

In many cases, selection focuses on higher maximum growth rates, which are best supported by keeping the cells under balanced growth conditions throughout the whole rbALE experiment. The time window of exponential growth depends on the inoculation density, the lag-phase and the specific growth rate and should become smaller with increasing number of beneficial mutations leading to faster cell population growth and earlier consumption of the usually fixed nutrient resources. Therefore, the application of conventional shaking flasks and offline OD measurements for monitoring biomass growth does not interface with appropriate standardization.

To overcome this limitation several microbioreactor systems are available that are based on shaken microtiterplates (MTPs), e.g. "GrowthProfiler" (EnzyScreen), "Bioscreen C" (Oy Growth Curves) or "BioLector" (m2p-labs), and employ (quasi)-continuous biomass measurement via integrated image analysis, optical density or backscatter (see[60] for an comprehensive overview). Additionally, MTPs enable a higher throughput to perform replicate ALE experiments under identical conditions[43, 61] and thus provide access to important quantities for analyzing the evolutionary process, e.g. in terms of beneficial mutation rates and the occurrence of fixed or converged mutations.[62] Noteworthy, the typical 1 mL working volume of standard MTP's is also sufficient to carry out further in-depth characterizations of evolved strains using available spectrophotometric assays[63] and quantitative omics technologies.[64]

293 However, when applied as stand-alone devices for rbALE, the above systems still require on

294 the manual passaging of cells from one MTP-well to another. Consequently, there is a high

295 demand for automation to optimally balance available resources (i.e., personnel, material,

296 and time) with the expected outcomes (i.e., strains with improved properties, new

297 knowledge on cellular metabolism, and targets for rational strain design).

298 As a first step in this direction, we recently established a workflow to perform rbALE

299 experiments in an automated manner.[43] By building on an existing microbial phenotyping

300 platform (i.e., Mini Pilot Plant integrating a BioLector system with liquid handling

301 robotics[65]), the workflow covers essential steps for autonomous rbALE, including

302 preparation of different media compositions, their cool temporary storage and the repeated

303 inoculation of individual MTP-cavities in the BioLector. The latter is supported by various

304 online measurements (i.e., backscatter, pO2, pH and fluorescence) and ensures an

305 instantaneous recording of metabolic adaptation events during ALE experimentation

306 (Figure 3.2). Moreover, the setup enables dynamic adjustments of passage sizes for keeping

307 genetic diversity, but (if required) also provides access to the full spectrum of applicable

308 selection pressures.

309

310 **6 Evolutionary engineering of small molecule production**

311 Redirecting cellular metabolism towards the production of small molecules is challenging,

312 because metabolic pathways have been evolved for tight control and our understanding of

313 their regulation is incomplete.[66] For ALE approaches, the difficulty lies in applying a

314 pressure that selects for production of the compound of interest. While traditional

315 approaches select for suppressor mutants, the development of transcription factor-based

316 biosensors enabled a direct coupling of product formation to an easily selectable output,

317 which resulted in the first reports of "synthetic evolution".[16, 67–69] For *C. glutamicum*, ALE

318   for increased small molecule production has been shown for fatty acids, L-ornithine,

319   putrescine, plasmid based proteins and L-valine (Table 1).

320

321   **6.1 Selection for suppressor mutants**

322   Early efforts on the improvement of small molecule production focused on the application

323   of random mutagenesis (UV- or chemical mutagenesis) and on selection schemes based on

324   the resistance to antimetabolites or product analogs inhibiting growth. Key enzymes of

325   biosynthetic pathways (e.g. amino acid biosynthesis) are typically feedback-inhibited by the

326   particular end-product of the pathway. Application of product analogous antimetabolites

327   enabled the efficient isolation of mutations causing feedback-resistance of key enzymes.[2]

328   Two groups have recently published work on improving small molecule production by

329   selecting for suppressor mutants. Takeno and co-workers used two different chemicals,

330   Tween40 and cerulenin, to select for mutants with a higher fatty acid production, using

331   multiple selection steps on agar plates.[70] In contrast, Jiang and co-workers applied rbALE

332   in media containing the desired product, L-ornithine, to select for mutants resistant to

333   product inhibition.[71] In both studies the final product titer could be improved with the

334   evolved strain, from 3.21 ± 0.06 mg l^{-1} to 279.95 ± 8.50 mg l^{-1} for fatty acids[70], and from

335   10.2 ± 0.2 g l^{-1} to 13.6 ± 0.5 g l^{-1} for L-ornithine.[71] While Jiang et al. did not perform whole

336   genome sequencing, Takeno et al. reported 3 mutations in genes responsible for fatty acid

337   synthesis. Takeno and co-workers repeated the selection step on cerulenin plates, and

338   identified one additional mutation.[72] Re-engineering of this mutation in the previously

339   evolved strain resulted in a 1.2-fold higher oleic acid titer. This shows how iterative

340   selection of a parental strain can be used to find different mutations that have an additive

341   effect on productivity. In contrast, the L-ornithine evolved strain was further engineered for

342   putrescine production.[73] Random mutagenesis in combination with rbALE in media

343   containing putrescine resulted in a strain showing a two-fold higher final putrescine titer

344 (111.42 ± 2.56 mM). Sequencing analysis revealed 78 SNPs, further analysis suggested that

345 a decrease in activity of the α-ketoglutarate decarboxylase OdhA and an increase in the

346 major facilitator superfamily permease CgmA, a putative putrescine permease, contributed

347 to a higher putrescine production.

348

349 **6.2 Biosensor guided evolution**

350 Biosensors can greatly increase the throughput and selectivity for ALE, and thereby

351 accelerate strain development (Figure 2). Initially, two studies reported on transcription

352 factor-based biosensors that were designed to induce *eyfp* expression in response to higher

353 intracellular amino acid concentrations in *C. glutamicum*. The pSensLys biosensor detects

354 amino acids with a basic side chain[13] and the Lrp biosensor detects L-methionine and

355 branched chain amino acids.[14] Both studies showed that random mutagenesis coupled to

356 FACS could be used to select for cells with higher productivity, illustrating the use of

357 biosensors to screen for producers strains. However, applying random mutagenesis results

358 in many mutations. Binder et al reported 268 SNPs [13] and a similar study reported 83 SNPs

359 [74]. This high number of mutations complicates the identification of the causal ones.

360 In a recent study, Mahr and co-workers describe a biosensor guided ALE approach based

361 on the native mutagenesis rate, by applying multiple rounds of selection using the Lrp

362 biosensor (Table 1).[75] The L-valine producer strain *C. glutamiucm* Δ*ace*, which produces

363 valine in stationary phase, was used as parental strain. Five iterative evolution steps were

364 performed and in each step the top 10% fluorescent cells were transferred by FACS. After

365 only five iterations, a single isolate showed a two-fold increase in L-valine production

366 compared to the parental strain. Only seven mutations were identified after whole genome

367 sequencing. This is in large contrast to the higher number of mutations found when random

368 mutagenesis is applied. The identified mutation *ureD*(E188*) resulted in a truncated

369 version of the urease accessory protein and was shown to increase the final L-valine titer

370 (44.2 mM for evolved strain) by more than 100%.[75] A low amount of mutations was also

371 the result of a simple biosensor ALE experiment, in which iterative FACS selection was done

372 with a strain containing a plasmid based *egfp*. Only one mutation was identified, a loss of

373 function of *parB*, which resulted in 10-fold higher plasmid copy numbers.[76] Further

374 examples for biosensor guided evolution approaches in *E. coli* are the coupling of mutation

375 rate to small molecule production[69] and the selection of improved producers via an

376 antibiotic based positive and negative selection strategy.[77]

377 The mutations identified by ALE can be further investigated by targeted mutagenesis

378 followed by biosensor mediated screening, which was shown for the *argB* gene using

379 pSensLys[78] and for the RBS sequence in *tktA* using the ShiR-based shikimate sensor.[79] An

380 interesting method was described by Binder et al., who designed a rapid recombineering

381 and screening approach, based on the RectT recombinase from prophage Rac and the

382 pSenLys sensor, which was applied to screen for mutations in *murE* with an impact on L-

383 lysine production.[80] Furthermore, studies in *E. coli* have demonstrated the use of

384 multiplexed genome engineering in combination with barcoding to increase mutation rates

385 and enable tracking of mutations.[81, 82] Altogether, these approaches demonstrate the

386 potential of transcription factor-based biosensors for the design of synthetic evolution

387 schemes to enable the improvement of microbial small molecule production.

388
389 **7 Conclusions**

390 In engineering disciplines, the possibility of process improvement via evolution is a feature

391 unique to biotechnology. But what do we learn from the comparison of different ALE

392 endeavours? Evolution of living organisms is shaped by the particular environmental

393 conditions (like media composition, gas supply, etc.), the genetic background and by the

394 mode of cultivation (batch, chemostat, solid media), including passage time and culture

395 volume. For example, LaCroix et al. achieved a significant increase of growth rates in shorter

396 time compared to the Lenski experiment, because batch cultures where propagated during

397 exponential phase rather than inoculated from stationary phase cultures.[20, 25] These

398 examples demonstrate that an ALE setup must be efficiently tailored to the particular

399 scientific aim. However, most studies only focus on one aspect of industrial performance

400 (e.g., productivity or stress tolerance). A more integrative approach could combine several

401 improvement targets into one experiment. Especially the development of biosensors could

402 aid in this field, as they allow for an easy coupling of selecting for producers under industrial

403 conditions. This could even be extended with high-throughput technologies enabling the

404 design of novel ALE strategies (Figure 3).

405 Several recent examples highlight the fact, that ALE or untargeted selection strategies

406 should be complemented by sequencing, and possibly by transcriptome analyses and re-

407 engineering to obtain novel information on enzyme properties or pathway regulation.

408 Especially in the absence of a random mutagenesis step, the amount of mutations is most of

409 the times low enough to allow for reverses engineering and an in-depth characterization of

410 specific mutations. This is in contrast to classical genome breeding approaches where a high

411 number of -induced mutations (e.g. via UV radiation) may result in the accumulation on non-

412 beneficial side mutation having a negative impact on genome stability. Often, different

413 mutations found in different studies can result in an additive effect, but it can be hard to

414 keep track of all described mutations. Recent database efforts such as the AleDB could aid

415 in improving our understanding of metabolic regulations .[62] A more systemic analysis of

416 ALE experiments combined with sophisticated data management will accelerate the

417 identification of causative key mutations and epistatic interactions resulting in improved

418 strain properties.

419

426   **Conflict of interest**

427   The authors declare no financial or commercial conflict of interest.

428

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565

566 **Table 1.** Overview of ALE studies with *C. glutamicum*.

ALE target	Experimental setup	ALE duration	Phenotype of evolved strain	Sequencing/transcriptomics	Proven causal mutations	Reference
Growth						
Accelerating growth on glucose minimal media	Repetitive batch in minimal media containing glucose as sole carbon source	appr. 630 generations	26% higher growth rate (0.67 ± 0.01 h^{-1})	WGS of two independently evolved cell lines, 70 mutations	mutational hotspots in *pyk*, *fruK,* and *corA*	[22]
Accelerating growth on glucose minimal media	Repetitive batch in minimal media containing glucose as sole carbon source	7 months, appr. 1500 generations	42% higher growth rate (0.64 ± 0.01 h^{-1})	WGS after different ALE times, 7 mutations and 1 genomic deletion (CGP3 element)	*gntR1*-E70K and *ramA*-A52V	[23]
Stress tolerance						
Increased growth at high temperatures	Repetitive batch in rich media, gradual increase in process temperature	65 days	Better performance at suboptimal temperatures, increased Tmax (41.5 °C)	WGS of three evolved isolates, deletion of 2 genomic regions, 295 total point mutations	*glmU*-E295K and *otsA*-R328H	[38]
Increased tolerance to inhibitors in corn stover hydrolysate	Repetitive batch in minimal media with corn stover hydrolysate	128 days	Higher degradation rate of several inhibitors, higher growth rate on media containing corn stover hydrolysate	WGS of evolved and parental culture, 7 mutations; transcriptome comparison between evolved and parental strain	Not determined	[39]

Increased tolerance to methanol	Repetitive batch in minimal media containing methanol	50 generations	Improved growth rate on minimal media with 500-2000 mM methanol	WGS of single isolate, 29 mutations found; transcriptome analysis using DNA microarrays	*metY*-A165T and *cat*-Q342*	[40]
Increased tolerance to H_2O_2	Chemostat with a dilution rate of 0.15 h^{-1}, growth on minimal media containing glucose, gradual increase of H_2O_2 concentration to 10 mM	1900 h (approx. 411 generations)	Better performance in media containing 10 mM H_2O_2	No sequencing performed; transcriptome analysis using RNAseq	Not determined	[83]
Substrate expansion						
Improved growth on cellobiose	Repetitive batch in minimal media containing cellobiose	25 days	Improved growth on cellobiose	WGS of two differently evolved strains, 10 shared mutations identified; transcriptome analysis using DNA microarrays	Not determined	[42]
Improved growth on D-xylose	Automated repetitive batch in minimal media containing D-xylose	13 days (35 generations)	2-fold increase in growth rate on D-xylose (0.26 ± 0.02 h^{-1})	WGS of multiple evolved strains	Upregulation of *iolT1*	[43, 45]

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co-utilization of xylose and methanol	Repetitive batch in minimal media with xylose and methanol	206 days (approx. 27 generations)	20 fold increase in growth rate (0.03 h^{-1})	WGS of three evolved isolates, six missense mutations; ^{13}C flux analysis	Not determined	[41]
Metabolic engineering to guide evolution						
Evolution of a PEP and pyruvate carboxylase-deficient strain towards growth on glucose	Repetitive batch in media containing yeast extract and glucose	13, 15 or 33 days	Improved growth rate to 59% of WT (0.23 ± 0.01 h^{-1})	WGS of three independently evolved mutants	*icd*-A94D, *icd*-R453C, and *icd*-G407S	[51]
Increasing small molecule production						
Increased fatty acid production	Sequential selection for suppressor mutants on agar plates containing Tween40 or cerulenin	Multiple days	Increased fatty acid production (322.23 ± 15.09 mg l^{-1})	WGS of best producing isolate, total of four mutations	*fasR*-S20D, *fasA*^{up}-C63G, *fasA*-A2623V and *accD3*-A433T	[70, 72]
Increased putrescine production	Repetitive batch in rich media containing putrescine, after random mutagenesis	11 days	Increased putrescine titer (111.42 ± 2.56 mM)	WGS of best producing isolate, 78 SNPs	Not determined	[73]

|						
Increased L-ornithine production	Repetitive batch in media containing glucose and L-ornithine	70 days	20% higher L-ornithine titer (13.6 ± 0.5 g l^{-1})	Not performed	Not determined	[71]
Biosensor guided evolution						
Increased L-valine production	Repetitive batch in minimal media containing glucose and acetate, selection via biosensor mediated FACS	6 days	higher growth rate (0.41 ± 0.02 h^{-1}) and 63 % increased L-valine titer (57 mM)	WGS of evolved culture and two isolates	*glxR*-T93S, *prpD*-T201I, *rpsP*-D30D and *ureD*-E188*	[75]
plasmid based protein production	Repetitive batch in minimal media containing glucose, selection via biosensor mediated FACS	8 days	Higher plasmid copy number	Only sequencing of plasmid backbone	Several inactivating mutations in *parB*	[76]

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569 **Figure legends**
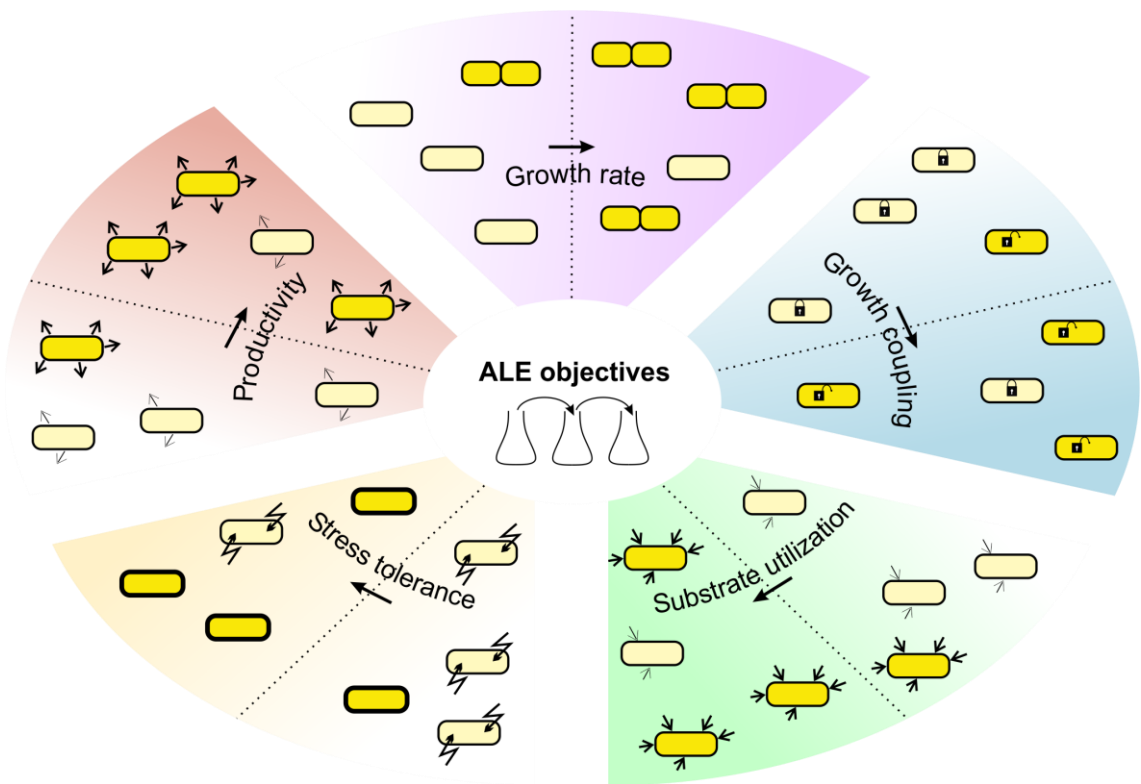
570

571 **Figure 1.**

572 **Overview of different objectives in *C. glutamicum* ALE studies.** ALE has

573 successfully been applied to increase the growth rate[22, 23], substrate utilization[43],

574 stress tolerance[39, 40] and small molecule production.[75] Growth coupling strategies

575 have been used to increase precursor supply and small molecule production.[51]

576

Fluorescence

Antibiotic resistance

Growth

Generations
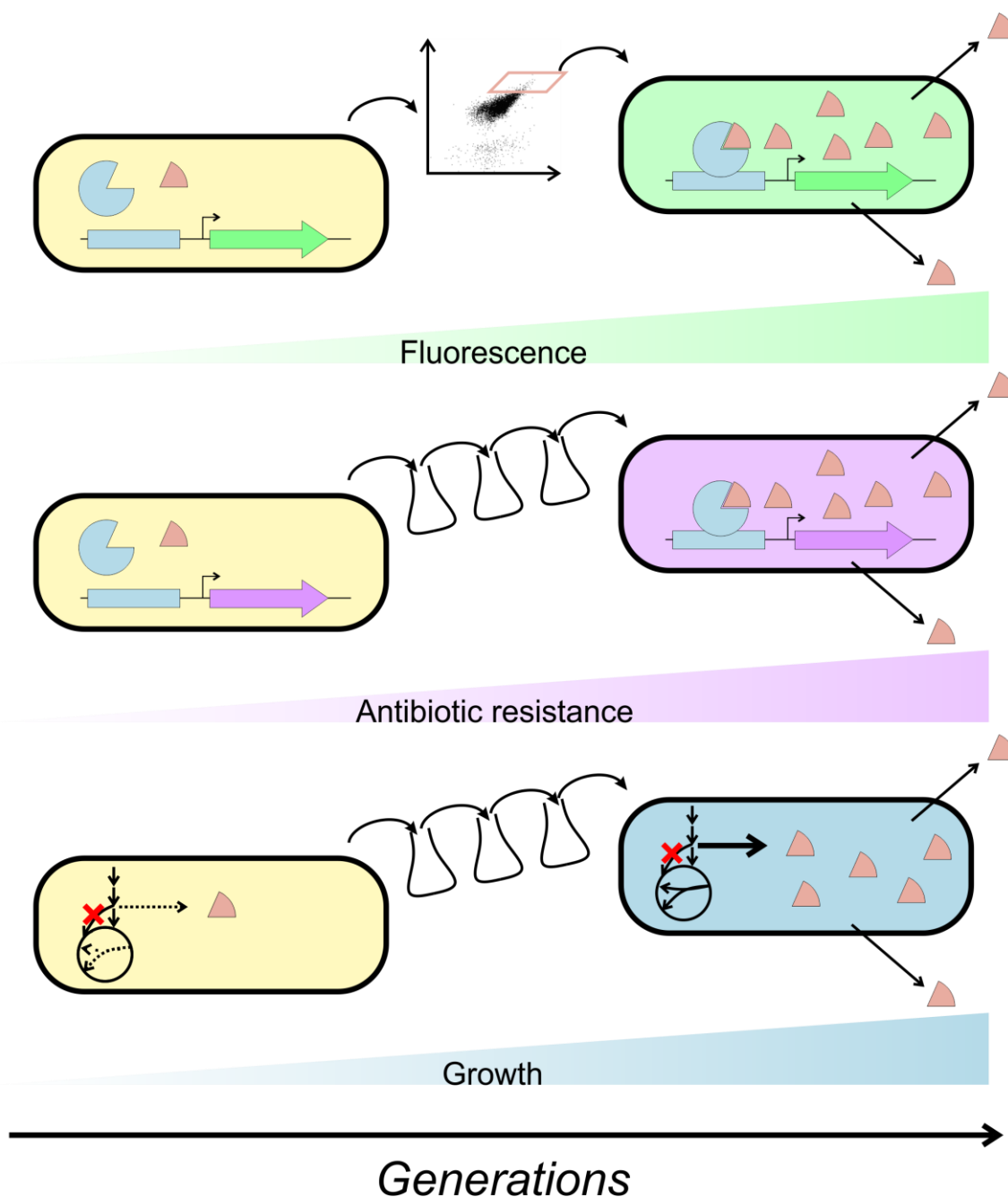
Figure 2.

Overview of different synthetic evolution approaches to select for increased small molecule production. Synthetic regulatory circuits can be constructed to couple intracellular product concentration to a selectable output, for example to fluorescent protein synthesis[75] which can be selected for by FACS, or to synthesis of an antibiotic or auxotrophic marker.[77] Metabolic engineering to guide evolution can be applied to increase small molecule production by selecting for growth rate.[51]
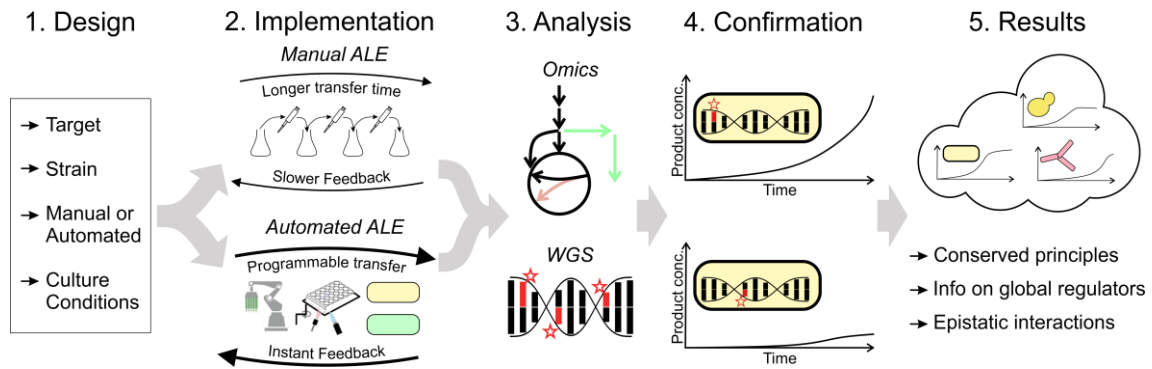
Figure 3.

Outlook on future ALE strategies. Future ALE studies could implement multiple selection pressures, to optimize industrial performance of microbial strains. Automated approaches allow for simultaneous cultivation of multiple cultures, programmable transfer-time points and instant feedback, especially in combination with biosensors. ALE should be followed by whole genome sequencing analysis and confirmation of causal mutations via re-engineering approaches. Finally, online databases can be used to gather ALE results for different microbial organisms to ultimately increase our knowledge on biological systems.