Review

Evolutionary engineering of *Corynebacterium glutamicum*

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Word count: 6295

Keywords: *Corynebacterium glutamicum*, adaptive laboratory evolution (ALE), evolutionary engineering, biosensors, laboratory automation

Abbreviations: ALE, adaptive laboratory evolution, **rbALE**, repeated batch ALE, **MTP**, microtiterplates

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

usually facilitated by repetitive batch cultivations or continuous cultivations, which can be simple (e.g. shake flasks) or more advanced (e.g. pH auxostats).[17, 19] When combined with next-generation sequencing and comprehensive omics analysis, ALE strategies can be used to obtain better production strains, but also to identify non-intuitive targets for strain engineering and ultimately to gain a comprehensive understanding of biological pathway regulation. Furthermore, advances in automation of laboratory workflows and microbial phenotyping are fostering a tremendous increase in throughput and efficiency.

This review gives an overview of evolutionary engineering of *C. glutamicum*, including approaches to improve growth on glucose or alternative carbon sources, stress tolerance, or small molecule production by growth coupling strategies and biosensor guided evolution (Figure 1, examples summarized in Table 1). We conclude by highlighting the potential of a smart combination of synthetic biology and workflow automation for the next-generation evolutionary engineering approaches.

2 Accelerating growth on glucose

The time needed for biomass production represents a key factor in the economic success of biotechnological processes. While ALE approaches have been intensively explored to accelerate growth of *E. coli*[20, 21], so far only two recent studies reported on the evolution of *C. glutamicum* towards higher growth rates on glucose minimal medium (Table 1).[22, 23] Interestingly, these studies revealed similar key targets for enhancing metabolic flux and increasing substrate uptake rates for both species indicating that microorganisms follow similar principles to adapt to fast growth.

Among the key targets improving growth on glucose, we recently described mutations in the *pyk* gene, encoding the pyruvate kinase (PK). Re-introduction of identified SNPs into the parental strain caused a significant decrease in PK activity (T12A, and A20V).[22] Furthermore, a mutation of alanine 271 to threonine (A271T) was enriched in one cell line.

Intriguingly, a PK enzyme carrying this mutation was previously described as being desensitized towards the allosteric activator fructose 1,6-bisphosphate (FBP), resulting in a reduced PK activity upon accumulation of the glycolytic intermediate FBP.[24] Mutations in the *pyk* gene were also identified in the prominent *E. coli* long-term evolution experiment that lasted for more than 25 years, and in a recent study by LaCroix et al. where cells were evolved towards fast growth on glucose.[25, 26] It is interesting to note that fast proliferating eukaryotic cells, like embryonic stem cells and cancer cells, also harbour a less active PKM2 isoform of the PK enzyme.[27, 28] From these findings, we can conclude that lowering the PK activity appears to be a conserved strategy of fast growing cells that need to exploit large amounts of glucose for anabolic pathways.

Further key targets identified by Pfeifer et al. were mutations in *fruK*, which encodes for 1-phosphofructokinase (Pfk1). In a previous study, deletion of the *fruK* gene was actually reported to enhance glucose uptake of *C. glutamicum*. The authors speculated that this was the result of an accumulation of F1P relieving *ptsG* repression via the SugR regulator.[29] However, further analyses are required to understand the impact of Pfk1 on the sugar phosphate pool and its impact on regulatory networks in these strains.

The *corA* gene, coding for a putative Mg^{2+}/Co^{2+} transport protein[30], represented an unexpected mutational hotspot in the *C. glutamicum* long-term evolution and was also identified, but not discussed, in the *E. coli* experiment of LaCroix et al..[22, 25] Mg^{2+} limitation was previously described to increase glucose consumption rates and metabolic flux of *E. coli* in the stationary phase. Here, the authors assumed that reduced Mg^{2+} levels might cause a block in pyruvate dehydrogenase (PDH) activity leading to an accumulation of upstream intermediates like PEP, which stimulate glucose uptake.[31] However, the availability of magnesium ions is known to affect a variety of different enzymes and to have an impact on the stability of polyphosphates.[30] For example, the biologically active form of ATP is a 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

temperature, to reduce cooling costs). An improved heat tolerance was reported for *C. glutamicum* after a repetitive batch ALE (rbALE) of 65 days, in which the temperature was gradually increased from 38 °C to 41.5 °C.[38] Sequencing of three evolved isolates revealed a surprisingly high amount of 295 point mutations and 2 genomic deletions in total. This was caused by an acquired mutator phenotype, which resulted in a mutation rate 40-fold to 80-fold higher than that of the parental strain. A genetic basis for this phenotype was not found. However, the combined reengineering of the mutations g*lmU*(E295K) and *otsA*(R328H) resulted in an improved heat tolerance and increased the maximum specific growth rate at 40.7 °C from 0.27 ± 0.02 h^{-1} to 0.33 ± 0.03 h^{-1}.

Two different studies applied rbALE to increase tolerance for inhibitors present in corn stover hydrolysate[39] and for methanol, which is an impurity found in certain glycerol waste streams.[40] A fixed amount of inhibitor was added, and cells were selected for growth. The corn stover evolved strain showed a higher degradation rate of the inhibitors furfural, HMF, vanillin, syringaldehyde, 4-hydroxybenzaldehyde and acetic acid.[39] When grown on corn stover hydrolysate, the evolved strain also showed a 68% higher glutamic acid titer and 35% higher yield than the parental strain. Increased glucose consumption rate probably resulted from a higher *ptsI* expression, which codes for a component of the phosphotransferase system (PTS). In the methanol study, tolerance was increased by using media with 50 mM methanol.[40] The evolved strain showed a higher growth rate under methanol concentrations between 500 and 2000 mM. Sequencing identified 29 mutations and combined engineering of two of them, *metY*(A165T) and *cat*(Q342*), increased the methanol tolerance of the parental strain to that of the evolved strain. Interestingly, both the reverse engineered strain and the evolved strain lost the ability to grow on ethanol. This showed that, in contrast to an observed beneficial cross-tolerance for isobutanol after ALE for increased temperatures[38], a single-target ALE approach is typically associated with significant negative trade-off effects.

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

potential of metabolic re-routing for the successful implementation of synthetic methylotrophy in established industrial platform organisms.

In the xylose and cellobiose study, targeted engineering was first applied to obtain two different strains that could grow on cellobiose as sole carbon source.[42] ALE was performed until growth and cellobiose consumption did not improve anymore. Sequencing analysis revealed a high number of mutations (36 and 300), which could not be explained. After the ALE for cellobiose, the xylose consumption pathway was introduced and co-utilization of xylose and cellobiose was shown.

An improved growth rate on only D-xylose was shown by Radek and co-workers, using a rationally engineered strain that was subsequently evolved by high throughput rbALE for 35 generations in only 13 days.[43] A best performer showed more than a doubling of the maximum specific growth rate, compared to the parental strain (0.26 ± 0.02 h^{-1} and 0.10 ± 0.02 h^{-1}, respectively). Sequencing analysis revealed putative loss-of-function mutations in the transcriptional regulator IolR, involved in myo-inositol uptake and degradation, and cg3388, encoding for a putative transcriptional regulator. In a subsequent study, the functional loss of IolR was shown to result in an upregulation of the gene *iolT1* (encoding for the myo-inositol/proton symporter IolT1), and additional reengineering could prove the importance of IolT1 for xylose uptake.[45] Interestingly, the identified mutations are different from those identified by the xylose-methanol ALE study.[41] Altogether, these examples once more highlight the importance of subsequent re-engineering of key mutations to ultimately expand our knowledge on biological systems.

4 Metabolic engineering to guide evolution

Combining evolutionary engineering with rationally engineered system perturbations is a promising approach for the identification of metabolic traits that can be beneficial for target molecule production.[46] Gene knockouts are frequently used to investigate the metabolic

and regulatory function of gene products[47, 48], but also for pre-defining the cell´s metabolic network with regard to precursor supply for biotechnological applications (Figure 2).[49] Growth defects that result from systems perturbations by introduction of gene knockouts provide a guided selection pressure towards fitness recovery. This can lead to alternative re-optimized metabolic states, for example by harnessing the underground metabolism by building on promiscuous enzyme activity of the particular species.[47, 50]

Schwentner et al. used the inactivation of anaplerotic reactions to evolve an alternative producer platform for the amino acid L-valine.[51] This effort was motivated by the fact that metabolic engineering of *C. glutamicum* for L-valine production is typically based on complete inactivation or downregulation of the pyruvate dehydrogenase complex (PDHC).[52, 53] Schwentner et al. evolutionary engineered an alternative route for increased pyruvate supply by inactivating the genes encoding the PEP and pyruvate carboxylase (PEPCx, *ppc* and PCx, *pyc*). The resulting strain was unable to grow on glucose as sole carbon source since the replenishment of the oxaloacetate pool was impaired.[54]. Whole genome sequencing of the evolved strains revealed *icd*, encoding isocitrate dehydrogenase (ICD), as a consistent mutation target in all evolved strains. Re-engineering of identified key mutations in *icd* revealed an upregulation of the glyoxylate shunt[55] as an alternative route to replenish the oxaloacetate pool in the Δ*pyc* Δ*ppc* background.[51] However, further studies are necessary to identify the molecular mechanisms underlying the activation of the glyoxylate shunt as a secondary effect of reduced ICD activity. In accordance with assumptions of Baumgart et al., the authors postulated that the reduced ICD activity and the resulting accumulation of isocitrate promote activation of the glyoxylate shunt.[51, 56] Based on a 1.9-fold increased intracellular L-valine level and a predicted increased flux through the PDHC, Schwentner et al. suggested that the re-engineered strains represent promising candidates for L-valine producers. Indeed, plasmid-based overexpression of L-valine

biosynthesis genes (pJC4*ilvBNCE*) led to a fourfold-enhanced L-valine product yield in comparison to the wild type.[51]

The study of Schwentner and co-workers demonstrated the potential of guided evolution strategies to improve glucose conversion to value-added compounds like L-valine. In contrast, the previously discussed guided evolution approaches of Lee et al. and Tuyishime and colleagues with pre-engineered *C. glutamicum* cells resulted in strains showing efficient co-utilization of alternative substrates such as xylose and methanol[41] or xylose and cellobiose[42] demonstrating the broad application spectrum of guided evolution strategies (Table 1).

McCloskey and colleagues intensively studied growth recovery of different *E. coli* knockout strains during ALE at systems level. The combination of ALE and multi-omics technologies provided deep insights into the versatile pathways allowing re-balancing of the metabolite and redox state.[47, 48] Further, growth coupling approaches with *E. coli* used the strong evolutionary driving force towards redox homeostasis for increasing fermentative production of lactic acid[49], 1-butanol[57] and linear higher alcohols[58] by inactivating competing NADH oxidizing enzymes. These examples highlight, that not only the experimental conditions but also the genetic strain background need to be streamlined for the particular aim of the ALE.

5 The benefit of automation

A number of different ALE approaches are already established that can roughly be classified according to the cultivation conditions (i.e., chemostat vs. repetitive batch culture), experimental throughput (i.e., lab-scale vs. micro-scale bioreactor systems) and degree of automation (i.e., manual vs. autonomous operation). See[17, 59] for a detailed discussion of 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]

However, when applied as stand-alone devices for rbALE, the above systems still require on the manual passaging of cells from one MTP-well to another. Consequently, there is a high demand for automation to optimally balance available resources (i.e., personnel, material, and time) with the expected outcomes (i.e., strains with improved properties, new knowledge on cellular metabolism, and targets for rational strain design).

As a first step in this direction, we recently established a workflow to perform rbALE experiments in an automated manner.[43] By building on an existing microbial phenotyping platform (i.e., Mini Pilot Plant integrating a BioLector system with liquid handling robotics[65]), the workflow covers essential steps for autonomous rbALE, including preparation of different media compositions, their cool temporary storage and the repeated inoculation of individual MTP-cavities in the BioLector. The latter is supported by various online measurements (i.e., backscatter, pO2, pH and fluorescence) and ensures an instantaneous recording of metabolic adaptation events during ALE experimentation (Figure 3.2). Moreover, the setup enables dynamic adjustments of passage sizes for keeping genetic diversity, but (if required) also provides access to the full spectrum of applicable selection pressures.

6 Evolutionary engineering of small molecule production

Redirecting cellular metabolism towards the production of small molecules is challenging, because metabolic pathways have been evolved for tight control and our understanding of their regulation is incomplete.[66] For ALE approaches, the difficulty lies in applying a pressure that selects for production of the compound of interest. While traditional approaches select for suppressor mutants, the development of transcription factor-based biosensors enabled a direct coupling of product formation to an easily selectable output, which resulted in the first reports of "synthetic evolution".[16, 67–69] For *C. glutamicum*, ALE

for increased small molecule production has been shown for fatty acids, L-ornithine, putrescine, plasmid based proteins and L-valine (Table 1).

6.1 Selection for suppressor mutants

Early efforts on the improvement of small molecule production focused on the application of random mutagenesis (UV- or chemical mutagenesis) and on selection schemes based on the resistance to antimetabolites or product analogs inhibiting growth. Key enzymes of biosynthetic pathways (e.g. amino acid biosynthesis) are typically feedback-inhibited by the particular end-product of the pathway. Application of product analogous antimetabolites enabled the efficient isolation of mutations causing feedback-resistance of key enzymes.[2] Two groups have recently published work on improving small molecule production by selecting for suppressor mutants. Takeno and co-workers used two different chemicals, Tween40 and cerulenin, to select for mutants with a higher fatty acid production, using multiple selection steps on agar plates.[70] In contrast, Jiang and co-workers applied rbALE in media containing the desired product, L-ornithine, to select for mutants resistant to product inhibition.[71] In both studies the final product titer could be improved with the evolved strain, from 3.21 ± 0.06 mg l^{-1} to 279.95 ± 8.50 mg l^{-1} for fatty acids[70], and from 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 genome sequencing, Takeno et al. reported 3 mutations in genes responsible for fatty acid synthesis. Takeno and co-workers repeated the selection step on cerulenin plates, and identified one additional mutation.[72] Re-engineering of this mutation in the previously evolved strain resulted in a 1.2-fold higher oleic acid titer. This shows how iterative selection of a parental strain can be used to find different mutations that have an additive effect on productivity. In contrast, the L-ornithine evolved strain was further engineered for putrescine production.[73] Random mutagenesis in combination with rbALE in media containing putrescine resulted in a strain showing a two-fold higher final putrescine titer

(111.42 ± 2.56 mM). Sequencing analysis revealed 78 SNPs, further analysis suggested that a decrease in activity of the α-ketoglutarate decarboxylase OdhA and an increase in the major facilitator superfamily permease CgmA, a putative putrescine permease, contributed to a higher putrescine production.

6.2 Biosensor guided evolution

Biosensors can greatly increase the throughput and selectivity for ALE, and thereby accelerate strain development (Figure 2). Initially, two studies reported on transcription factor-based biosensors that were designed to induce *eyfp* expression in response to higher intracellular amino acid concentrations in *C. glutamicum*. The pSensLys biosensor detects amino acids with a basic side chain[13] and the Lrp biosensor detects L-methionine and branched chain amino acids.[14] Both studies showed that random mutagenesis coupled to FACS could be used to select for cells with higher productivity, illustrating the use of biosensors to screen for producers strains. However, applying random mutagenesis results in many mutations. Binder et al reported 268 SNPs [13] and a similar study reported 83 SNPs [74]. This high number of mutations complicates the identification of the causal ones.

In a recent study, Mahr and co-workers describe a biosensor guided ALE approach based on the native mutagenesis rate, by applying multiple rounds of selection using the Lrp biosensor (Table 1).[75] The L-valine producer strain *C. glutamiucm* Δ*ace*, which produces valine in stationary phase, was used as parental strain. Five iterative evolution steps were performed and in each step the top 10% fluorescent cells were transferred by FACS. After only five iterations, a single isolate showed a two-fold increase in L-valine production compared to the parental strain. Only seven mutations were identified after whole genome sequencing. This is in large contrast to the higher number of mutations found when random mutagenesis is applied. The identified mutation *ureD*(E188*) resulted in a truncated version of the urease accessory protein and was shown to increase the final L-valine titer

(44.2 mM for evolved strain) by more than 100%.[75] A low amount of mutations was also the result of a simple biosensor ALE experiment, in which iterative FACS selection was done with a strain containing a plasmid based *egfp*. Only one mutation was identified, a loss of function of *parB*, which resulted in 10-fold higher plasmid copy numbers.[76] Further examples for biosensor guided evolution approaches in *E. coli* are the coupling of mutation rate to small molecule production[69] and the selection of improved producers via an antibiotic based positive and negative selection strategy.[77]

The mutations identified by ALE can be further investigated by targeted mutagenesis followed by biosensor mediated screening, which was shown for the *argB* gene using pSensLys[78] and for the RBS sequence in *tktA* using the ShiR-based shikimate sensor.[79] An interesting method was described by Binder et al., who designed a rapid recombineering and screening approach, based on the RectT recombinase from prophage Rac and the pSenLys sensor, which was applied to screen for mutations in *murE* with an impact on L-lysine production.[80] Furthermore, studies in *E. coli* have demonstrated the use of multiplexed genome engineering in combination with barcoding to increase mutation rates and enable tracking of mutations.[81, 82] Altogether, these approaches demonstrate the potential of transcription factor-based biosensors for the design of synthetic evolution schemes to enable the improvement of microbial small molecule production.

7 Conclusions

In engineering disciplines, the possibility of process improvement via evolution is a feature unique to biotechnology. But what do we learn from the comparison of different ALE endeavours? Evolution of living organisms is shaped by the particular environmental conditions (like media composition, gas supply, etc.), the genetic background and by the mode of cultivation (batch, chemostat, solid media), including passage time and culture volume. For example, LaCroix et al. achieved a significant increase of growth rates in shorter time compared to the Lenski experiment, because batch cultures where propagated during

exponential phase rather than inoculated from stationary phase cultures.[20, 25] These examples demonstrate that an ALE setup must be efficiently tailored to the particular scientific aim. However, most studies only focus on one aspect of industrial performance (e.g., productivity or stress tolerance). A more integrative approach could combine several improvement targets into one experiment. Especially the development of biosensors could aid in this field, as they allow for an easy coupling of selecting for producers under industrial conditions. This could even be extended with high-throughput technologies enabling the design of novel ALE strategies (Figure 3).

Several recent examples highlight the fact, that ALE or untargeted selection strategies should be complemented by sequencing, and possibly by transcriptome analyses and re-engineering to obtain novel information on enzyme properties or pathway regulation. Especially in the absence of a random mutagenesis step, the amount of mutations is most of the times low enough to allow for reverses engineering and an in-depth characterization of specific mutations. This is in contrast to classical genome breeding approaches where a high number of -induced mutations (e.g. via UV radiation) may result in the accumulation on non-beneficial side mutation having a negative impact on genome stability. Often, different mutations found in different studies can result in an additive effect, but it can be hard to keep track of all described mutations. Recent database efforts such as the AleDB could aid in improving our understanding of metabolic regulations .[62] A more systemic analysis of ALE experiments combined with sophisticated data management will accelerate the identification of causative key mutations and epistatic interactions resulting in improved strain properties.

Acknowledgement

The authors acknowledge funding from the Helmholtz Association (grant W2/W3-096). Further funding was obtained from Bioeconomy Science Center (BioSC, Grant. No. 325-40000213) as part of the Focus FUND project "HyImPAct – Hybrid processes for important precursor and active pharmaceutical ingredients".

Conflict of interest

The authors declare no financial or commercial conflict of interest.

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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]

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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Figure legends

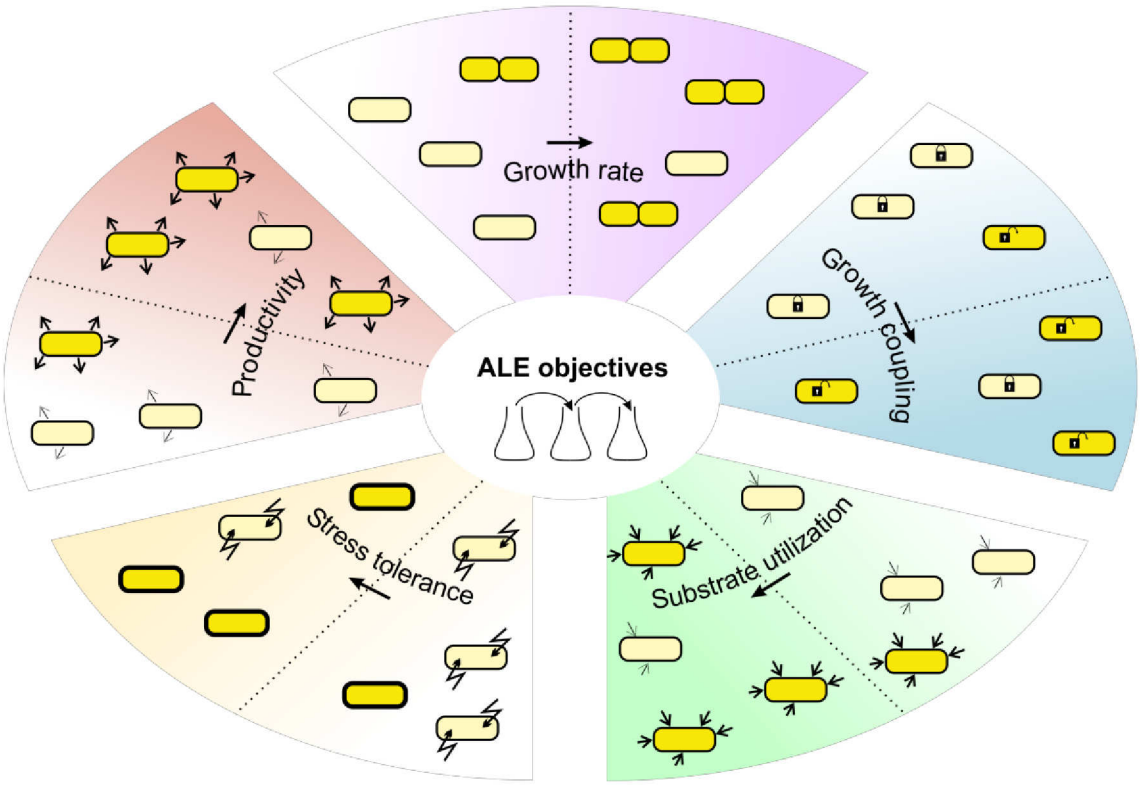

Figure 1.

Overview of different objectives in *C. glutamicum* ALE studies. ALE has successfully been applied to increase the growth rate[22, 23], substrate utilization[43], stress tolerance[39, 40] and small molecule production.[75] Growth coupling strategies have been used to increase precursor supply and small molecule production.[51]

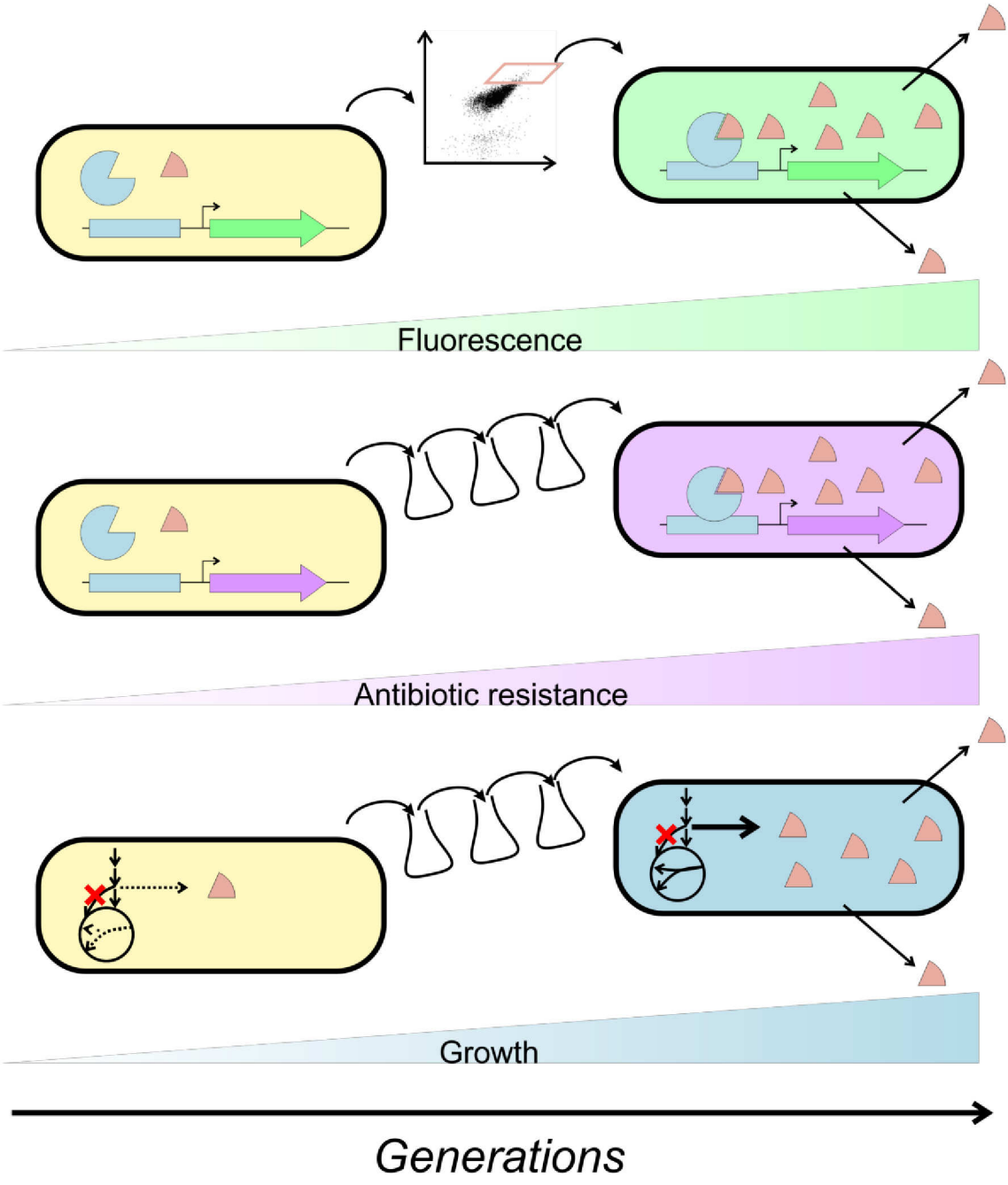

Figure 2.

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]

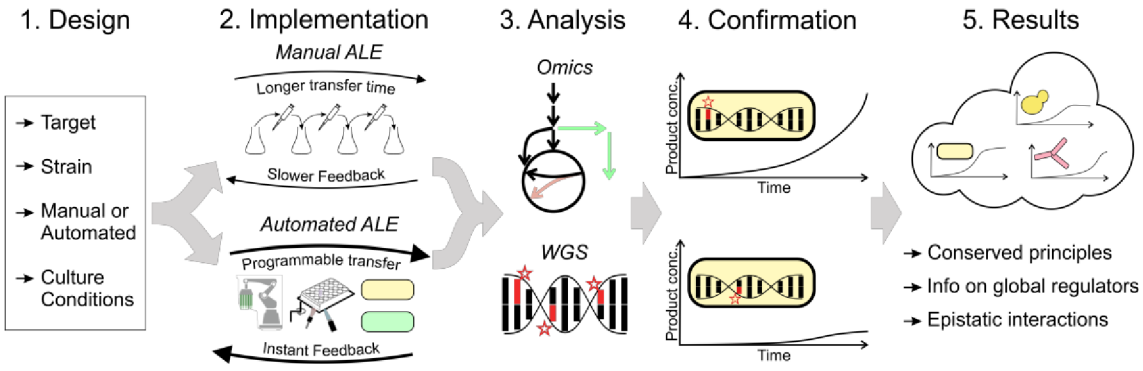

Figure 3.

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.