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Metabolically engineered *Corynebacterium glutamicum* for bio-based production of chemicals, fuels, materials, and healthcare products

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ABSTRACT

Corynebacterium glutamicum is a major workhorse in industrial biotechnology. For the past several decades, this soil bacterium has been used to produce the amino acids L-glutamate and L-lysine at a level of 6 million tons per year. Utilizing novel genome editing methods and advanced tools of systems and synthetic biology, the portfolio of *C. glutamicum* has exploded from classical food and feed products to the chemical industry, cosmetics and healthcare applications. Within the past five years, the number of industrial products has almost doubled to approximately 70 natural and non-natural compounds. This review summarizes the state-of-the-art regarding metabolically engineered cell factories of *C. glutamicum*, illustrates recent key technological developments and provides examples of the major industrial applications of this important microbe.

1. Introduction

Corynebacterium glutamicum is an essential pillar of white biotechnology. Its development as one of the most important industrial workhorses is due to its ability to produce and secrete large amounts of amino acids (Becker and Wittmann, 2015, 2017b). The global competition in this multibillion dollar business has steadily driven innovation and progress. Consequently, the major players in the amino acid market made substantial investments in research and development. Today, *C. glutamicum* is not only the heart of many industrial production processes but also one of the best-studied model organisms (Becker and Wittmann, 2017b; Heider and Wendisch, 2015; Ma et al., 2017). The toolbox for quantitative analysis of metabolism (Becker and Wittmann, 2018; Matsuda et al., 2017) has likewise expanded, as have methods to manipulate the genome in a desired manner (Lee and Wendisch, 2017). Cutting-edge technologies of the last decade, such as transcriptomics (Hayashi et al., 2002; Pfeifer-Sancar et al., 2013; Wendisch and Polen, 2013), metabolomics (Bolten et al., 2007; Krömer et al., 2005; Luo et al., 2007; Wittmann and Heinzle, 2001b), ^{13}C fluxomics (Kiefer et al., 2004; Quek et al., 2009; Schwechheimer et al., 2018; Wittmann, 2007; Wittmann and De Graaf, 2005; Wittmann and Heinzle, 2001a, 2002; Wittmann et al., 2004a, 2004b), metabolic modelling (Kjeldsen and Nielsen, 2009; Melzer et al., 2009; Wittmann and Heinzle, 2001c) and their integrative application (Buschke et al., 2013a; Hüser et al., 2005; Kohlstedt et al., 2014; Krömer et al., 2004; Silberbach et al., 2005), played a leading role in the systems metabolic engineering of *C. glutamicum* for the production of amino acids (Becker et al., 2011; Lee and Wendisch, 2017), diamines (Becker and Wittmann, 2017a; Kind et al., 2010a; Mimitsuka et al., 2007; Schneider and Wendisch, 2010), vitamins (Hüser et al., 2005), pyrazines (Dickschat et al., 2010), organic acids (Inui et al., 2004b; Okino et al., 2008a, 2008b) and alcohols (Blombach et al., 2011; Inui et al., 2004a; Smith et al., 2010; Yamamoto et al., 2013). In addition, substrate assimilation routes in *C. glutamicum* were studied (Kiefer et al., 2002; Neuner and Heinzle, 2011; Neuner et al., 2013; Peng et al., 2011) and engineered (Buschke et al., 2011; Kawaguchi et al., 2008; Rittmann et al., 2008) to improve production and to allow the use of non-native renewable raw materials (Buschke et al., 2013b; Wendisch et al., 2016). Fig. 1 summarizes the bio-based production routes that have been established for streamlined cell factories of *C. glutamicum* to date. In this review, we will outline the application of the microbe for the production of chemicals, fuels, materials, and healthcare products. We mainly cover a research period of approximately 20 years and include the technological developments and metabolic engineering approaches used to develop new or optimized production strains for the synthesis of both natural and non-natural products.

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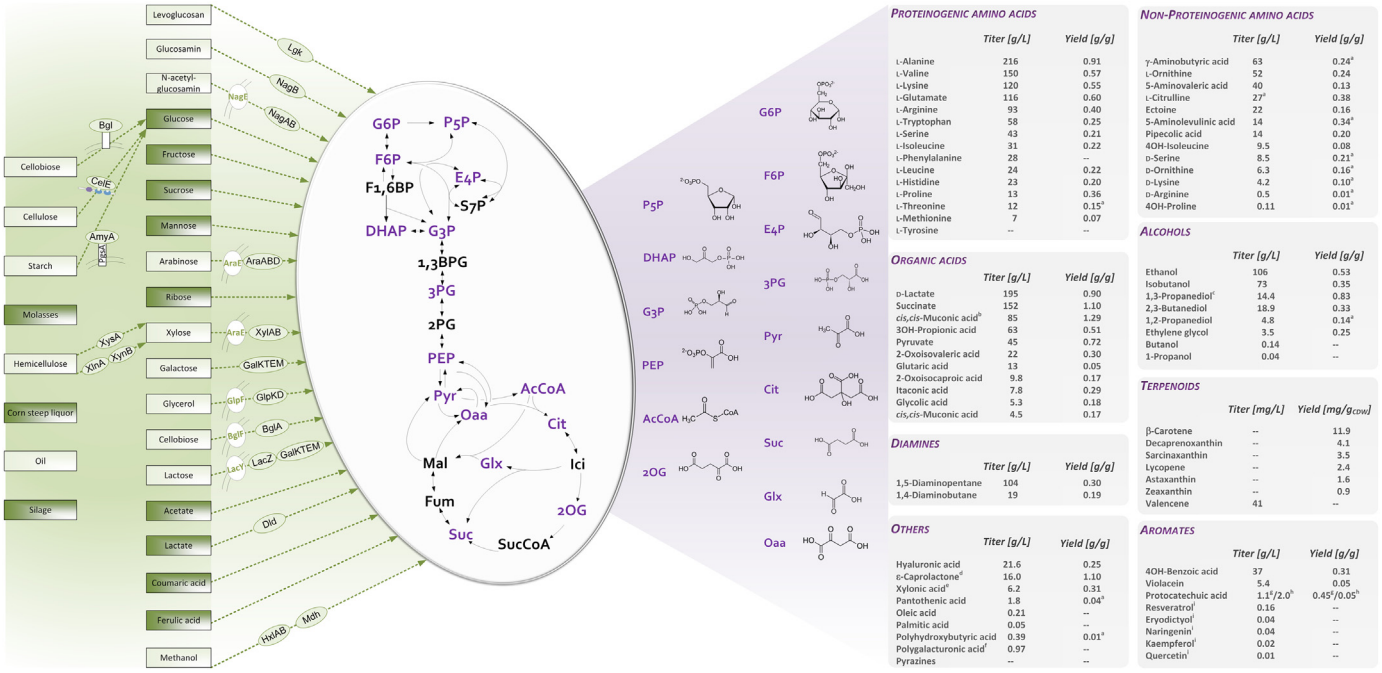
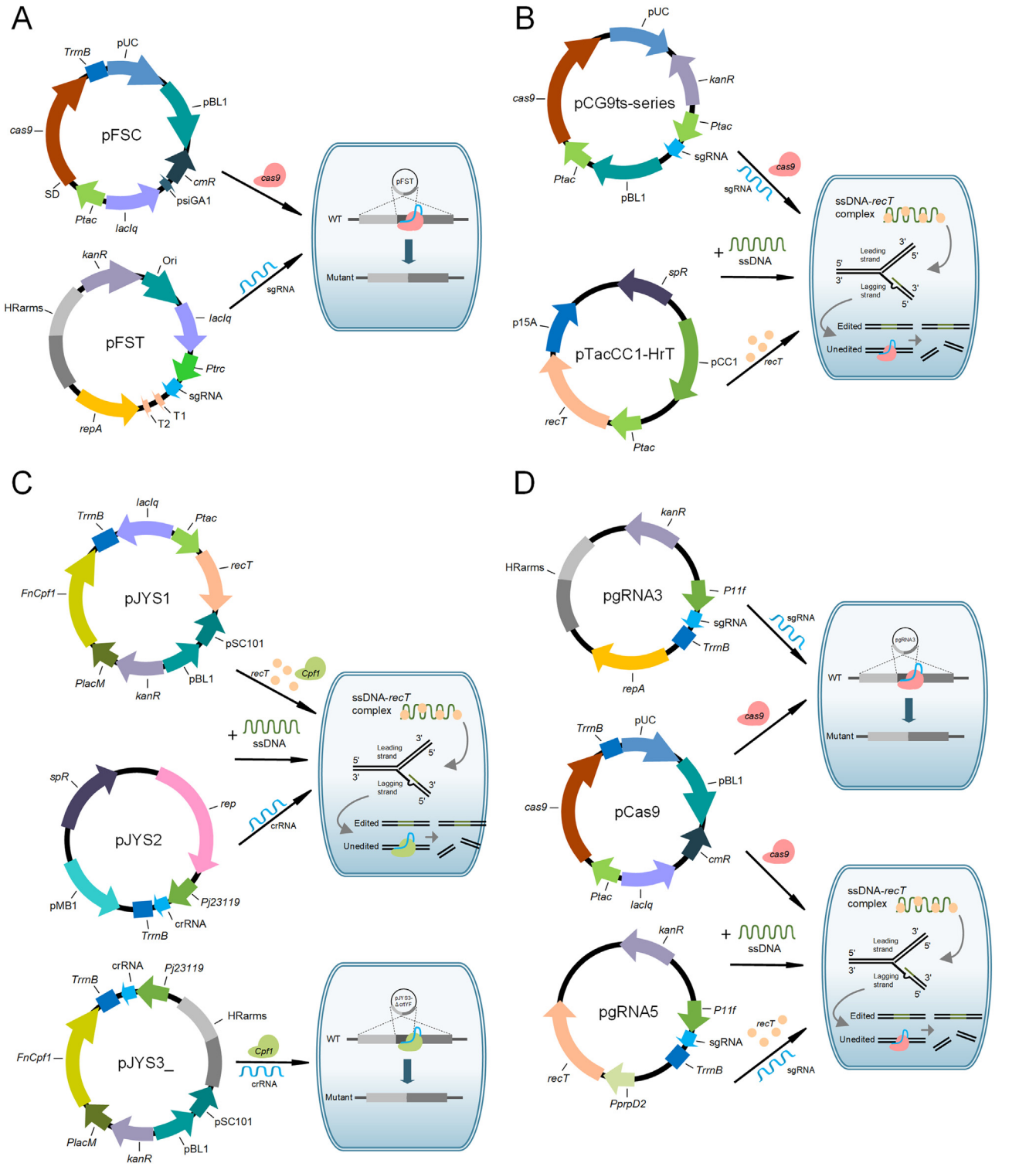


Fig. 1. Bio-based production routes established in *C. glutamicum* for manifold natural and non-natural products from renewable feedstocks. Natural (dark green) and non-natural (light green) carbons sources are metabolized via central key intermediates (purple) into the desired products. Data are taken from (Becker et al., 2011, 2016, 2018; Bückle-Vallant et al., 2014; Chen et al., 2016, 2017; Cheng et al., 2017; Chung et al., 2017; Colon et al., 1995; Dickschat et al., 2010; Doo et al., 2009; Eberhardt et al., 2014; Hasegawa et al., 2013; Heider et al., 2014; Henke et al., 2017; Huang et al., 2017; Hüser et al., 2005; Ikeda, 2003; Ikeda and Katsumata, 1999; Ikeda et al., 2009; Jensen and Wendisch, 2013; Jojima et al., 2010, 2015; Joo et al., 2017; Jorge et al., 2017a; Kallscheuer and Marienhagen, 2018; Kallscheuer et al., 2016, 2017; Kim et al., 2015b; Kitade et al., 2018; Krause et al., 2010; Li et al., 2016; Ma et al., 2016; Mizukami et al., 1994; Okai et al., 2017; Okai et al., 2016; Otten et al., 2015; Park et al., 2014; Perez-Garcia et al., 2017a; Perez-Garcia et al., 2017b; Rados et al., 2015; Schneider et al., 2012; Shi et al., 2016; Shin et al., 2016, 2018; Siebert and Wendisch, 2015; Song et al., 2013; Stäbler et al., 2011; Sun et al., 2016; Takeno et al., 2013; Taniguchi et al., 2017; Tsuge et al., 2015a; Vogt et al., 2014; Wieschalka et al., 2013; Wu et al., 2010; Yamamoto et al., 2013; Yang et al., 2015, 2016; Yi et al., 2014; Yim et al., 2017; Zahoor et al., 2014; Zhu et al., 2015). a: estimated from reference; b: biotransformation from catechol; c: biotransformation from glycerol; d: biotransformation from cyclohexanone; e: biotransformation from xylene; f: given as flocculating activity in $U\,L^{-1}$; g: biotransformation from ferulic acid; h: *de novo* synthesis from glucose; i: biotransformation from phenylpropanoids. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2. Next-generation toolboxes

2.1. Genome editing

The isolation of natural *C. glutamicum* plasmids and the development of the first DNA transfer technologies were milestones for strain engineering (Becker and Wittmann, 2017b). Today, routine genome manipulation, including knockin and knockout of genes, the mutation of single nucleotides, or the introduction of insertions, relies on the conditionally lethal levansucrase (*sacB*) marker (Jäger et al., 1992). This method uses a non-self-replicating suicide vector, which integrates into the genome, followed by a second recombination to remove the vector backbone for scarless genetic modification (Becker et al., 2005; Schäfer et al., 1994). However, the frequent occurrence of false-positive clones and the need for two successive rare double crossover events (Kirchner and Tauch, 2003; Nesvera and Patek, 2011) still limit the speed and throughput of strain engineering of *C. glutamicum*. In recent years, clustered regularly interspaced short palindromic repeats (CRISPR) with their CRISPR-associated proteins (Cas) (Jinek et al., 2012) emerged as a key enabling technology for genome editing of humans (Cho et al., 2013a), animals (Cho et al., 2013b), bacteria (Jiang et al., 2013b) and yeast (DiCarlo et al., 2013), and this technology has been established for *C. glutamicum* by several different research studies (Cho et al., 2017; Jiang et al., 2017; Liu et al., 2017b; Peng et al., 2017) (Fig. 2). In short, the class II CRISPR/Cas9 system for genome editing of prokaryotes recruits the Cas9 nuclease from *Streptococcus pyogenes*. Cas9 is activated through binding of CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA), which – to facilitate genetic engineering – can be fused into a single guide RNA (sgRNA) (Jinek et al., 2012). The resulting ribonuclease protein (RNP) complex then binds to a specific DNA sequence – known as a protospacer – with the specific protospacer-adjacent motif (PAM) NGG and introduces a double-strand break (DSB) in the target DNA (Jinek et al., 2012). The DSB can be repaired by homologue-directed DNA repair (HDR) while introducing the desired genomic modification at the targeted locus, or by the nonhomologous end-joining (NHEJ) mechanism. As efficient NHEJ does not occur in *C. glutamicum* (Cho et al., 2017; Liu et al., 2017b; Peng et al., 2017), the system appears ideal for targeted genome manipulation. However, adaptation of the system for *C. glutamicum* was challenging (Cho et al., 2017; Jiang et al., 2017). The first report on genome editing of *C. glutamicum* using a CRISPR-related system thus made use of the Cpf1 endonuclease of *Francisella novicida* (Jiang et al., 2017). The authors developed both a double-plasmid-based system for small genome alterations and an all-in-one system for larger gene deletions and insertions. The double-plasmid approach uses (i) an expression vector for Cpf1 additionally encoding the *Escherichia coli* prophage recombinase (RecT) and (ii) a crRNA-expressing plasmid. Recombination was mediated by single-strand (ss) donor DNA that was cotransformed with the crRNA plasmid (Jiang et al., 2017). For the all-in-one-plasmid approach, the genes for Cpf1 and crRNA were combined with the donor DNA in a single vector. Beyond gene deletion, the double-plasmid-CRISPR-Cpf1 system was also applied for codon saturation mutagenesis of *proB*, encoding γ-glutamyl kinase, which successfully released the enzyme from feedback inhibition by proline (Jiang et al., 2017). Because of the T-rich PAM sequence of Cpf1, the further development of Cas9, recognizing a GC-rich PAM sequence, remained highly attractive for use in GC-rich *C. glutamicum*. Peng and coworkers developed a CRISPR/Cas9 system based on two plasmids, one carrying the *cas*9 gene



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under control of the IPTG-inducible P_{tac} promoter followed by a SD sequence (Peng et al., 2017). Temperature sensitivity of the vector backbone allowed fast and straightforward curing after genome editing. The second vector contained the P_{trc}-transcribed sgRNA and a homologous repair template (Peng et al., 2017). The optimal length of the repair arms thereby depended on the desired modification and had significant influence on the modification efficiency (Peng et al., 2017). The system was also successfully applied to introduce point mutations and gene insertions (Peng et al., 2017). Another two-vector system was developed in parallel (Cho et al., 2017). While one vector mediated RecT expression from an unregulated P_{tac} promoter, the other vector mediated IPTG-inducible expression of sgRNA and Cas9opt, an enzyme variant that was codon-optimized for Actinomycetes (Tong et al., 2015). In this system, RecT expression was essential to obtain positive transformants. As an editing template, ssDNA was used for scarless knockout of one or more genes in a sequential and iterative manner (Cho et al., 2017). Adaptation of the transformation protocol was crucial to achieve an adequate transformation efficiency. After genome

Fig. 2. Overview of CRSPR/Cas9 and CRISPR/Cpf1 assisted genome editing strategies developed for *C. glutamicum*. A *Cas9* is expressed under control of the IPTG-inducible P_{tac} promoter on the pFSC plasmid. sgRNA cassette is expressed under control of the IPTG-inducible P_{trc} promoter from the pFST plasmid also comprising homologous regions (HRarms). Cas9 introduced double strand break of DNA is fixed through homology-directed repair (Peng et al., 2017). B Vector pTacCC1-HrT expresses *recT* under control of the P_{tac} promoter and is transformed prior to the ssDNA editing templates and pCG9ts-series vectors, expressing *cas9* and sgRNA under control of P_{tac} (Cho et al., 2017). C Upper part: Double-plasmid-based CRISPR/Cpf1 system using pJYS1 plasmid for *cpf1* expression from under control of P_{lacM} promoter and *recT* expression under control of the P_{tac} promoter, and pJYS2 plasmid containing crRNA under control of the constitutive P_{j23119} promoter (Jiang et al., 2017). ssDNA is used as editing template. Lower part: All-in-one CRISPR/Cpf1 plasmid. *Cpf1* under control of P_{lacM} promoter, crRNA under control of *Pj23119* promoter and HRarms are introduced via the pJYS3 plasmid. The Cpf1-crRNA complex introduces a double strand break at the targeted locus, which is fixed through homology directed repair (Jiang et al., 2017). D Upper part: *Cas9* is expressed from the pCas9 plasmid using P_{tac}. The pgRNA3 plasmid contains HRarms and the sgRNA cassette, controlled by the constitutive promoter *P11f*. Genome editing takes place upon double strand break by Cas9-sgRNA-ribonucleoprotein complex and subsequent repair relying on plasmid-borne repair templates (Liu et al., 2017b). Lower Part: ssDNA-directed recombineering using CRISPR/Cas9 and *recT*. Recombinase *recT* under control of promotor *PprpD2* and sgRNA under control of *P11f* are expressed from vector pgRNA5 prior to introduction of ssDNA and plasmid pCas9 containing *cas9* under control of P_{tac} (Liu et al., 2017b). *CmR*: Chloramphenicol resistance gene; *FnCpf1*: *cpf1* derived from *F. novicida*; HRarms: homologous regions, repair DNA; *kanR*: kanamycin resistance gene; *lacIq*: *lac* repressor of *E. coli*; *recT*: recombinase from *E. coli*; *rep*: replication origin of *C. glutamicum* derived from pGA1 replicon; *repA*: temperature sensitive replicon; SD: Shine Dalgarno sequence; *spR*: spectinomycin resistance gene; TrrnB: transcription terminator from *E. coli*.

editing, plasmids were cured by growing the cells on antibiotic-free plates at 37 °C (Cho et al., 2017). Multiplex gene knockout was feasible with this approach, although at low efficiency. Similarly, Liu and coworkers developed a two-plasmid CRISPR-Cas9 toolbox (Liu et al., 2017b). Their system, however, could work independently of RecT. Key to the success of this system were the decoupled control of Cas9 and sgRNA expression and the use of plasmid-borne editing templates. Cas9 expression was thereby strictly controlled by using the IPTG-inducible P_{tac} promoter, whereas sgRNA was constitutively expressed under the control of the promoter P_{11F} (Liu et al., 2017b). Moreover, optimized transcription termination was required for the feasibility of CRISPR-Cas9-based counter-selection (Liu et al., 2017b). With this system, knockin and knockout of genes was successfully realized, although at rather low efficiency. By recruiting P_{prpD2}-controlled RecT expression, ssDNA could be used as an editing template with substantially higher editing efficiency for single or multiple nucleotide exchanges. Due to the higher efficiency, the combination of CRISPR-Cas9 with RecT and ssDNA was also applicable for double-locus modification (Liu et al., 2017b). Overall, the CRISPR-based system appears highly attractive, as it promises highly efficient, fast, nonbiased and multiplex genome editing and – in theory – only produces positive transformants, as Cas9 is lethal in *C. glutamicum* when HDR templates are not incorporated. In practice, however, the results are somewhat different. Admittedly, editing efficiency can be as high as 100% (Cho et al., 2017; Jiang et al., 2017; Peng et al., 2017), but this is rather an exception. Several drawbacks remain that limit the application of CRISPR-based genome editing in *C. glutamicum*. First, the expression of Cas9 is highly critical, as high levels might lead to a lack of transformants due to lethality. Low levels of Cas9, however, can trigger the survival of false positives (Cho et al., 2017; Jiang et al., 2017; Liu et al., 2017b; Peng et al., 2017). In addition, Cas9 expression suffers from rather high mutation rates during replication, resulting in nonsense mutations, insertions or even full gene deletions from the plasmid and thus failure of the system (Liu et al., 2017b). Moreover, the editing efficiency can be influenced by several other factors, including knockout size (Jiang et al., 2017; Liu et al., 2017b), overall length of homologous arms, relative length of homologous arm to insert gene (Peng et al., 2017), and design of the sgRNA (Cho et al., 2017; Peng et al., 2017). For the latter, efficiency deviations of between 0% and 100% have been reported for a single gene (Peng et al., 2017). Even replicates might show high deviations and are not reproducible (Cho et al., 2017). Nevertheless, substantial improvement has already been achieved, including multiplex engineering (Cho et al., 2017; Liu et al., 2017b) and fast sequential gene knockout (Cho et al., 2017), encouraging further research to speed up strain engineering.

2.2. Engineering of gene expression

Whereas the knockout of genes and related gene function is rather straightforward, fine-tuned up- and downregulation relies on the use of specific genetic elements with regulatory function. In this regard, strong and constitutive natural promoters are valuable in specifically increasing the expression of a gene of interest in *C. glutamicum* (Becker et al., 2016, 2007; Patek et al., 2013a; Vasicova et al., 1999). Promoter selection for the engineering of gene expression is complemented by expression control at the translational level using start codon engineering (Becker et al., 2010, 2009; Jorge et al., 2016; Li et al., 2018) and by varying the gene copy number, the latter being tunable by genome-based integration of several gene copies (Becker et al., 2011) or by plasmid selection, e.g., the use of plasmids with low or high copy number (Choi et al., 2018; Nesvera et al., 1997; Patek and Nesvera, 2013b). With regard to the promoter site, heterologous promoters have also been adapted for use in *C. glutamicum*. For instance, IPTG-inducible expression was realized by reconstruction of the *E. coli* T7 RNA polymerase expression system, allowing tightly controllable yet high levels of protein expression (Kortmann et al., 2015). For more finely tuned expression regulation, the natural promoter set of *C. glutamicum* was extended by synthetic promoter libraries (Rytter et al., 2014; Yim et al., 2013). Yim and coworkers synthesized a randomized promoter region by PCR using a 70-bp-long forward primer of degenerate oligonucleotide N (with a 25% probability of A, G, C, or T at each position). The synthetic promoters were coupled to GFP for screening, promoter strength analysis, and cell sorting. The relative fluorescence intensity varied between 200 (low strength) and 1200 (high strength) (Yim et al., 2013). Another approach was based on the known −10 consensus sequences of *C. glutamicum* (Patek et al., 2003) combined with the −35 consensus sequence of *E. coli* and an additional AT-rich region upstream of −35 to make four general promoter designs (Rytter et al., 2014). These designs were then randomized by PCR and characterized according to their ability to drive β-galactosidase expression. The resulting promoter strengths, given in Miller units, varied by several orders of magnitude (Rytter et al., 2014). The method was additionally used to establish an IPTG-inducible promoter library for *C. glutamicum* (Rytter et al., 2014). Despite the progress in generating synthetic promoter libraries, research is ongoing to deepen the understanding of native promoters in *C. glutamicum* (Albersmeier et al., 2017), which is substantially supported by high-throughput sequencing of cDNA libraries. Similar to promoter libraries, synthetic ribosome binding site (RBS) libraries have been created for *C. glutamicum* to extend the molecular engineering toolbox (Zhang et al., 2015a). These synthetic RBSs were completely chemically synthesized using a general seeding sequence of $AAAGG(N)_{6–9}$, which is based on a common RBS feature and the regularly found nucleotide spacing between an RBS and its adjacent start codon. The synthetic RBS libraries with variable sequences and spacer lengths were tailored for the expression of the *aro* genes using fusion proteins comprising GFP and *aroGBDE*. A combinatory approach was then used to optimize the linear aromatic pathway for shikimate production (Zhang et al., 2015a). As previously described (Schneider

et al., 2012), the length of the spacer sequence between the RBS and the start codon had a substantial impact on gene expression and proved to be a valuable tool for optimizing the production of putrescine (Schneider et al., 2012) and astaxanthin (Henke et al., 2016) in *C. glutamicum*. The availability of promoters and RBS sequences of different strengths and their combinatory assembly provide a vast array of molecular biology tools for the engineering of finely-tuned expression in *C. glutamicum*. Moreover, bicistronic expression systems have been developed for reliable and efficient gene expression in *C. glutamicum* (Liu et al., 2017c; Zhao et al., 2016). This strategy borrows the genetic structure of bacterial polycistronic expression cassettes, which allow the coordinated expression of proteins from closely arranged genes (Liu et al., 2017c). Such bi- or polycistronic expression cassettes were successfully used for heterologous gene expression in *C. glutamicum* and provided strains that produced ectoine (Becker et al., 2013), aminovalerate (Rohles et al., 2016) or violacein (Sun et al., 2016). Recent studies reported the design of bicistronic genetic structures for modulating gene expression. These structures consist of a promoter, a 5′-UTR with an embedded SD sequence, a DNA sequence encoding a short peptide, and a second SD sequence containing a stop codon (TAA) overlapping the start codon (ATG) directly in front of the gene of interest by 1 bp (Liu et al., 2017c; Zhao et al., 2016). The bicistronic expression elements allowed high-level expression of eGFP, which was increased by 1.4–790-fold (Zhao et al., 2016) and 2.2-fold (Liu et al., 2017c). Beyond the classical genetic elements, CRISPR interference (CRISPRi, CRISPR/dCas9) offers a new way to manipulate the expression of specific genes (Bikard et al., 2013; Larson et al., 2013). This approach involves the recruitment of a deactivated Cas9 variant (dCas9) that retains its RNA-guided DNA binding ability but lacks nuclease activity. This variant can be used to block RNA polymerase binding and thus repress gene expression. CRISPRi was established in *C. glutamicum* before CRISPR-based genome editing (Cleto et al., 2016). D*cas*9 and sgRNA were expressed from independent replicating plasmids, and their expression was controlled by IPTG induction using the P_{tac} promoter. When targeting the genes *pgi*, encoding phosphoglucoisomerase, *pck*, encoding phosphoenolpyruvate carboxykinase, and *pyk*, encoding pyruvate kinase, their expression was efficiently repressed by 98% (*pgi*, *pck*) and 97% (*pyk*) (Cleto et al., 2016). The timeline of CRISPRi was only three days, indicating the value of this approach for gene repression. Park and coworkers developed another two-plasmid CRISPRi system, in which dCas9 was expressed under the control of the *tetA* promoter and sgRNA was constitutively expressed (Park et al., 2018). For single genes, repression of between 75% and almost 100% was achieved. These authors also reported simultaneous double-gene repression. Here, reduced mRNA levels were obtained for both genes (Park et al., 2018). However, the degree of repression was influenced by the order of the sgRNA sequences in the plasmid. Moreover, the results from double-gene repression did not fully match those from single-gene experiments, considering the correlation between the mRNA levels and the observed phenotypes of the mutants. Nevertheless, with some refinements, the system promises quick and high-throughput multitarget gene repression for strain engineering (Park et al., 2018).

2.3. Engineering through adaptive evolution

Adaptive laboratory evolution is a classical and powerful method based on the selection of advantageous cellular properties through a process combining iterative genetic diversification (spontaneous or induced mutation) with selective pressure that mimics natural selection (Sauer, 2001; Wang et al., 2018). This method has proven valuable in improving cellular properties without detailed knowledge regarding metabolic and regulatory traits (Portnoy et al., 2011). It is especially useful when addressing characteristics that cannot be linked to a specific gene function, such as tolerance, growth rate and the overall fitness of a strain (Buschke et al., 2013b). Several studies have already bred *C. glutamicum* strains with increased tolerance towards oxidative stress (Lee et al., 2013), thermal and solvent stress (Oide et al., 2015), and lignocellulose derived inhibitors (Wang et al., 2018) and strains exhibiting faster growth (Pfeifer et al., 2017) and high plasmid copy numbers (Choi et al., 2018). In recent years, an increasing number of approaches have sought to improve concrete metabolic features, such as substrate utilization (Lee et al., 2016; Radek et al., 2017) and product formation (Jiang et al., 2013a; Li et al., 2018; Mahr et al., 2015). Coutilization of cellobiose and xylose was achieved in the formerly cellobiose- and xylose-negative strain *C. glutamicum* ATCC 13032 by a combined approach involving metabolic engineering and adaptive evolution (Lee et al., 2016). First, the codon-optimized *Neurospora crassa* genes *cdt*-1, encoding a cellobiose transporter, and *gh*1-1, encoding beta-glucosidase, were expressed in *C. glutamicum*. The resulting strains *Cg-Cello01* (*cdt*-1$^+$, *gh*1-1$^+$) and *Cg-Cello02* (*gh*1-1$^+$) were evolved in serial batches in mineral salt medium with 2% cellobiose until the evolved strains were able to consume the supplied cellobiose within 12 h. Interestingly, analysis of the *Cg-Cello01(evo)* strain revealed the loss of the *cdt*-1 gene, indicating that the heterologous transporter was not suitable for cellobiose utilization. Sole expression of beta-glucosidase was sufficient for growth on cellobiose as a unique carbon source. Whole-genome sequencing of the evolved strains *Cg-Cello01(evo)* and *Cg-Cello02(evo)* revealed multiple mutations. Selection of mutations occurring in both evolved strains combined with additional transcriptome data narrowed the relevant modifications to a consortium of transporters and metabolic and auxiliary proteins that are responsible for cellobiose uptake (Lee et al., 2016). By additionally introducing the *E. coli xylA* and *xylB* genes, encoding xylose isomerase and xylulokinase, simultaneous cofermentation of xylose and cellobiose was achieved (Lee et al., 2016). With regard to products, laboratory evolution was applied to improve the production of ornithine (Jiang et al., 2013a) and valine (Mahr et al., 2015). For the latter, a biosensor-aided approach was used to sort cells with the desired property, i.e., increased valine production, from iterative rounds of evolutive adaptation of the basic valine producer *C. glutamicum* Δ*aceE* (Mahr et al., 2015). The biosensor was based on the transcriptional regulator Lrp (Lange et al., 2012), which triggers a measurable fluorescent output as a response to intracellular valine detection (Mahr et al., 2015). Within five evolution steps, valine production by the evolved consortium was increased by 25%, which was accompanied by a 3- to 4-fold reduction in the formation of the by-product alanine (Mahr et al., 2015). Genome sequencing and re-engineering of the parental strain *C. glutamicum* Δ*aceE* identified a loss-of-function mutation in the gene *ureD* (urease accessory protein) as being responsible for an increase of up to 100% in valine production (Mahr et al., 2015). This study clearly demonstrates the power of biosensor-driven laboratory evolution for easy and straightforward selection of strains with desired metabolic traits among billions of mutants. Beyond the use of biosensors for the online monitoring and selection of improved strains, miniaturization and automation bear a huge potential for laboratory evolution by increasing throughput and standardization, and robotic workstations were recently applied to improve xylose utilization in *C. glutamicum* (Radek et al., 2017). The system is highly flexible and can perform adaptation processes for different microbes and conditions (Radek et al., 2017).

2.4. Biosensors

As mentioned above, the use of biosensors can be of vast help for adaptive evolution (Mahr et al., 2015). Moreover, biosensors can be applied for high-throughput screening (Eggeling et al., 2015; Liu et al., 2017a), optimization and regulation of heterologous pathways (Michener et al., 2012), live cell imaging (Mustafi et al., 2014), and dynamic regulation (Liu et al., 2017a; Zhang et al., 2015c). In principle, biosensors can be classified as (i) transcription factor (TF) sensors, (ii) riboswitch sensors, and (iii) protein function biosensors (protein-protein interaction (FRET), artificial proteins) (Eggeling et al., 2015; Liu et al., 2017a; Zhang et al., 2015c). Beyond the abovementioned Lrp-

based valine biosensor (Lange et al., 2012), other TF biosensors have been developed for *C. glutamicum* to sense cAMP (Schulte et al., 2017) and shikimate (Liu et al., 2017a). The shikimate sensor consisted of the LysR-type transcriptional regulator ShiR coupled to eGFP expression. The new sensor was used for real-time monitoring of shikimate production and for high-throughput screening of a RBS library coupled to FACS-based selection of strains with improved shikimate production (Liu et al., 2017a). The cAMP sensor relied on the global transcriptional regulator GlxR and the strongly GlxR-repressed promoter of the cg3195 gene, with eYFP as the reporter gene. The sensor was designed to screen libraries of single mutant cells with an altered cAMP level (Schulte et al., 2017). In addition to the TF biosensors, a toolbox of FRET-based biosensors for rapid L-lysine analysis was developed for *C. glutamicum* (Steffen et al., 2016). To construct the sensor, an optimized binding protein for L-lysine, L-arginine and L-ornithine (LAO-BP) from *E. coli* was flanked by two fluorescent proteins (eCFP, Citrine). Through circular permutation, the sensitivity of the sensor was substantially increased. The novel cpLAO-BP protein was then used to construct a sensor toolbox using different flexible and rigid linkers placed between cpLAO-BP and the fluorescent protein (Steffen et al., 2016). As a proof-of-concept, the sensor was used for the detection and quantification of L-lysine in supernatant samples. Table 1 gives an overview of the biosensors established for *C. glutamicum*.

2.5. Chassis strains

Despite the great advances in metabolic engineering in recent decades, the complexity of cellular metabolism and its regulation remains a challenge in strain development. Indeed, the genomes of microbes encode a vast quantity of proteins with regulatory, metabolic or signaling function that support survival in a harsh natural environment with continuously changing conditions. Moreover, there are recombinogenic and mobile DNA fragments or cryptic virulence genes encoded in the genome. However, for biotechnological purposes, these genes and proteins are unnecessary, with some being simply dispensable (Gao et al., 2010) while others may even be harmful (Choi et al., 2015). The ideal producer strain is equipped only with genes that support robust and fast growth on industrially relevant carbon sources and a streamlined pathway to synthesize the desired product. The minimal gene set ideally ensures full predictability, and none of the inserted gene functions should be influenced by the host (Unthan et al., 2015). Hence, several strains with reduced genomes have already been created (de Lorenzo, 2015; Gao et al., 2010; Lieder et al., 2015; Reuss et al., 2017). For *C. glutamicum,* two chassis strains were developed lacking insertion sequence (IS) elements of the major IS*Cg*1 and IS*Cg*2 families (Choi et al., 2015). These were shown to impair recombinant protein production, here GFP, by causing genetic rearrangements of the GFP coding region within the expression plasmid. The IS-cured strains WJ004 (IS*Cg*1-cured) and WJ008 (IS*Cg*2-cured) were then tested for their production potential for three models: GFP, poly(3-hydroxybutyrate) (PHB), and γ-aminobutyrate (GABA). For all products, WJ008 exhibited the best production properties, with 30% higher production of GFP, 38% higher accumulation of PHB, and 15% higher GABA production than the wild type strain (Choi et al., 2015). Another study reported the reduction of the *C. glutamicum* genome by approximately 6% through the elimination of prophage sequences (Baumgart et al., 2013). The resulting strain MB001 had a significantly increased transformation efficiency and an up to 30% improved production performance for heterologous proteins (Baumgart et al., 2013). MB001 then underwent additional genome reduction using a top-down approach for the identification and deletion of irrelevant gene clusters (Unthan et al., 2015). Overall, this approach identified 26 gene clusters that are not required to maintain the biological fitness of *C. glutamicum*. Even strains with some combinations thereof grew comparably to the wildtype. However, some combinations of gene cluster deletions were found to be incompatible or resulted in strains with reduced biological fitness (Unthan et al., 2015). Proceeding with the combinatorial deletion of the identified irrelevant gene cluster created a library of 28 strains, from which the final chassis strain C1* was selected (Baumgart et al., 2018). Despite a genome reduction of 13.4%, the growth performance of this chassis strain was similar to that of the wild type, including in long-term cultivation (Baumgart et al., 2018). Such genome-reduced chassis strains were successfully applied as hosts for the coproduction of astaxanthin and L-lysine (Henke et al., 2018). Beyond the creation of chassis strains with a generally reduced genome, *C. glutamicum* chassis strains with specific metabolic or structural features have been constructed. Two recent studies report the implementation of bacterial microcompartments (BMCs) in *C. glutamicum* (Baumgart et al., 2017; Huber et al., 2017). BMCs have evolved as distinct reaction chambers within the cell to protect the organism from toxic intermediates or to maintain specific reaction conditions for some enzyme functions, i.e., increased CO_2 levels. In principle, they are classified as carboxysomes with anabolic function, generally involving carboxylating reactions, and as metabolosomes with catabolic function (Huber et al., 2017). Introduction of the carboxysomal gene cluster of *Halothiobacillus neapotitanus* into *C. glutamicum* resulted in the formation of BMC-like structures and functional expression of RuBisCO (Baumgart et al., 2017). Using the large subunit of RuBisCO as a leader protein, the model protein eYFP could be successfully targeted to the carboxysome (Baumgart et al., 2017). A distinct microcompartment could also be

Table 1
Biosensors developed for screening and life-cell imaging of *Corynebacterium glutamicum.*

Analyte	Type	Sensor	Source	Signal	Description	Ref.
L-Arginine	TF	LysG	*C. glutamicum*	eYFP	Screening of mutant libraries for feedback-deregulated ArgB, LysC and HisG for the production of L-arginine, L-lysine and L-histidine	(Schendzielorz et al., 2014)
cAMP	TF	GlxR	*C. glutamicum*	eYFP	Screening of mutant libraries for altered cAMP levels	(Schulte et al., 2017)
L-Lysine	TF	LysG	*C. glutamicum*	eYFP	Screening of mutant libraries from chemical mutagenesis for increased L-lysine production and identification of new targets for improvement by genome sequencing	(Binder et al., 2012)
L-Lysine	FRET	LAO-BP	*E. coli*	eCFP Citrine	Fine-tuning of sensor sensitivity and construction of a sensor toolbox. Detection and quantification of L-lysine in supernatant	(Steffen et al., 2016)
Shikimate	TF	ShiR	*C. glutamicum*	eGFP	Screening of RBS libraries and FACS-based selection of improved shikimate producers	(Liu et al., 2017a)
L-Valine	TF	Lrp	*C. glutamicum*	GFP	Sensor for L-methionine and branched-chain amino acids. Preference for L-valine. Monitoring of production heterogeneity and screening of mutant libraries for increased production of L-valine	(Mustafi et al., 2012)
L-Valine	TF	Lrp	*C. glutamicum*	eYFP	Screening and FACS of cells with improved L-valine production obtained by adaptive evolution. Sequencing guided target identification	(Mahr et al., 2015)
L-Valine	TF	Lrp	*C. glutamicum*	eYFP	Monitoring of single-cell productivity of L-valine producing *C. glutamicum*	(Mustafi et al., 2014)

ArgB: N-acetyl-l-glutamate kinase; (e)CFP: (enhanced) cyan fluorescing protein; (e)GFP: (enhanced) green fluorescing protein (e)YFP: (enhanced) yellow fluorescing protein; FRET: Förster (or fluorescence) resonance energy transfer; Lrp: transcriptional regulator; LysC: Aspartokinase; LysG: lysine export transcriptional regulatory protein; HisG: ATP phosphoribosyltransferase; RBS: ribosome binding site; ShiR: lysR-type transcriptional regulator; TF: transcription factor.

established by expressing optimized gene clusters of the *Citrobacter freundii* propanediol utilization bacterial compartment (Huber et al., 2017). Empty compartments could be equipped with eYFP through C-terminal targeting of the protein with synthetic scaffold interaction partners as well as with a nonnative peptide (Huber et al., 2017). These microcompartmented chassis strains can be of great value for future production processes. Other approaches for the creation of chassis strains focused on the metabolism of *C. glutamicum* rather than on its cellular structure. Throughout evolution, Nature has generated metabolic pathway synergies for the production of metabolic compounds, which have been recruited in recent years for biotechnological production processes. One example is the systems metabolic engineering of *C. glutamicum* for the production of L-lysine (Becker et al., 2011) and L-lysine-derived compounds (Kind et al., 2014; Rohles et al., 2016). The core of these studies was a streamlined production strain for L-lysine, which serves both as workhorse for L-lysine production (Becker et al., 2011), and as a metabolic chassis for the production of diaminopentane (Kind et al., 2014), aminovalerate and glutarate (Rohles et al., 2016). The optimized L-lysine strain was constructed in twelve iterative rounds whereby the metabolism was optimized with regard to L-lysine biosynthesis, the supply of oxaloacetate and NADPH, and the reduction of carbon loss through competing pathway reactions (Becker et al., 2011). Another study reported the generation of a platform producer for carotenoids. Here, *C. glutamicum* was engineered for the overproduction of lycopene by plasmid-borne expression of the lycopene gene cluster and elimination of the downstream metabolic pathway. This strain was then used as a chassis for the expression of lycopene-processing genes to obtain producers of various C50 carotenoids (Heider et al., 2014). Other examples include 2-ketoisovalerate-producing *C. glutamicum* as a chassis for isobutanol (Blombach et al., 2011), ornithine-derived putrescine production (Schneider and Wendisch, 2010), the delAro^4 strain, lacking the catabolism of aromatic compounds, for the production of aromatics (Kallscheuer et al., 2016), and glutamate-derived γ-aminobutyrate (GABA) production (Shi and Li, 2011).

3. Systems metabolic engineering of *C. glutamicum*

3.1. Proteinogenic amino acids

Amino acid production can be considered the “core business” of *C. glutamicum*. Starting with the production of L-glutamate in the 1950s (Kinoshita et al., 1957), the amino acid market has continuously grown over the past decades to become a highly competitive billion dollar business (Becker and Wittmann, 2012b; Eggeling and Bott, 2015; Leuchtenberger et al., 2005). Of the large collection of proteinogenic amino acids that are now accessible by fermentation of *C. glutamicum* (Fig. 1), recent achievements in the production of L-lysine, L-isoleucine and L-methionine are highlighted in this review. Their biosynthetic pathways are depicted in Fig. 3A.

3.1.1. L-Lysine

L-Lysine is an essential amino acid that is mainly applied as a feed additive for pigs and poultry. Within the global amino acid market, L-lysine production is the fastest growing segment (Eggeling and Bott, 2015), with a current production of approximately 2.4 million tons per year (Lee and Wendisch, 2017). In the field of metabolic engineering, substantial effort has been made to improve the producer strains for industrial application, focusing on (i) the supply of carbon building blocks, (ii) NADPH supply, (iii) biosynthesis and export and (iv) competing pathways (Becker et al., 2016; Eggeling and Bott, 2015; Wittmann and Becker, 2007). In recent years, the use of alternative feedstocks has become a focus of research, and several strains have been created that produce L-lysine from starch (Seibold et al., 2006; Tateno et al., 2007a, 2007b), xylose (Meiswinkel et al., 2013a), arabinose (Schneider et al., 2011), glycerol (Meiswinkel et al., 2013b), cellobiose (Adachi et al., 2013), and mannitol (Hoffmann et al., 2018). A key study in metabolic engineering used the design-based approach to generate the L-lysine hyper-producing strain *C. glutamicum* LYS-12 (Becker et al., 2011). Upon the introduction of twelve carefully selected genetic modifications, a wild type strain was transformed into an industrially competitive producer achieving a production performance of 120 g L^{-1} L-lysine within 30 h, with a yield of 55% (Becker et al., 2011). Most recent engineering studies also focused on the abovementioned key targets. NADPH-supply and thus L-lysine production were improved by the construction of strains with a NADPH-providing glycolytic chain utilizing a heterologous NADP-dependent glyceraldehyde dehydrogenase from either *Streptococcus mutans* (GapN) (Takeno et al., 2010) or *Clostridium acetobutylicum* (GapC) (Xu et al., 2014) or a native but engineered GapA enzyme variant from *C. glutamicum* (Bommareddy et al., 2014). Use of GapN even allowed L-lysine production independently of the oxidative pentose phosphate pathway (Takeno et al., 2016). Engineering of the NADPH supply was also the key to success for efficient L-lysine production from the third-generation substrate mannitol (Hoffmann et al., 2018). In the first round of engineering, the mannitol-consuming and L-lysine-producing strain SEA-1, lacking the mannitol repressor MtlR, was equipped with a heterologous fructokinase to push carbon towards the pentose phosphate pathway (Hoffmann et al., 2018). This pushing strategy slightly increased the PPP flux and improved L-lysine production. The remaining high glycolytic flux suggested the use of the NADP-dependent GapN variant to couple glycolysis to NADPH production. This strategy improved L-lysine production by 60% (Hoffmann et al., 2018). For improved oxaloacetate supply, PEP carboxylase was rationally engineered to remove allosteric regulation (Chen et al., 2014). Introduction of the point mutation N917G improved L-lysine production by 37% (Chen et al., 2014). Moreover, reduced expression of pyruvate dehydrogenase (Buchholz et al., 2013), isocitrate dehydrogenase (Becker et al., 2009) and citrate synthase (van Ooyen et al., 2012; Yanase et al., 2016) increased L-lysine production. Upon pyruvate kinase deletion, however, different effects were observed (Becker et al., 2008; Gubler et al., 1994; Shiio et al., 1987) depending – as recently demonstrated (Yanase et al., 2016) – on the genetic background of the strain. An interesting study reported the coupling of the tricarboxylic acid (TCA) cycle with L-lysine biosynthesis via the common intermediate succinyl-CoA, a strategy that improved L-lysine production by 60% (Kind et al., 2013). Though well known as key enzyme for L-lysine production, the aspartokinase enzyme remains an engineering target of high interest and is still subject for deregulation (Chen et al., 2011; Schendzielorz et al., 2014). Despite its obviously prominent role in L-lysine production, diaminopimelate metabolism was engineered only recently. Various mutations in the *murE* gene, encoding UDP-N-acetylmuramoyl-L-alanyl-D-glutamate:meso-diaminopimelate ligase led to increased L-lysine production (Binder et al., 2012, 2013; Hochheim et al., 2017). The classical aerobic fermentation processes for L-lysine have been complemented in the last decade by proof-of-concept studies on anaerobic L-lysine production, the feasibility of which was first demonstrated by Takeno and coworkers using agar piece assays for L-lysine production under anoxic conditions with nitrate respiration (Takeno et al., 2007). Anaerobic L-lysine production was also possible via electro-fermentation (Xafenias et al., 2017). Production was thereby mainly influenced by the gas environment (with CO_2 being superior to nitrogen), electrode potential (with 2-fold and 4-fold higher titer and yield under reductive conditions), and use of redox mediators (Xafenias et al., 2017). Using an anodic approach with ferricyanide as an extracellular electron carrier (Vassilev et al., 2018), 2.9 mM L-lysine was produced by *C. glutamicum* under anaerobic conditions. The setup also supported cell growth, indicating an appropriate energy supply under oxidizing conditions (Vassilev et al., 2018).

3.1.2. L-Isoleucine

L-Isoleucine is an essential branched-chain amino acid that serves as a major supplement in infusions and special diets. Its biosynthesis is closely interlinked with that of other amino acids, including L-lysine, L-

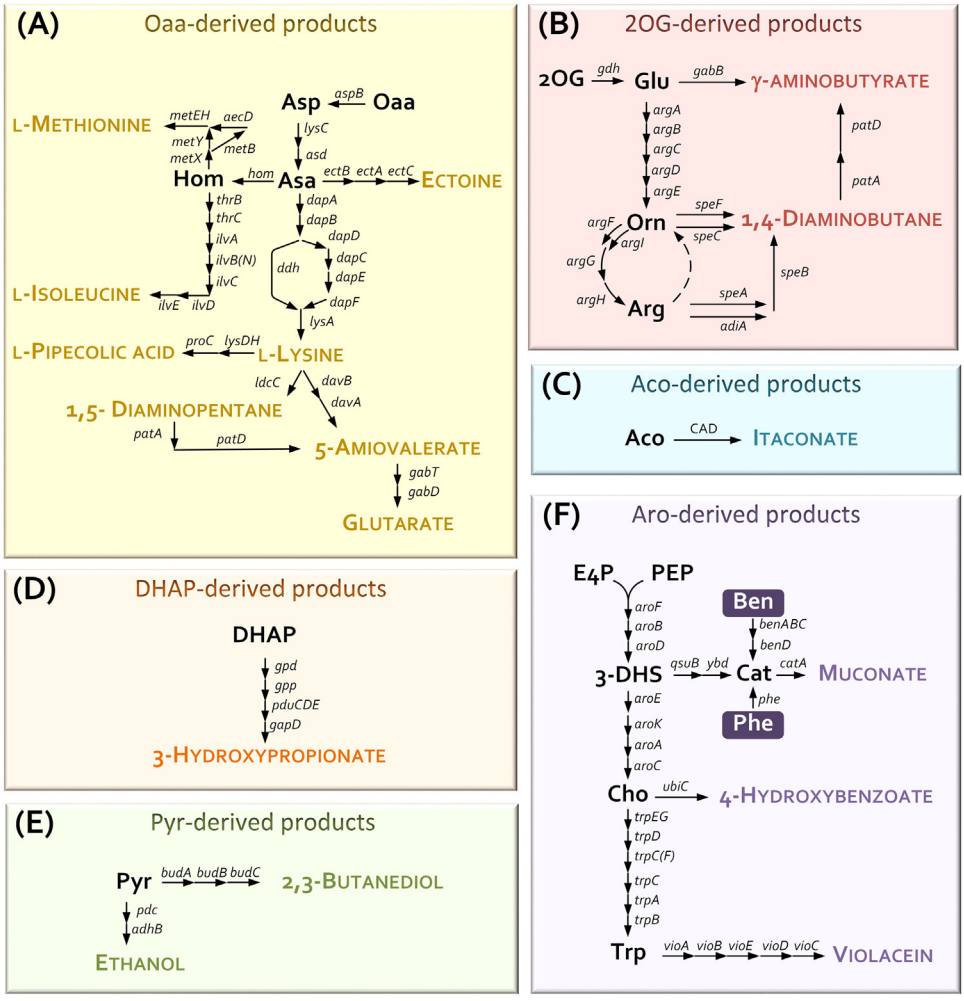

Fig. 3. Biosynthetic routes established in *C. glutamicum* for the production of (A) Oaa-derived products: 1,5-diaminopentane, 5-aminovalerate, ectoine, glutarate, L-isoleucine, L-lysine, L-methionine, L-pipecolic acid; (B) 2OG-derived products: 1,4-diaminobutane, γ-aminobutyric acid; (C) Aco-derived products: itaconic acid; (D) DHAP-derived products: 3-hydroxypropionate; (E) Pyr-derived products: 2,3-butanediol, ethanol; (F) Aro-derived products: 4-hydroxybenzoate, *cis*,*cis*-muconic acid, violacein. The biotransformation substrate benzoate and phenol are highlighted in purple. Metabolite abbreviation: 2OG: 2-oxoglutarate; 3-DHS: 3-dehydroshikimate; Aco: aconitate; Arg: L-arginine; Asa: L-aspartate semialdehyde; Asp: L-aspartate; Ben: benzoate Cat: catechol; Cho: chorismate; DHAP: dihydroxyacetone phosphate; E4P: erythrose 4-phosphate, Glu: L-glutamate; Hom: homoserine; Orn: L-ornithine; PEP: phosphoenolpyruvate; Pyr: pyruvate; Trp: L-tryptophan. Enzymes are encoded as follows: *adhB*: alcohol dehydrogenase; *adiA*, arginine decarboxylase; *aecD*: cystathionine β-lyase; *argA*, *argB*, acetyl-glutamate kinase; *argC*, N-acetylglutamylphosphate reductase; *argD*, acetyl-ornithine aminotransferase; *argE*, acetyl-ornithinase; *argF*, ornithine carbamoyltransferase; *argG*, arginino-succinate synthase, *argH*, arginine-succinate lyase; *argI*, bifunctional ornithine/N-acetylglutamate acetyltransferasase; *aroA*: 3-phosphoshikimate 1-carboxyvinyltransferase; *aroB*: 3-dehydroquinate synthase; *aroC*: chorismate synthase; *aroD*: 3-dehydroquinate dehydratase; *aroE:* shikimate 5-dehydrogenase*; aroF*: 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase; *aroK*: shikimate kinase; *asd*: aspartate semialdehyde dehydrogenase; *aspB*: aspartate aminotransferase; *benABC*: benzoate 1,2-dioxygenase; *benD*: benzoate diol dehydrogenase; *budA*: α-acetolactate decarboxylase; *budB*: α-acetolactate synthase; *budC*: acetoin reductase; CAD: aconitate decarboxylase; *catA*: muconate cycloisomerase; *dapA*: dihydrodipicolinate synthase; *dapB*: dihydrodipicolinate reductase; *dapD*: tetrahydrodipicolinate succinylase; *dapC*: succinyl-amino-ketopimelate transaminase; *dapE*: succinyl-diaminopimelate desuccinylase; *dapF*: diaminopimelate epimerase; *davA*: 5-aminovaleramidase; *davB*: lysine-monooxygenase; *ddh*: diaminopimelate dehydrogenase; *gabB*: glutamate decarboxylase; *gabD*: glutarate semialdehyde dehydrogenase; *gabT*: 5-aminovalerate transaminase; *gapD*: aldehyde dehydrogenase; *gdh*: glutamate dehydrogenase; *gpd*: glycerol 3-phosphate dehydrogenase; *gpp*: glycerol 3-phosphate phosphatase; *hom*: homserine dehydrogenase; *ilvA*: threonine dehydratase; *ilvBN*: acetohydroxyacid synthase; *ilvC*: acetohydroxyacid isomeroreductase, *ilvD*: dihydroxy acid dehydratase; *ilvE*: branched chain amino acid transaminase; *ldcC*: lysine decarboxylase; *lysA*: diaminopimelate decarboxylase; *lysC*: aspartokinase; *lysDH*: lysine 6-dehydrogenase; *metB*: cystathionine γ-synthase; *metEH*: methionine synthases; *metX*: homoserine acetyltransferase; *metY*: O-acetlyhomoserine sulfhydrolase; *patA*: putrescine/cadaverine transaminase; *patD*: γ-aminobutyraldehyde dehydrogenase; *pdc*: pyruvate decarboxylase; *pduCDE*: diol dehydrogenase; *phe*: phenol 2-monooxygenase; *proC*: pyrroline 5-carboxylate reductase; *qsuB*: 3-dehydroshikimate dehydratase; *speA*: arginine decarboxylase; *speB*: agmatine ureo-hydrolase; *speC*: ornithine decarboxylase; *speF*: ornithine decarboxylase; *thrB*: homoserine kinase*; thrC:* threonine synthase*; trpAB*: tryptophan synthase, *trpC*: indole-3-glycerol phosphate synthase, *trpCF*: phosphoribosyl anthranilate isomerase, *trpD*: anthranilate phosphoribosyl transferase, *trpEG*: anthranilate synthase; *ubiC*: chorismate:pyruvate lyase; *vioA*: tryptophan oxidase; *vioB*: chromopyrrolate synthase; *vioC*: deoxyviolaceinate synthase; *vioD*: protoviolaceinate synthase; *vioE*: prodeoxyviolacein synthase; *ybd*: protocatechuate decarboxylase.

threonine, L-valine and L-leucine (Fig. 3A). Indeed, there is only one enzyme, threonine dehydratase (TD), that is unique for L-isoleucine biosynthesis (Li et al., 2017), which presents a major challenge for strain engineering. Production hosts for L-isoleucine were mainly L-threonine- and L-lysine-producing mutants of *C. glutamicum* with feedback-insensitive enzyme variants of aspartokinase, homoserine dehydrogenase (HD), homoserine kinase and TD (Guillouet et al., 2001; Sahm et al., 1996). Because of its key function in L-isoleucine production, TD has been a major subject for engineering. For example, heterologous expression of the *E. coli* TD variant, encoded by *tdcB*, increased L-isoleucine production by 4- to 12-fold compared to overexpression of the native enzyme, encoded by *ilvA* (Guillouet et al., 1999; Guillouet et al., 2001). In addition, various enzyme variants of TD were created with improved catalytic properties to increase L-isoleucine production (Guo et al., 2015b; Yin et al., 2012). In addition to TD, acetohydroxy acid synthase (AHAS) also plays an important role in flux control in L-isoleucine biosynthesis. Similarly to TD, different enzyme variants, mutated in the catalytic or regulatory subunit, were tested for L-isoleucine production (Guo et al., 2015a). Coexpression of feedback-resistant AHAS (P176S, A426G, L575W) and TD (F383V) in a classical L-isoleucine producer led to a 132% increase in production. In fed-batch fermentation, the strain accumulated 31 g L^{-1} L-isoleucine (Yin et al., 2012), the highest titer so far reported for this amino acid. As mentioned, many L-isoleucine producers were developed from L-lysine- or L-threonine-producing strains and were thus often impaired by by-product formation. One strategy for improved production involved attenuating L-lysine production through the deletion of *ddh* and *lysE*, encoding diaminopimelate dehydrogenase and lysine exporter. Deletion of these genes reduced by-product formation by 86%; however, only a small amount of the "gained" carbon was redirected to L-isoleucine

production, with the remainder stimulating the formation of other by-products, including L-alanine and L-glutamate (Dong et al., 2016). Another study reported the introduction of defined point mutations in the background of L-lysine-producing *C. glutamicum* MH20–22B. These mutations removed feedback inhibition of TD ($ilvA^{V323A}$) and HD (hom^{G378E}) and generated promoter variants for modulated expression of *ilvA*, *hom* and *dapA*. The best combination of genomic mutations created a strain that produced 14 g L^{-1} L-isoleucine without substantial by-product formation (Vogt et al., 2015). Beyond the engineering strategies described here in detail, additional engineering was carried out targeting the biosynthetic pathway (Wang et al., 2013), supply of NADPH (Ma et al., 2016; Shi et al., 2013b), transport processes (Xie et al., 2012; Yin et al., 2013), and proteins with global function (Yin et al., 2013; Zhao et al., 2015). Notably, *C. glutamicum* is also able to produce L-isoleucine via an L-threonine-independent route (Krömer et al., 2006a); however, this route has not been engineered to date.

3.1.3. L-Methionine

The sulfur-containing amino acid L-methionine has one of the largest shares in the global amino acid market (Becker and Wittmann, 2012b). Interestingly, it is the only feed amino acid that is not produced by fermentation, with its production largely relying on the chemical synthesis of the racemate D,L-methionine (Becker et al., 2012). However, bio-based pure L-methionine is provided by the company CheilJedang (CJ) in a two-step process comprising fermentative production of succinyl-homoserine or acetyl-homoserine and subsequent enzymatic conversion of these precursors into L-methionine (Hong et al., 2016; Li et al., 2017). The reason for the lack of a fully fermentative production route lies in the inherent complexity of the biosynthetic pathway regulation, which involves metabolic inhibition and repression mediated by L-methionine and several pathway intermediates (Lee and Hwang, 2003). Moreover, the global transcriptional regulator McbR plays the primary role in controlling the expression of the L-methionine synthetic genes (Krömer et al., 2008; Rey et al., 2005, 2003). Still, pathway simulations to elucidate the production potential of *C. glutamicum* are promising (Krömer et al., 2006b), especially considering the use of reduced sulfur sources, such as methanethiol (Bolten et al., 2010). Production of L-methionine was achieved in *C. glutamicum* by introducing a feedback-deregulated HD, obtained by random mutagenesis, in the L-lysine-producing strain MH20–22B (Park et al., 2007). To avoid L-threonine accumulation, the *thrB* gene, encoding homoserine kinase, was deleted. The resulting strain produced 2.9 g L^{-1} L-methionine. The major product was, however, L-lysine (24 g L^{-1}) (Park et al., 2007). In recent studies, the L-methionine titer was substantially increased to 6.9 g L^{-1} (Li et al., 2016) and 6.3 g L^{-1} (Qin et al., 2015). The overall pathway engineering of the best strain is displayed in Fig. 4. Starting from the wild type ATCC 13032, the global regulator *mcbR* was deleted to release the L-methionine pathway from transcription control, and *thrB* was deleted to reduce carbon loss by L-threonine formation (Qin et al., 2015). Moreover, feedback-resistant aspartokinase and homoserine dehydrogenase, as well as the export protein complex BrnFE, were overexpressed from a plasmid (Qin et al., 2015). Li and coworkers used a combined approach of metabolic engineering and random mutagenesis (Li et al., 2016). First, L-methionine uptake was disabled by deleting *metD*. Subsequently, a strain that was resistant to the L-methionine analogs ethionine, norleucine and methionine methylsulfonium chloride was isolated and further modified by deleting *thrB* and introducing $lysC^{T311I}$ (to prevent the feedback inhibition of biosynthesis), pyc^{P458S} (to improve the supply of oxaloacetate (Ohnishi et al., 2002)), zwf^{A243T} and gnd^{S361F}, the latter being responsible for improved NADPH supply (Becker et al., 2007; Ohnishi et al., 2005). In addition, the start codons of *dapA* (A → G) and *pyc* (G → A) were exchanged to modulate expression. The major product in this strain was 6.9 g L^{-1} L-methionine, with some remaining synthesis of L-lysine (~ 1 g L^{-1}) and L-aspartate (~ 1 g L^{-1}) as by-products (Li et al., 2016).

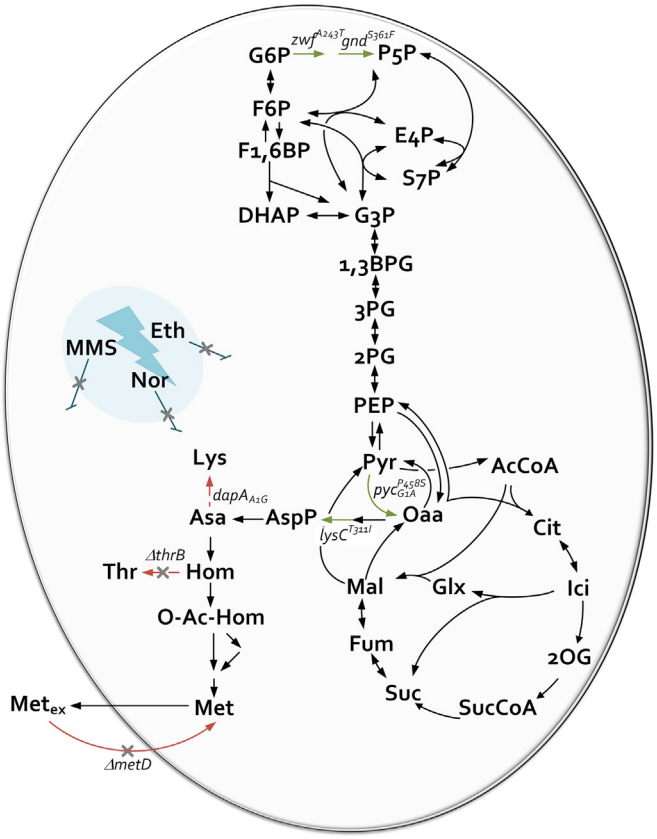

Fig. 4. Systems metabolic engineering of *C. glutamicum* for the production of L-methionine. A combined approach of random mutagenesis and metabolic engineering was applied (Li et al., 2016). Reactions with increased flux through amplified expression or removal of feedback inhibition are highlighted in green. Red denotes reactions with reduced or abolished fluxes. Crosses indicate (i) resistance to the methionine analogs ethionine (Eth), norleucine (Nor) and methionine methylsulfonium chloride (MMS) and (ii) gene deletion with loss of the corresponding gene function. $dapA_{A1G}$: gene encoding dihydrodipicolinate synthase with start codon exchange (ATG→GTG); gnd^{S361F}: gene, encoding 6-phosphogluconate dehydrogenase with amino acid exchange serine → phenylalanine at position 361; $lysC^{T311I}$: gene, encoding aspartokinase with amino acid exchange threonine → isoleucine at position 311; *metD*: gene, encoding methionine uptake system; pyc^{P458S}_{G1A}: gene encoding pyruvate carboxylase with start codon exchange (GTG → ATG) and amino acid exchange proline → serine at position 458; *thrB*: gene, encoding homoserine kinase; zwf^{A243T}: gene, encoding glucose 6-phosphate dehydrogenase with amino acid exchange alanine → threonine at position 243. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Nonproteinogenic amino acids

In addition to the proteinogenic amino acids, the nonproteinogenic amino acids ornithine (Udaka and Kinoshita, 1958) and citrulline (Ikeda et al., 2009) are classical products of *C. glutamicum* and are still the subject of many research studies (Eberhardt et al., 2014; Hwang and Cho, 2014; Kim et al., 2015a, 2015b; Lubitz et al., 2016; Schneider et al., 2011; Zhang et al., 2017). Here, we focus on a selection of nonproteinogenic amino acids that are not naturally produced by *C. glutamicum*.

3.2.1. γ-aminobutyric acid

GABA is a widely used nonproteinogenic amino acid and can serve as a building block for pharmaceuticals, foods and biodegradable plastics (Jorge et al., 2016). Its production is closely connected to the L-glutamate pathway (Fig. 3B) and can be easily implemented in *C. glutamicum* through heterologous expression of the glutamate

decarboxylase *gabB* from either *Lactobacillus brevis* (Shi and Li, 2011) or *E. coli* (Okai et al., 2014; Takahashi et al., 2012). Coexpression of the two alleles *gabB1* and *gabB2* combined with an optimized urea supply improved the production to 26 g L^{-1} after 60 h of fed-batch fermentation (Shi et al., 2013a). Increased supply of the GABA precursor L-glutamate was achieved by deleting the *pknG* gene encoding serine/threonine protein kinase G (Okai et al., 2014), which controls the activity of the 2-oxoglutarate dehydrogenase complex (ODHC) (Niebisch et al., 2006), a key enzyme in L-glutamate production (Asakura et al., 2007; Kim et al., 2009). Upon *pknG* deletion, GABA production was increased by 2.3-fold (Okai et al., 2014). Similarly, direct deletion of *odhA*, encoding a subunit of ODHC, improved GABA production (Wang et al., 2015). L-Glutamate supply and thus GABA production were also enhanced by deleting the gene encoding pyruvate carboxylase (*pyc*) (Wang et al., 2015). The increased expression of *ppc*, encoding PEP carboxylase, *icd*, encoding isocitrate dehydrogenase, and *gdh*, encoding glutamate dehydrogenase, entailed by *pyc* deletion, was likely responsible for the improved production performance (Wang et al., 2015). Aside from the L-glutamate route, GABA production was established in 1,4-diaminobutane-producing *C. glutamicum* via expression of the putrescine transaminase (PatA) and γ-aminobutyraldehyde dehydrogenase (PatD) genes of *E. coli* (Jorge et al., 2016) (Fig. 3B). The base strain was then further improved by deleting the genes *gabT* and *gabD*, encoding γ-aminobutyrate aminotransferase and succinate-semialdehyde dehydrogenase, respectively, which catalyze GABA degradation, and *gapP*, encoding a GABA-specific importer that is responsible for GABA uptake from the medium (Jorge et al., 2016). In addition, the supply of the precursor 1,4-diaminobutane was improved by abolishing N-acetyl-diaminobutane formation. The resulting strain produced 8 g L^{-1} GABA. In a further study, GABA production by this strain was enhanced by engineering L-glutamate metabolism (Jorge et al., 2017a). Here, consumption of the L-glutamate precursor 2-oxoglutarate by ODHC was reduced by start codon engineering (ATG→TTG) of the E1 subunit of ODHC. Moreover, a T15A mutation was introduced into the ODHC inhibitory protein OdhI, preventing its phosphorylation and thus maintaining OdhI in its inhibitory form. Consequently, the GABA titer was increased to 63 g L^{-1} (Jorge et al., 2017a). Moreover, the substrate spectrum for GABA production was extended to xylose, glucosamine and N-acetyl-glucosamine (Jorge et al., 2017a).

3.2.2. Aminovalerate

Several species are capable of degrading L-lysine via the 5-aminovalerate (5AVA) pathway, which involves reactions mediated by L-lysine-monooxygenase, encoded by *davB*, and 5-aminovaleramidase, encoded by *davA* (Fig. 3A). 5AVA is an interesting building block for bio-based plastics and other chemical synthesis routes and is thus of industrial interest. The first system for *de novo* bio-based production of 5AVA in *C. glutamicum* was established via stable genome-based integration of the *Pseudomonas putida davBA* gene cluster under the control of a strong constitutive promotor in the background of the L-lysine hyper-producing strain LYS-12 (Rohles et al., 2016). The remaining L-lysine secretion in the basic producer was successfully eliminated by deleting the L-lysine exporter gene *lysE*. Related to the intrinsic *gabTD* genes, 5AVA was partly metabolized to glutarate. Elimination of the initial reaction of the glutarate pathway generated an optimized 5AVA cell factory that exhibited an excellent production performance, with a titer of 28 g L^{-1} and a productivity of 0.9 g L^{-1} h^{-1} (Rohles et al., 2016). Another study reported plasmid-borne expression of codon-optimized *davB* and His-tagged *davA* in the background of the industrial L-lysine producer BE, whereby different synthetic promotors as well as various origins of replication were tested (Shin et al., 2016). The best expression system, containing codon-optimized *davBA* under the control of the H_{36} promotor, allowed the production of 33 g L^{-1} of 5AVA in a *gabT* deletion mutant of BE (Shin et al., 2016). To further enhance 5AVA production, the best L-lysine producer was chosen from different classically optimized industrial strains and used as a host for the expression of the native *davBA* genes of *P. putida* or codon-optimized *davBA* genes (Joo et al., 2017). Interestingly, the native gene variant gave the best production, with a final titer of 40 g L^{-1} 5AVA (Joo et al., 2017). This strain was also used to produce 12.5 g L^{-1} 5AVA directly from *Miscanthus* hydrolysates (Joo et al., 2017). An alternative route to 5AVA in *C. glutamicum* originated from diaminopentane (Fig. 3A) and relied on the expression of cadaverine/putrescine transaminase, encoded by *patA*, and γ-aminobutyraldehyde dehydrogenase, encoded by *patD*, from *E. coli* (Jorge et al., 2017b). Following this strategy, a final titer of 5.1 g L^{-1} was achieved after eliminating by-products such as DAP, N-acetyl-DAP and glutarate (Jorge et al., 2017b). The strain was further engineered to utilize different substrates, including xylose (by expressing *xylA* from *Xanthomonas campestris* and *xylB* from *C. glutamicum*), arabinose (by expressing the *E. coli* arabinose operon *araBAD*), starch (by expressing *amyA* from *Streptomyces griseus*) and glucosamine (by expressing *nagB* from *C. glutamicum*) (Jorge et al., 2017b).

3.2.3. Pipecolic acid

The nonproteinogenic amino acid L-pipecolic acid (L-PA) can be used as a precursor of immunosuppressants or peptide antibiotics (Perez-Garcia et al., 2016). As L-PA is synthesized from L-lysine (Fig. 3A), the genome-reduced L-lysine-producing strain GRLys1 (Unthan et al., 2015) was chosen as the production host to establish L-PA production in *C. glutamicum* (Perez-Garcia et al., 2016). Introduction of a synthetic operon containing the L-lysine 6-dehydrogenase gene *lysDH* from *Silicibacter pomeroyi* and the endogenous pyrroline 5-carboxylate reductase *proC* enabled L-PA production from glucose with a yield of 0.09 g g^{-1}, while L-lysine remained the major product in this strain. L-PA formation was enhanced by deleting the L-lysine exporter *lysE*, and L-lysine was simultaneously eliminated as an undesired by-product (Perez-Garcia et al., 2017a). Further improved expression levels of *lysDH* and *proC* in fed-batch fermentation increased L-PA production up to a titer of 14.4 g L^{-1}, with increased productivity of 0.21 g L^{-1} h^{-1} (Perez-Garcia et al., 2017a). Moreover, additional metabolic engineering of substrate assimilation pathways allowed L-PA production from alternative carbon sources, including glycerol, xylose, starch and glucosamine (Perez-Garcia et al., 2017a).

3.2.4. Ectoine

Ectoine is a cyclic nonproteinogenic amino acid and naturally serves as an osmoprotectant in halophilic bacteria (Kunte et al., 2014). It is regularly used in the cosmetics industry, with current production at the ton scale. Its biosynthesis shares aspartate semialdehyde as a common intermediate with L-lysine biosynthesis (Fig. 3A), enabling ectoine production in the background of an L-lysine-producing base strain of *C. glutamicum* by the heterologous expression of the *Pseudomonas stutzeri* ectoine gene cluster *ectABCD* (Becker et al., 2013). The integration locus *ddh*, encoding the diaminopimelate dehydrogenase branch of L-lysine biosynthesis, reduced carbon flux from the common intermediate aspartate-semialdehyde towards L-lysine. Full elimination of by-product formation, however, relied on deletion of the L-lysine exporter LysE (Becker et al., 2013). The use of the constitutive promoter P_{tuf} decoupled ectoine production from the corrosive high-salinity conditions that are typically required by native producers (Becker et al., 2013). In a fed-batch process, the optimized strain ECT-2 produced 4.5 g L^{-1} ectoine, with a yield of 0.24 g g^{-1} and a productivity of 6.7 g L^{-1} d^{-1} (Becker et al., 2013). Another study reported ectoine production by heterologous expression of the *ectABC* gene cluster of *Chromohalobacter salexigens* in the background of a more advanced L-lysine producer, comprising the modifications $lysC^{T311I}$, pyc^{P458S} and hom^{V59A}, with additional deletion of the sugar regulator *sugR* and *ldhA*, encoding lactate dehydrogenase (Perez-Garcia et al., 2017b). Production was thereby increased to 22 g L^{-1}, with a productivity of 7.7 g L^{-1} d^{-1} (Perez-Garcia et al., 2017b). However, the yield was somewhat lower (0.16 g g^{-1}) than previously reported, likely because of the high carbon

loss that resulted from the formation of the by-product L-lysine (~ 12 g L^{-1}). Interestingly, deletion of *lysE* did not improve ectoine production and instead resulted in reduced titers and perturbed growth (Perez-Garcia et al., 2017b).

3.3. Diamines

3.3.1. 1,5-Diaminopentane

Diaminopentane (DAP) is a carbon-5 diamine that is formed in several species as an intermediate in L-lysine degradation (Fig. 3A). Its production has become attractive because of its potential function as a polymer building block (Kind and Wittmann, 2011). DAP production was first demonstrated in *C. glutamicum* by heterologous expression of the L-lysine decarboxylase *cadA* from *E. coli* (Mimitsuka et al., 2007). However, expression of *ldcC,* an *E. coli* L-lysine decarboxylase with a working optimum at neutral pH, proved to be much more beneficial (Kind et al., 2010a). The expression efficiency of *ldcC* was further increased by using a codon-optimized *ldcC* gene under the control of the strong constitutive promoter P_{tuf} (Kind et al., 2010a). The relevance of *ldcC* expression was also demonstrated in a study that tested a set of different synthetic promoter sequences, in which the production of DAP ranged from 0 g L^{-1} to 28 g L^{-1} according to the promoter (Oh et al., 2015). Detailed investigation of DAP-producing *C. glutamicum* with a combined approach of metabolome and transcriptome analysis led to the identification of the DAP-associated by-product N-acetyl-DAP and elimination of its production through the identification and deletion of the gene encoding the responsible N-acetyltransferase (Kind et al., 2010b). The product yield was further enhanced upon the identification and overexpression of a previously unknown DAP exporter, which resulted in a 20% increased yield (Kind et al., 2011). Improved secretion of DAP was also achieved by heterologous expression of the *E. coli* DAP-L-lysine-antiporter *cadB* (Li et al., 2014). Combination of specific beneficial modifications (Fig. 5) in the L-lysine-producing chassis strain Lys-12 generated the super-producing cell factory DAP-16, exhibiting a glucose-based conversion yield of 50% (Kind et al., 2014). During fed-batch fermentation, a titer of 88 g L^{-1} DAP was achieved, with a space-time yield of 2.2 g L^{-1} h^{-1}. Subsequent purification of DAP and co-polymerization with either succinic acid or sebacic acid produced the first fully bio-based polyamides, PA5.4 and PA 5.10 (Kind et al., 2014). Most recently, several randomly created industrial L-lysine producers were tested as chassis for DAP-production using *ldcC* of *E. coli* expressed from various synthetic promoters combined with *lysE* deletion. This increased DAP production to 104 g L^{-1} (Kim et al., 2018). In addition to glucose, a broad range of alternative substrates have been shown to be feasible for DAP production (Buschke et al., 2013b). Coexpression of *Streptococcus bovis* 148 α-amylase and *E. coli* L-lysine decarboxylase enabled production of 5 g L^{-1} DAP from starch (Tateno et al., 2009). Starch was additionally made available for DAP production by using consortia of starch-fermenting *E. coli* strains and DAP-producing strains of *C. glutamicum* (Sgobba et al., 2018). To shift the production from food raw materials to industrial waste streams, a *C. glutamicum* DAP cell factory was engineered to metabolize xylose, the most abundant sugar in wood-borne hemicellulose (Buschke et al., 2011). In a first step, xylose assimilation was implemented through the expression of the *xylAB* genes from *E. coli*, encoding xylose isomerase and xylulokinase (Buschke et al., 2011). A comprehensive approach of ^{13}C metabolic flux analysis and transcriptome analysis identified important bottlenecks in the pentose phosphate pathway and the tricarboxylic acid cycle (Buschke et al., 2013a). Through subsequent systems-level engineering, a xylose-based DAP-producer with 100% increased productivity was constructed. In fed batch fermentation, the strain produced 103 g L^{-1} DAP-HCl from xylose, with a yield of 32% (Buschke et al., 2013a). Furthermore, xylo-oligosaccharides were successfully used for DAP production by cell surface display of β-xylosidase from *Bacillus subtilis*

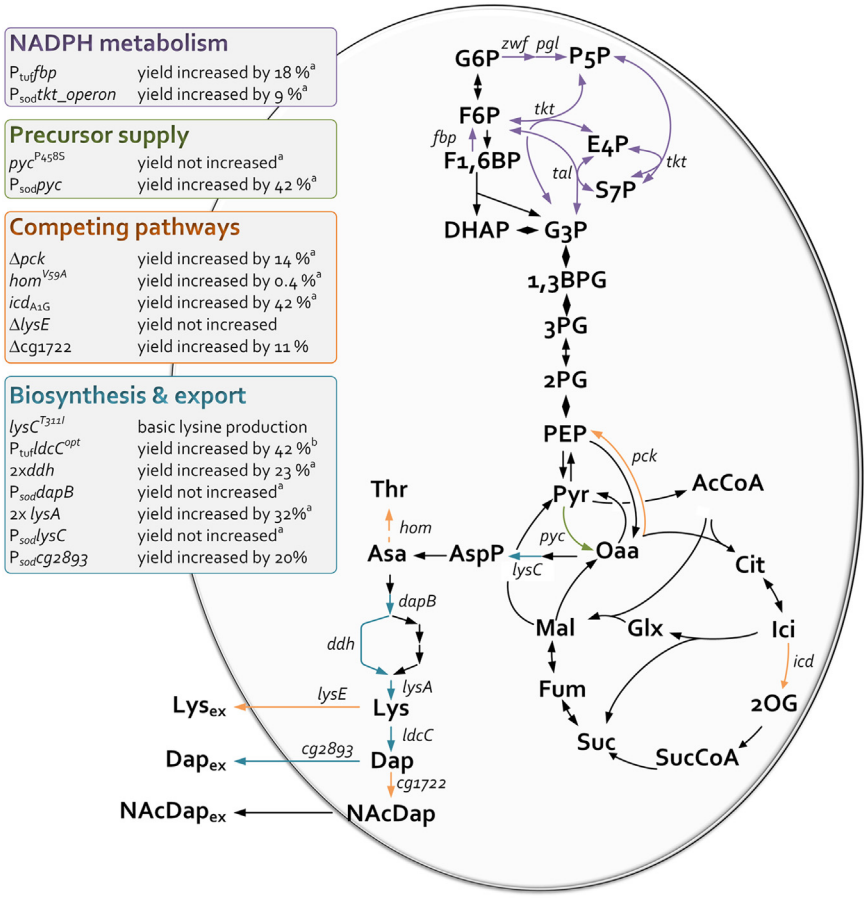

Fig. 5. Systems metabolic engineering of *C. glutamicum* for the production of 1,5-diaminopentane. The central metabolic pathway of *C. glutamicum* was streamlined for DAP production through engineering of targets for improving the supply of carbon precursor (green) and NADPH (purple), for reducing or abolishing reactions to competing pathways (orange) and engineering of biosynthesis and export (blue) (Kind et al., 2014). Enzymes are encoded as follows: cg1722: N-acetyl transferase; cg2893: major facilitator permease; *dapB*: dihydrodipicolinate reductase; *ddh*: diaminopimelate dehydrogenase; *fbp*: fructose 1,6-bisphosphatase; *hom*^{V59A}: homserine dehydrogenase with amino acid exchange valine → alanine at position 59; *icd*$_{A1G}$: isocitrate dehydrogenase with start codon exchange ATG→GTG; *ldcC*^{opt}: *E. coli* lysine decarboxylase with codon-usage optimized for expression in *C. glutamicum*; *lysA*: diaminopimelate decarboxylase; *lysC*^{T311I}: aspartokinase with amino acid exchange threonine → isoleucine at position 311; *lysE*: lysine exporter; *pck*: phosphoenolpyruvate carboxykinase; *pgl*: 6-phosphogluconolactonase; P_{sod}: promoter of the *sod* gene, encoding superoxide dismutase; P_{tuf}: promoter of the *tuf* gene, encoding elongation factor tu; *pyc*^{P458S}: pyruvate carboxylase with amino acid exchange proline → serine at position 458; *tal*: transaldolase; *tkt*: transketolase; *tkt*_operon: expression unit comprising the genes *tkt*, *tal*, *zwf*, and *pgl*; *zwf*: glucose 6-phosphate dehydrogenase. a: yield increase during optimization of Lys-12 chassis strain, b: compared to L-lysine-producing chassis strain. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Imao et al., 2017). In addition, coassimilation of methanol was achieved in DAP-producing *C. glutamicum* by heterologous reconstruction of the ribulose monophosphate pathway (Lessmeier et al., 2015).

3.3.2. 1,4-Diaminobutane

The diamine putrescine is an intermediate of L-arginine and L-ornithine degradation in several species (Becker and Wittmann, 2017a). In *C. glutamicum*, putrescine production was first established by expressing the *E. coli* genes encoding the L-ornithine and L-arginine decarboxylases (Fig. 3B) (Schneider and Wendisch, 2010). Deletion of the L-arginine repressor *argR* and low-level activity of ornithine-carbamoyl transferase supported production (Schneider et al., 2012; Schneider and Wendisch, 2010). The best strain produced 19 g L^{-1} 1,4-diaminobutane from glucose, with a yield of 0.16 g g^{-1} and a productivity of 0.55 g L^{-1} h^{-1} (Schneider et al., 2012). Production was further increased by *snaA* deletion. The *snaA* gene encodes an N-acetyltransferase that is responsible for the formation of acetylated putrescine as a by-product, and its deletion improved production by two-fold and provided a yield of 0.21 g g^{-1} (Nguyen et al., 2015b). Further improvement up to a yield of 0.26 g g^{-1} was achieved by attenuating ODHC activity to increase the precursor supply and by expressing a feedback-resistant N-acetylglutamate kinase (Nguyen et al., 2015a). A very recent study reported a combined approach of systems metabolic engineering and adaptive evolution to generate a strain with optimized putrescine production (Li et al., 2018). Among seven ornithine decarboxylase genes, the authors identified *speC1* of *Enterobacter cloacae* as the most suitable. Further engineering focused on the NADPH supply and blocking of putrescine-modifying reactions (Li et al., 2018). Through evolution of the basic strain, putrescine production was almost doubled. Comparative genome sequencing and proteome analysis identified the pentose phosphate pathway, glyoxylate shunt and ornithine biosynthesis as potential up-regulation targets, whereas other amino acid pathways and fatty acid metabolism were considered to be competing reactions (Li et al., 2018). Production of 1,4-diaminobutane was also realized from the nonnative carbon sources xylose (Meiswinkel et al., 2013a) and glycerol (Meiswinkel et al., 2013b).

3.4. Organic acids

Although the production of organic acids by *C. glutamicum* does not have a long history, several production processes have been established for products including D-lactate (Inui et al., 2004b; Okino et al., 2008b), succinate (Inui et al., 2004b; Okino et al., 2008a), glycolate (Zahoor et al., 2014), oxo-isocaproate (Bückle-Vallant et al., 2014), pyruvate (Wieschalka et al., 2012) and oxo-isovalerate (Krause et al., 2010). The most impressive titers were achieved for D-lactate (195 g L^{-1}) (Tsuge et al., 2015b) and succinate (152 g L^{-1}) (Chung et al., 2017). Achievements in their production have been reviewed recently (Becker et al., 2015; Tsuge et al., 2015a; Wieschalka et al., 2013). Hence, we will focus on the production of other organic acids.

3.4.1. Glutarate

As a dicarboxylic acid, glutarate can be used as a building block for bio-based polyamides and polyesters. Moreover, hydration of glutarate provides 1,5-pentanediol, which is used as an emollient in the plastics industry. Glutarate production in *C. glutamicum* requires filling the metabolic gap between L-lysine and 5-aminovalerate (Fig. 3A). As described in detail above, this can be realized by introducing the *davBA* gene cluster (Joo et al., 2017; Rohles et al., 2016; Shin et al., 2016) or via the DAP pathway with additional recruitment of *patAD* (Jorge et al., 2017b). Due to endogenous reactions catalyzed by 5-aminovalerate transaminase and glutarate semialdehyde dehydrogenase, 5AVA is then metabolized to glutarate (Rohles et al., 2016). Although not specifically engineered for glutarate production, such a strain can produce up to 140 mmol of glutarate per mol of glucose (Rohles et al., 2016). Indeed, in 5AVA production processes, glutarate accumulates at levels of up to 7 g L^{-1} (Rohles et al., 2016) and 13.4 g L^{-1} (Shin et al., 2016) as a by-product. This finding is highly encouraging for further development towards the construction of a streamlined glutarate cell factory.

3.4.2. Itaconic acid

Itaconic acid is a carbon-5 unsaturated dicarboxylic acid with a market size of about US$ 400 million in 2017 (Hajian and Yusoff, 2015). Its biosynthesis starts from the TCA cycle intermediate *cis*-aconitate (Fig. 3C). Itaconate production was established in *C. glutamicum* by heterologous plasmid-based expression of the codon-optimized *Aspergillus terreus* CAD1 gene, encoding *cis*-aconitate decarboxylase (Otten et al., 2015). Production was improved by protein fusion of CAD and the maltose-binding protein MalE of *E. coli*, which resulted in increased CAD activity, probably due to protein stabilization. However, CAD activity was still a limiting factor for production efficiency. Higher expression and up to ten-fold increased enzyme activity was achieved during growth under nitrogen-limited conditions. Additional strain engineering focused on the supply of the precursor *cis*-aconitate. This was addressed by introducing the rare start codons GTG and TTG into the *icd* gene, encoding isocitrate dehydrogenase. The best strain accumulated 7.8 g L^{-1} itaconate within 72 h (Otten et al., 2015).

3.4.3. 3-hydroxypropionic acid (3HP)

The bifunctional chemistry of 3HP, comprising a hydroxyl and a carboxyl group, makes it particularly useful for the synthesis of biodegradable plastics (Becker and Wittmann, 2015). Moreover, it serves as a platform chemical for the production of acrylic acid and 1,3-propanediol. *C. glutamicum* was only recently engineered for the production of 3HP via the glycerol pathway (Fig. 3D). The strategy combined the *Saccharomyces cerevisiae* biosynthetic gene cluster for glycerol synthesis (*gpd*, *gpp*) with a synthetic 3HP production pathway comprising diol dehydrogenase (*pduCDE*) and its activator (*pduGH*) from *Klebsiella pneumoniae* and a mutated aldehyde dehydrogenase ($gapD^{E209Q/E269Q}$) from *Cupriavidus necator* (Fig. 6) (Chen et al., 2017). The latter showed superior activity to that of other aldehyde dehydrogenases that were tested (AldH from *E. coli* and PuuC from *K. pneumoniae*). At 21 g L^{-1} 3HP production was already efficient in this strain; however, it was associated with substantial formation of lactate and acetate as by-products. Hence, the genes *ldhA*, *poxB*, *ptaA* and *ackA*, encoding lactate dehydrogenase, pyruvate:quinone oxidoreductase, phosphate acetyltransferase and acetate kinase, were deleted from the genome (Fig. 6). To further reduce carbon flux through the lower glycolytic pathway, expression of *gapA*, encoding glyceraldehyde dehydrogenase, was weakened by start codon exchange (ATG → GTG). In addition, *ptsH*, encoding a subunit of the phosphotransferase system for sugar uptake, was deleted. As a substitute for glucose uptake, inositol permease (IolT1) and glucokinase (Glk) were overexpressed in combination with deletion of the regulator IolR (Fig. 6). Overall, this approach increased production to 39 g L^{-1} in batch fermentation. Additional implementation of the *xylAB* operon from *E. coli* and *araE* from *C. glutamicum* ATCC31831 (Fig. 6) enabled 3HP production from xylose (35 g L^{-1}) and glucose/xylose mixtures (36 g L^{-1}). Applying fed-batch fermentation, the strain was able to produce up to 62.6 g L^{-1} 3HP, with a yield of 0.51 g g^{-1} glucose. On glucose/xylose mixture, the titer (54.8 g L^{-1}) and yield (0.49 g g^{-1} sugar) were somewhat lower but still considerable (Chen et al., 2017).

3.4.4. cis,cis-Muconic acid (MA)

The bio-based production of MA has become highly attractive as it provides a renewable route to adipic acid and terephthalic acid, two of the most important building blocks in the plastics industry (Xie et al., 2014). Production can be achieved either by *de novo* synthesis from sugars (Johnson et al., 2016; Zhang et al., 2015b) or by biotransformation from aromatic compounds (Bang et al., 1996; Barton et al., 2018; Kohlstedt et al., 2018). The attraction of the biotransformation approach lies in the valorization of a so far largely

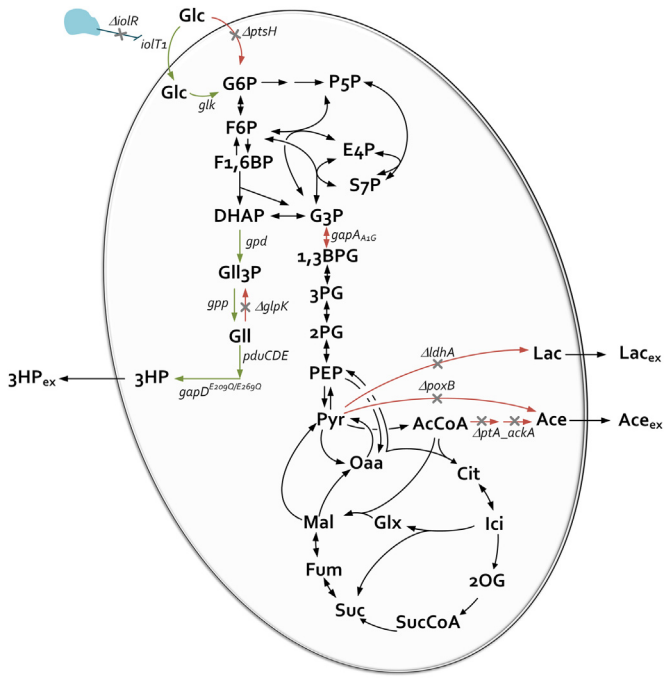

Fig. 6. Systems metabolic engineering of *C. glutamicum* for the production of 3-hydroxypropionate (Chen et al., 2017). Reactions with increased flux through amplified expression, novel gene function or removal of feedback inhibition are highlighted in green. Red denotes reactions with reduced or abolished fluxes. Crosses indicate (i) removal of repression and (ii) gene deletion with loss of the corresponding gene function. Enzymes are encoded as follows: *ackA*: acetate kinase; $gapA_{G1A}$: glyceraldehyde dehydrogenase with start codon exchange ATG → GTG; $gapD^{E209Q/E269Q}$: aldehyde dehydrogenase with amino acid exchange glutamate → glutamine at position 209 and 269 from *Cupriavidus necator; glk*: glucose kinase; *gpd*: glycerol 3-phosphate dehydrogenase from *Saccharomyces cerevisiae; gpp*: glycerol 3-phosphate phosphatase from *Saccharomyces cerevisiae; iolR*: repressor of inositol transporter; *iolT1*: inositol transporter; *ldhA*: lactate dehydrogenase; *pduCDE*: diol dehydrogenase from *Klebsiella pneumonia; poxB*: pyruvate:quinone oxidoreductase; *pta:* phosphate acetyltransferase*; ptsH*: phosphocarrier protein of phosphotransferase system. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

unused biomass constituent, namely, lignin (Kohlstedt et al., 2018; Vardon et al., 2015), which additionally comes at a highly attractive yield. In *C. glutamicum*, MA production from lignin-derived aromatics was very recently realized by interrupting the catechol branch of the catabolic β-ketoadipate pathway (Becker et al., 2018). Upon deletion of the *catB* gene, encoding muconate cycloisomerase, the *C. glutamicum* MA-1 strain produced MA from benzoate, catechol and phenol with a molar yield of 100%. Subsequently, the expression of *catA*, encoding the key enzyme catechol dioxygenase, was decoupled from the natural induction by benzoate using the strong constitutive promoter P_{tuf}. The resulting strain MA-2 exhibited a substantially increased specific production rate. In a fed-batch process, using catechol as the biotransformation substrate, *C. glutamicum* strain MA-2 accumulated 85 g L^{-1} MA, the highest titer so far reported for microbial MA production (Becker et al., 2018). Moreover, the strain survived the toxic environment provided by aromatic mixtures obtained through lignin depolymerization, which allowed a cascaded chemical and biochemical process for the conversion of lignin into MA (Becker et al., 2018). Embedded in a biorefinery concept, this bio-based approach towards MA production adds substantial value to the so far unused lignin-containing waste streams. In addition to the aromatic-derived route for MA production, *C. glutamicum* was engineered to produce MA from glucose (Shin et al., 2018). The base strain inha103, carrying the deletions Δ*aroE*, Δ*pcaGH* and Δ*catB* in the genes encoding shikimate 5-dehydrogenase, protocatechuate deoxygenase, and muconate cycloisomerase, was additionally modified by the expression of protocatechuate decarboxylase YBD, allowing production of 3.5 g L^{-1} MA in the newly constructed strain inha108. Production was improved by engineering glucose uptake via the non-PTS transporter IolT and by additional overexpression of *qsuB*, encoding 3-dehydroshikimate dehydratase. The best strain, P30, produced 4.5 g L^{-1} MA, with a molar yield of 22% within three days (Shin et al., 2018).

3.5. Alcohols

The high tolerance of *C. glutamicum* is an ideal prerequisite for using it as a production host for alcohols. The feasibility of this approach has been impressively demonstrated by the generation of a strain that produced 73 g L^{-1} of isobutanol in a fermentation setup with continuous solvent extraction (Yamamoto et al., 2013). In addition to its use in isobutanol production, *C. glutamicum* has been engineered for the production of ethanol (Inui et al., 2004a), 1,2-propanediol (Lange et al., 2017; Siebert and Wendisch, 2015), 1-propanol (Siebert and Wendisch, 2015) and ethylene glycol (Chen et al., 2016). A recent study reported the production of 14 g L^{-1} 1,3-propanediol using a biotransformation approach with glycerol as the substrate and cofactor recycling via L-glutamate fermentation (Huang et al., 2017). In the following section, we will give further details regarding the genetic engineering of *C. glutamicum* for the production of 2,3-butanediol and ethanol.

3.5.1. 2,3-butanediol (2,3-BDO)

Microbial production of the diol 2,3-BDO is of increasing interest as a renewable route for producing this valuable platform chemical (Celinska and Grajek, 2009). Though *C. glutamicum* is genetically equipped for producing 2,3-BDO from pyruvate (Fig. 3E), efficient production is prevented by the lack of *budA*, encoding α-acetolactate decarboxylase, an enzyme that plays a key role in 2,3-BDO fermentation in *K. pneumoniae* (Yang et al., 2015). Plasmid-based expression of the *budA* from *K. pneumoniae* in the wild type strain of *C. glutamicum* was sufficient to enable production of 1.76 g L^{-1} 2,3-BDO (Yang et al., 2015). To further improve production, heterologous expression of the *K. pneumoniae budB* and *budC* genes, encoding α-acetolactate synthase and acetoin reductase, was introduced despite *C. glutamicum* possessing homologous gene variants with the respective functions. While *budB* expression increased production by 1.8-fold, *budC* expression was detrimental. Fermentation of the *budAB* strain at an elevated substrate concentration allowed the production of 18.9 g L^{-1} 2,3-BDO from 80 g L^{-1} glucose. Production from molasses and cassava powder was somewhat lower (12 g L^{-1}) (Yang et al., 2015). Although 2,3-BDO was the major product, the strain accumulated lactate, succinate, acetate and acetoin as by-products (Yang et al., 2015). Reduction of by-product formation was addressed in another study (Rados et al., 2015) by deleting *ldhA*, *aceE* (encoding the E1 subunit of the pyruvate dehydrogenase complex), *pqo* (pyruvate:quinone oxidoreductase) and *mdh* (malate dehydrogenase). In this strain background, the *Lactococcus lactis* 2,3-BDO pathway was assembled by expressing the genes *als* (α-acetolactate synthase), *aldB* (α-acetolactate decarboxylase) and *butA* (butanediol dehydrogenase, BDH) from the IPTG-inducible expression plasmid pEKEx2 (Rados et al., 2015). To increase BDH activity, the *butA* gene was then expressed from the strong constitutive promoter P_{tuf}. Fermentation of the strain was a two-stage process. First, cells were grown under aerobic conditions using acetate as the carbon source, as the Δ*aceE* modification disabled growth from glucose. For 2,3-BDO production, the process was then shifted to glucose-based anaerobic fermentation, yielding 6.3 g L^{-1} 2,3-BDO at a yield of 0.3 g g^{-1} glucose (Rados et al., 2015).

3.5.2. Ethanol

Under oxygen deprivation, *C. glutamicum* produces significant amounts of ethanol when equipped with a plasmid for the expression of

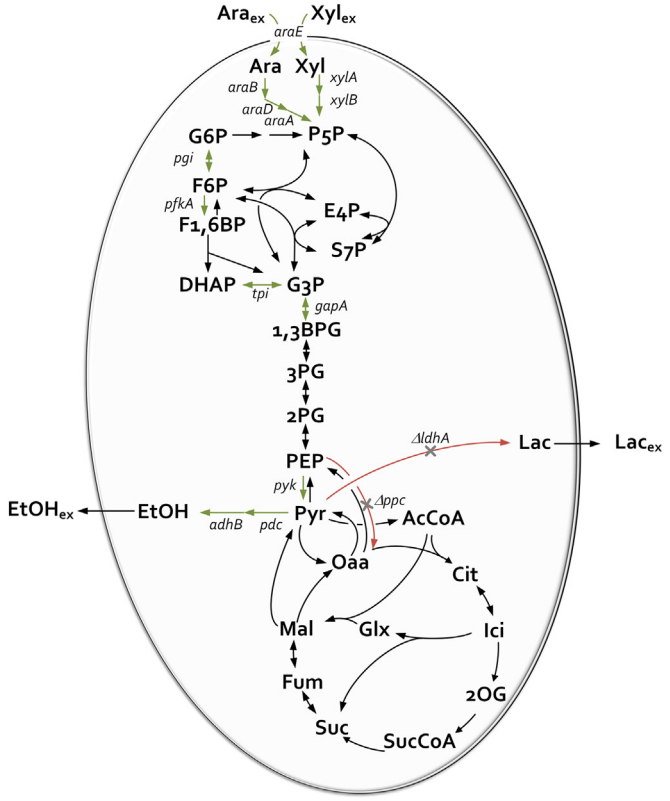

Fig. 7. Systems metabolic engineering of *C. glutamicum* for the production of ethanol (Jojima et al., 2015). Reactions with increased flux through amplified expression, novel gene function or removal of feedback inhibition are highlighted in green. Red denotes reactions with reduced or abolished fluxes. Crosses indicate gene deletion with loss of the corresponding gene function. Enzymes are encoded as follows: *adhB*: alcohol dehydrogenase from *Zymomonas mobilis;* *araA*: arabinose isomearse; *araB*: ribulose kinase; *araD*: ribulose 5-phosphate 4-epimerase; *araE*: pentose transporter; *gapA*: glyceraldehyde 3-phosphate dehydrogenase; *ldhA*: lactate dehydrogenase; *pdc*: pyruvate decarboxylase from *Zymomonas mobilis;* *pfkA*: phosphofructokinase; *pgi*: phosphoglucoisomerase, *ppc*: phosphoenolpyruvate carboxylase; *pyk*: pyruvate kinase; *tpi*: triosephosphate isomerase; *xylA*: xylose isomerase; *xylB*: xylulokinase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the *Zymomonas mobilis* genes encoding pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adhB*) (Fig. 3E) (Inui et al., 2004a). Production occurred in growth-arrested cells and was accompanied by lactate and succinate formation. Deletion of *ldhA* abolished lactate formation while increasing ethanol production by 3-fold. Additional deletion of *ppc*, encoding phosphoenolpyruvate carboxylase, drastically decreased succinate formation; however, ethanol production was strongly impaired and required the addition of pyruvate or acetaldehyde to the medium (Inui et al., 2004a). Indeed, pyruvate feeding also had a positive effect on ethanol production in the ancestor strains (Inui et al., 2004a). Hence, to improve pyruvate supply in *C. glutamicum*, a strain with a streamlined glycolytic chain was constructed by markerless overexpression of *pgi*, encoding phosphoglucoisomerase, *pfkA*, encoding phosphofructokinase, *gapA*, encoding glyceraldehyde dehydrogenase, *tpi*, encoding triose phosphate isomerase and *pyk*, encoding pyruvate kinase from the P_{tac} promoter (Fig. 7) (Jojima et al., 2015). This almost doubled productivity, while the yield remained unchanged. Using a high-copy number plasmid for *pdc* and *adhB* expression then increased the yield at a constant production rate. The overall production process was then divided into aerobic growth and anaerobic production phases, which finally yielded 106 g L^{-1} ethanol (Jojima et al., 2015). When the strain was additionally equipped with the xylose (*xylAB*) and arabinose (*araBAD*) operons from *E. coli* and the pentose transporter *araE* from *C. glutamicum* ATCC31831, fermentation from sugar mixtures of glucose, xylose and arabinose was realized (Jojima et al., 2015).

3.6. Aromatics

The group of aromatic compounds is highly diverse with regard to both its biochemical complexity and range of applications. It equally comprises not only rather simple structures, such as aromatic amino acids and protocatechuic acid, but also more complex molecules, including violacein, flavonoids and stilbenes. Some of these molecules have shown promising bioactivity for anticancer, antibacterial, antiviral and anti-inflammatory treatment and, thus, have become a focus of research for recombinant production in *C. glutamicum*. For instance, stilbenes (resveratrol, pinosylvin, piceatannol), (2S)-flavanones (naringenin, eriodictyol) and flavonols (kaempferol, quercetin) were recently produced from phenylpropanoid precursors by using plant-derived, codon-optimized genes for the respective pathways (Kallscheuer et al., 2016, 2017). Other studies reported the production of protocatechuic acid, applicable as polymer building block and food constituent, through biotransformation from ferulic acid (Okai et al., 2017) and by *de novo* synthesis from glucose (Kallscheuer and Marienhagen, 2018; Okai et al., 2016). In addition, various isomers of hydroxybenzoate were accessible by fermentation of *C. glutamicum* (Kallscheuer and Marienhagen, 2018; Kitade et al., 2018). Here, we will focus on the *de novo* production processes for violacein and 4-hydroxybenzoate.

3.6.1. Violacein

Violacein is a tryptophan derivative (Fig. 3F) with antiviral and anticancer activity and naturally occurs in pathogenic *Chromobacterium violaceum* and *Janthinobacterium lividum* (Rodrigues et al., 2012). To produce crude violacein (a mixture of violacein and deoxyviolacein), the tryptophan-producer *C. glutamicum* ATCC21850 was chosen as the chassis strain. Production was first realized using a self-replicating plasmid carrying the *vio* operon of *C. violaceum* under the control of a constitutive promoter (Sun et al., 2016). This approach already allowed the production of 532 mg L^{-1} of crude violacein. In addition, an IPTG-inducible plasmid system was tested for expression of the *vio* operon of *J. lividum*, resulting in a 1.2-fold improvement (Sun et al., 2016). As the improvement in production was attributed to the inducible promoter, control of the expression of the *C. violaceum* genes was changed to the P_{trc} promoter. Subsequently, the promiscuous overlapping gene structure of the natural operon was untangled, providing each of the genes with an individual RBS in *C. glutamicum* (Sun et al., 2016). For further optimization, the induction time, concentration of the inducer IPTG, and cultivation temperature were varied to identify the optimal fermentation conditions for violacein production. In fed-batch fermentation, the best strain, carrying the *vio* operon of *C. violaceum* with the *C. glutamicum* RBSs, produced 5.4 g L^{-1} of crude violacein within 115 h (Sun et al., 2016), which is the highest titer and productivity reported so far. Direct comparison to previous studies in *E. coli* (Rodrigues et al., 2014, 2013) is, however, impossible, as violacein and deoxyviolacein were not discriminated in this study.

3.6.2. 4-hydroxybenzoic acid (4-HBA)

4-HBA is mainly applied for the production of parabens, which are used as preservatives in the cosmetics and food industries. As 4-HBA is synthesized via the shikimate pathway (Fig. 3F), the engineering strategy for 4-HBA production involved the stepwise overexpression of all seven enzymes of this pathway (Kitade et al., 2018). In particular, multicopy integration of the *C. glutamicum* genes *aroC*, encoding chorismate synthase, *aroK*, encoding shikimate kinase, and *aroB*, encoding 3-dehydroquinate synthase, improved 4-HBA formation. Moreover, the use of a mutated *aroG* gene from *E. coli*, encoding 3-deoxy-d-

arabinoheptulosonic acid 7-phosphate (DAHP) synthase, greatly increased production, and thus, multiple gene copies were integrated into the genome of *C. glutamicum* (Kitade et al., 2018). Screening for a feedback-resistant chorismate: pyruvate lyase, catalyzing the final step of 4-HBA biosynthesis, identified UbiC from the intestinal bacterium *Providencia rustigianii* as highly promising. Hence, the encoding gene was expressed in *C. glutamicum* to complement the 4-HBA pathway (Kitade et al., 2018). The formation of by-products (1,3-dihydroxyacetone and pyruvate) was then reduced by deleting *hdpA*, encoding haloacid dehalogenase superfamily phosphatase, and *pyk*. In an aerobic growth-arrested fermentation, production of 36.6 g L^{-1} 4-HBA, with a productivity of 1.5 g L^{-1} h^{-1} and a yield of 0.41 mol mol^{-1}, was achieved, which is the highest recombinant 4-HBA production reported so far (Kitade et al., 2018). Another study reported 4-HBA production in the *C. glutamicum* delARO^5 strain, which lacks five gene clusters responsible for the catabolism of aromatic compounds (Kallscheuer and Marienhagen, 2018). The strain was additionally optimized for the production of aromatics by overexpressing transketolase to improve the supply of erythrose 4-phosphate and by engineering glucose uptake via the inositol transporter as a substitute for the glucose PTS to improve the phosphoenolpyruvate supply. In addition, citrate synthase was downregulated, and the *E. coli aroH* gene, encoding 3-deoxy-d-arabinoheptulosonate-7-phosphate synthase, was introduced to enhance the shikimate pathway. The last step of 4-HBA biosynthesis relied on the expression of the *E. coli* UbiC enzyme, allowing the production of 3.3 g L^{-1} 4-HBA within 48 h (Kallscheuer and Marienhagen, 2018).

4. Conclusion

Fermentative production using metabolically engineered strains of *C. glutamicum* is a research field that is evolving at an ever-increasing rate. Processes for existing products are steadily improved, and novel processes are continuously established, leading to an explosion of the product portfolio. Within only the last five years, the number of products has more than doubled from approximately 35 (Becker and Wittmann, 2012a) to more than 70 (Fig. 1). The timeline of developing an industrial strain from scratch has shortened tremendously since the advent of industrial biotechnology. The development of efficient and fast genome editing methods such as CRISPR/Cas promises even faster strain engineering through multiplex genome editing (Cho et al., 2017). Moreover, the automation of strain engineering pipelines, combining targeted metabolic engineering and adaptive evolution, is rapidly developing. Biosensors for screening and selection (Eggeling et al., 2015) as well as robot-aided miniaturized systems (Radek et al., 2017) are waiting to be used. Given this progress, *C. glutamicum* will retain its leading role as a fermentation workhorse in industrial biotechnology and will make a significant contribution to the globally growing bioeconomy.

Conflict of interests

Authors declare no competing interests.

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