Chapter ##

Genome-reduced *Corynebacterium glutamicum* fit for biotechnological applications

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ABSTRACT: Genome minimization ultimately leads to the smallest genome sustaining life of a given cell, however, growth of this cell may be very slow and may require multiple supplements e.g. to overcome amino acid auxotrophies. By contrast, genome reduction of industrially relevant bacteria such as *Corynebacterium glutamicum* does not aim at generating minimal cells. Rather chassis cells are developed that are as fit as the wild type with respect to a target function: for example growth of *C. glutamicum* in glucose minimal medium. Thus, a balance between reducing the burden of expressed genes while maintaining fast growth with glucose without the requirement for supplements such as amino acids is required. Here, the application of this concept to *C. glutamicum* is discussed. Moreover, an outlook on how the advent of genome editing by CRISPR-Cas9 or CRISPR-Cpf1 impacts genome reduction and how highly parallel genome editing must be met by highly parallel strain characterization is presented. Finally, metabolic engineering approaches for the overproduction of amino acids, organic acids, terpenoids and diamines making use of genome-reduced *C. glutamicum* strains are detailed.

Keywords: *Corynebacterium glutamicum*; genome reduction; amino acid production; metabolic engineering; fine chemicals; two-step homologous recombination, CRISPR/Cas9

##.1 *Corynebacterium glutamicum*: one of the pillars of biotechnology

##.1.1 Role of C. glutamicum in the bioeconomy

It is believed that bioeconomy will play an important role in the world's future. White biotechnology, also known as industrial biotechnology, makes use of biotechnology for the sustainable processing and production of chemicals, materials and fuel [1]. *Corynebacterium glutamicum* is a central pillar of white biotechnology. *C. glutamicum* has a history of more than fifty years of safe production of food and feed amino acids, an industrial process which operates at the million-ton scale per annum [2] and shows a compound annual growth rate of 5.6% over 2017-2022 reaching US$25.6 billion by 2022 [3].

Strain development for *C. glutamicum* has embraced and driven technological development in the classical [4-5], genetic engineering [6-7], systems biology [8], synthetic biology [2,9], and systems metabolic engineering eras [2, 10-11]. Currently, this is obvious by the application and further development of CRISPR interference [12], CRISPR-Cas9 [13] and CRISPR-Cpf1 [14] genome editing and CRISPR multiplexing [15], biosensor-driven strain selection [16-22] and flux control [23-24], new process concepts such as co-production [25] and synthetic consortia [26] that have been applied to *C. glutamicum*.

##.1.2 C. glutamicum as host for a multitude of production processes

C. glutamicum has been engineered for the production of a broad spectrum of value-added compounds including specialty amino acids [27-28] such as N-alkylated amino acids [29-31] and omega-amino acids [32-35], diamines such as putrescine and cadaverine [36-37], organic acids such as pyruvate [38], succinate [39-42], glutarate [43] and itaconate [44], alcohols such as isobutanol [45-46] and n-propanol [47], aromatic compounds such as PHBA [48-50], 7-chloro-L-tryptophan [51], phenylpropanoids [52] and anthocyanine [53], vitamins such as

pantothenate [54] and riboflavin [55], terpenoids such as patchoulol [56] and astaxanthin [57], polymers such polyhydroxyalkanoates [58], hyaluronic acids [59], chondroitin [60] and proteins [61-62]. To facilitate biorefinery applications a flexible carbon feedstock concept has been realized for production processes from various second generation feedstocks without competing uses in human and animal nutrition [63-64].

##.1.3 C. glutamicum genome and genome-scale tools

C. glutamicum possesses a single circular chromosome with 3.3 Mb [65-66] and more than 3,000 protein encoding sequences (CDS). Genome-scale methods have been developed early [67-68]. Based on the complete genome sequence [65], genome-scale metabolic models were reconstructed. The first genome-scale metabolic models followed the approach for the *E. coli* genome-scale metabolic model [69] and comprised 446 and 502 reactions, respectively, involving 441 and 423 metabolites, respectively [70-71]. The genome-scale model *iEZ475* added balances for protons and water (https://www.13cflux.net/models/Corynebacterium_glutamicum/index.jsp) and contains 475 metabolic reactions involving 408 metabolites (340 intra- and 68 extracellular) that could be grouped to central carbon metabolism (about 42 reactions), amino acid synthesis (about 110 reactions) as well as to oxidative phosphorylation, membrane lipid metabolism, nucleotide salvage pathway, cofactor biosynthesis, biomass formation, alternate carbon metabolism, and about 90 transport reactions (https://www.13cflux.net/models/Corynebacterium_glutamicum/index.jsp). Biosynthesis reactions leading to protein, DNA, RNA and cell-wall components were accounted for based on their weight fraction of the biomass. The most advanced model (iCW773) has recently been described and reconstructs 773 genes, 950 metabolites, and 1207 reactions, of which 252 are

transport reactions [72]. Although all these models are named genome-scale, only about 26% of all ORFs are covered by the most advanced model. These stoichiometric models were complemented by a regulatory model involving 97 transcriptional regulator proteins and 1,432 regulatory interactions which later was extended to include other corynebacterial species and *E. coli* [73].

Transcriptomics was developed for *C. glutamicum*, first based on DNA microarrays [67], later by RNAseq [74]. A landscape RNAseq study helped to refine genome annotation with a re-annotation of 200 gene starts and the finding that among the 2,000 transcriptional start sites identified, about 33% belonged to leaderless transcripts [74]. Differential RNAseq is nowadays used to compare global gene expression patterns [75-78]. Proteomics for cytoplasmic proteins, membrane fraction proteins, cell wall-associated proteins, and secreted proteins are now available [79-83]. This, for example, led to the discovery of pupylation as post-translational modification that is relevant for iron release from the iron storage protein ferritin independent of degradation [84-85]. Metabolomics has been developed for *C. glutamicum* [86-87] and, for example, helped to identify a new pathway involving γ-glutamyl transpeptidase and γ-glutamyl dipeptides (γ-Glu-Glu, γ-Glu-Gln, γ-Glu-Val, γ-Glu-Leu, γ-Glu-Met) were detected by HPLC-MS in concentrations from 0.15 to 0.4 mg/g CDW [88].

##.2 Prophage cured strains

##.2.1 MB001 derived from wild type ATCC 13032

The *C. glutamicum* genome contains three prophage DNA islands (CGP1, CGP2, and CGP3). CGP1 comprises genes cg1507 to cg1524 (13.5 kbp), CGP2 genes cg1746 to cg1752 (3.9 kbp), and CGP3 is the largest prophage region with 187.3 kbp (comprising genes cg1890 to cg2071 [65, 89]. The activity of bacteriophages and phage-related mobile elements is a major source

for genome rearrangements and genetic instability of their bacterial hosts. Genome-wide expression analysis often revealed differential expression of phage genes [90-91]. Moreover, the large prophage CGP3 has recently been shown to be excised under SOS-response-inducing conditions [89]. Single-cell analyses with transcriptional fusions of promoters of phage genes (Pint2 and Plysin) to fluorescent protein reporter genes revealed that 0.01 to 0.08% of the cells grown in standard minimal medium induced CGP3 spontaneously, which reduced their viability. Apparently, spontaneously occurring DNA damage induced the SOS response and as consequence prophage induction [92]. This process required actively proliferating cells, whereas sporadic SOS induction was still observed in resting cells [93]. The prophage CGP3-encoded nucleoid-associated protein CgpS binds AT-rich DNA as prevails in the entire CGP3 prophage region, but is scarce throughout the rest of the genome. In its absence a significantly increased induction frequency of the CGP3 prophage resulted, whereas a strain lacking the CGP3 prophage displayed stable growth [94]. Based on the properties of the prophages and the resulting genetic instability, the first target for genome reduction was the deletion of these prophage DNA islands [95].

Deletion of the three prophage DNA islands reduced the genome size of *C. glutamicum* ATCC 13032 by 6% and resulted in strain MB001. Its growth properties were unchanged under standard and stress conditions. Under SOS-response-inducing conditions that trigger CGP3 induction in the *C. glutamicum* wild type, strain MB001 fared better than the wild type showing improved growth and fitness. In addition, strain MB001 exhibited increased transformation efficiency. This was attributed to the loss of the restriction-modification system (cg1996-cg1998) located within CGP3. Furthermore, plasmid copy number appeared to be increased since production of a heterologous model protein (enhanced yellow

fluorescent protein, eYFP) was 30% higher than in the wild type. Similarly, deletion of the genes for restriction-modification system (cg1996-cg1998) improved eYFP production [95].

These results characterized MB001 as an intermediate strain to be improved by further genome reduction (s. below), e.g. by targeting mobile IS elements, and as a suitable strain for metabolic engineering (stable, growing as fast as wild type on glucose minimal medium, higher plasmid copy number and better transformation efficiency). *C. glutamicum* MB001 was used as host for the production of various value-added compounds: amino acids [96-100], phenylpropanoids [49,52,101], isoprenoids [25,57,102-104], alcohols [105], carboxylic acids [100,106], and proteins [107-110]. In addition, MB001 and derivatives have been used to study Mu-transposition [111], assembly of the septal cell envelope [112], infection with phages ϕ673 and ϕ674 phages [113], identification of an isoprenoid pyrophosphate-dependent transcriptional regulator [114], cAMP phosphodiesterase CpdA [115] and cryptic prophages [94], as basis for ALE towards higher growth rates on glucose minimal medium [116] and to assemble bacterial microcompartments [117].

##.2.2 Prophage-cured lysine producing model strain GRLys1

The concept of prophage island DNA deletion was transferred from the wild type (see above) to the lysine producing model strain DM1933 [118]. The prophage DNA sequences of the three phages CGP1 CGP and CGP3 were deleted from the base strain DM1933 that contained the following genomic modifications promoting lysine overproduction: Δ*pck, pyc*^{P458S}*, hom*^{V59A}*, 2 copies of lysC*^{T311I}*, asd, dapA, dapB, ddh, lysA,* and *lysE* [118]. Derivatives of GRLys1 were used to overproduce L-pipecolic acid (L-PA) [27-28,119], 5-aminovaleric acid (5AVA) [34], glutarate [43], and for the coproduction of astaxanthin with lysine [25].

##.3 IS element free strain

##.3.1 MB001 derived IS element free strain CR099

All copies of IS elements ISCg1 and ISCg2 were deleted from the genome of strain MB001. In addition, it contains mutation A468T in Cg1720 which was inadvertently introduced. Cg1720 encodes the ATPase component of an uncharacterized ABC transporter. This strain was used to characterize synthetases and a hydrolase of the small alarmone (pp)pGpp [120-121]. In a similar approach two IS element-free *C. glutamicum* strains were derived from ATCC 13032: one lacking IS elements IS*Cg1a*, IS*Cg1b*, IS*Cg1c,* IS*Cg1e* and another lacking ISCg2b, ISCg2c, ISCg2e, ISCg2f [122]. Increased protein production was demonstrated in the IS element free strains [122].

##.4 C. glutamicum chassis strain C1* derived from ATCC 13032

A chassis strain based on *C. glutamicum* ATCC 13032 was constructed in a targeted top-down approach. As target function uncompromised growth in glucose minimal medium was chosen. *C. glutamicum* MB001 was used as starting strain. Next, genes were classified as known to be non-essential from prior experiments, likely non-essential based on transposon mutagenesis screens, unclassifiable or likely essential due to high conservation (Fig. ##.1). From these, genomic clusters with genes classified as (likely) non-essential were chosen for deletion from the genome of MB001. The generated deletion mutants were evaluated with respect to growth in glucose minimal medium. This phenotyping step proved crucial to identify non-essential gene clusters that are irrelevant for maintaining the biological fitness of the wild type (WT). A total of 26 gene clusters were found to be non-essential and their individual deletions shown not to compromise growth in glucose minimal medium.

Based on this mutant collection, combinatorial deletions of these gene clusters was performed resulting in a library of 28 strains. After statistical analysis of a thorough phenotypic screen and one genetic correction, the final chassis strain C1* exhibiting a genome reduction of 13.4% (412 deleted genes; Fig. ##.2), but showing wild-type-like growth behavior in glucose minimal medium, robustness against several stresses (including oxygen limitation) and long-term growth stability in defined and complex growth media, was selected [123].

Notably, genome sequencing of the penultimate strain, named C1, revealed a mutation in the promoter-region of regulatory gene *ramA* [124], i.e. a promoter-down mutation (TGCACT instead of the conserved −10-region TACACT). Moreover, this mutation is located in the SugR binding sites overlapping the −10 region [125-126]. A transcriptome analysis revealed 6 fold reduced *ramA* RNA levels and reduced RNA levels for several genes of the *ramA* regulon. Therefore, this point mutation in *C. glutamicum* C1 was reversed to yield the chassis strain *C. glutamicum* C1* [123].

C. glutamicum C1* showed slightly impaired growth with some alternative carbon sources such as acetate, pyruvate, arabitol and gluconate. These results are possible since the target function chosen was uncompromised growth with glucose as sole carbon and energy source. However, these physiological peculiarities have to be remembered when constructing and evaluating C1* derived strains for production purposes. As in the case of reversion of the *ramA* promoter down mutation present in C1, other SNPs may have to be reverted to allow for fast growth with acetate, pyruvate, gluconate or arabitol.

For all glucose-based production purposes, *C. glutamicum* C1* is an ideal starting point for metabolic engineering as a biotechnologically relevant chassis.

##.5 Applications of genome reduced strains

##.5.1 Applications of prophage-cured strain MB001 and derivatives

C. glutamicum MB001 found manifold biotechnological applications (Table ##.1). Derivatives of this prophage-cured strain were used for the production of proteins [107], citrulline [96-97], proline [98], lysine [99], decaprenoxanthin [102-103], astaxanthin [25,57], ciprofloxacin-triggered glutamate and oxoglutarate production [100], valencene [104], 3-hydroxypropionic acid [106], coproduction of 1,3-propanediol and glutamate [105], and phenylpropanoids [49,52,101].

As an example, the construction and use of strain MB001(DE3) for protein production based on an IPTG-inducible T7 expression system will be discussed. Part of the DE3 region from the protein production host *E. coli* BL21(DE3) including the T7 RNA polymerase *gene 1* driven by the *E. coli* lacUV5 promoter, which also is active in *C. glutamicum*, was integrated into the chromosome of *C. glutamicum* MB001 [107]. The corresponding expression vector pMKEx2 was developed to express a) the *lacI* gene encoding *E. coli lac* repressor and b) genes of interest under the control of a T7 promoter followed by *lacO1* for induction by IPTG [107]. The inducibility of the system was shown to be 450 fold when expression of the fluorescence protein reporter gene *eyfp* was analyzed. Fully IPTG-induced T7 RNA polymerase-dependent expression was about 3.5 times higher than expression from the fully IPTG-induced *tac* promoter in a control strain with the endogenous RNA polymerase. Importantly, fully IPTG-induced T7 RNA polymerase-dependent expression led to a uniform population with 99% of all cells showing high fluorescence as shown by flow cytometry [107]. As an impressive application example, overexpression of the endogenous pyruvate kinase gene *pyk* was demonstrated. The already very high *pyk* gene expression in the wild type (leading to a specific pyruvate kinase activity of 2.6 U/mg) was boosted about 50 fold (135 U/mg) [107].

##.5.2 CORYNEX

Besides, *C. glutamicum* strain ATCC13869 was commercialized as a protein expression system under the trademark CORYNEX® by the Japanese company Ajinomoto. When using the CORYNEX® strain YDK010, secretion of the Fab fragment of human anti-HER2 was low. Deletion of the genes encoding penicillin-binding protein (PBP1a), which is involved in cell wall peptidoglycan synthesis, and the surface (S)-layer protein CspB, showed a synergistic effect allowing efficient Fab production using the CORYNEX® system. This indicated at least two major permeability barriers to Fab secretion, i.e. peptidoglycan and the S-layer [127].

##.5.3 Applications of prophage-cured strain GRLys1 and derivatives

Derivatives of GRLys1 were used to overproduce 5AVA [34], L-PA [27-28,119], glutarate [43], and for the coproduction of astaxanthin with lysine [25].

As example, glutarate production based on the prophage-cured lysine model producer strain GRLys1 will be discussed (Fig. ##.1). Systems metabolic engineering included flux enforcement, which refers to coupling a biosynthetic production pathway to a metabolite pathway required for growth. This strategy has previously been applied to amino acid production by *E. coli* and *C. glutamicum*. Coupling of a production pathway involving a 2-oxoglutarate dependent hydroxylase to growth by deletion of 2-oxoglutarate dehydrogenase subunit gene *sucA* has first been shown for 4-hydroxy-L-isoleucine production by *E. coli* [128] and later for 4-hydroxy-L-proline production [129]. Thus, these production pathways became part of an artificial TCA cycle. This concept was extended in succinyl-CoA synthetase-negative (Δ*sucCD*), lysine producing *C. glutamicum* strains. In this case, the succinylase branch of L-lysine production metabolically complemented the TCA cycle disrupted due to the *sucCD* deletion [130]. Also coupling of the major ammonium assimilating enzyme glutamate

dehydrogenase to transamination reactions were used for flux enforcement when cadaverine/putrescine transaminase PutA and GABA/5AVA amino transferase GabT introduced for glutarate production metabolically complemented for the absence of glutamate dehydrogenase [43]. This prophage-cured, flux enforced strain in addition required expression of a heterologous gene for lysine decarboxylase for glutarate production. In this five step synthetic pathway, lysine was decarboxylated to cadaverine by lysine decarboxylase, and cadaverine converted to glutarate by two transamination (catalyzed PutA, GabT) and two oxidation steps (catalyzed by PutD and GabD) to the targeted product glutarate [43].

##.6 Outlook on construction and testing of new genome-reduced strains

Targets for gene deletions relevant for genome reduction can be scored by CRISPR interference [131] as applied first to *C. glutamicum* with respect to lysine production [12]. Evaluation of groups of genes for combined deletion can be done by multiplex CRISPRi [132]. Sequential or parallel targeted genome deletions and replacements in *C. glutamicum* by CRISPR genome editing is facile since this bacterium lacks efficient non-homologous end-joining. Although genome reduction in *C. glutamicum* has until now relied on genome editing by two-step homologous recombination using the conditionally lethal levansucrase (*sacB*) for positive selection [133], genome editing by CRISPR/Cas9 or CRISPR/Cpf1 as developed for *C. glutamicum* [13-15, 134-136] will find application in further genome streamlining.

Highly parallel strain characterization relies on microbioreactor systems that are based either on shaken microtiter plate cultivation devices or on down-scaled stirred tank reactors [137]. These systems allow for optical, non-invasive, online monitoring of important process parameters such as biomass concentration, dissolved oxygen, pH, or reporter protein

fluorescence. Their use is potentiated by combination with liquid handling robots for automatization of operation procedures. On-line and off-line strain phenotyping under industrially relevant conditions enables identification of the optimal combination of producer strain and bioprocess control strategy. Of course, the strain collections generated in genome reduction projects can be scored very well using microbioreactor systems as has been shown for characterizing growth [138], protein secretion [108-109] or amino acid production [17,95,118,123,139].

Table ##.1 Biotechnological applications using genome-reduced *C. glutamicum* strains

Product	Base strain	Production parameter(s)	Reference
3-hydroxy-propionic acid			[106]
arginine	MB001	Y: 0.30 $g \cdot g^{-1}$	[98]
astaxanthin	MB001	C: 1.7 mg g^{-1} DCW;V: 0.4 mg L^{-1} h^{-1};	[57,25]
noreugenin	MB001	T: 53 mg/L	[140]
Citrulline	MB001	T: 44.1 ± 0.5 mM; Y: 0.38 ± 0.01 $g \cdot g^{-1}$; P: 0.32 ± 0.01 $g \cdot l^{-1} \cdot h^{-1}$	[96-97]
coproduction of 1,3-propanediol and glutamate			[105]
coproduction of astaxanthin with glutamate	MB001	Astaxanthin: T: 2.33 $mg \cdot L^{-1}$; Y= 2.22 $g \cdot g^{-1}$; P: 0.12 $mg \cdot L^{-1} \cdot h^{-1}$ Glutamate: T: 0.05 $g \cdot L^{-1}$; Y: 0.13 $g \cdot g^{-1}$; V: 005 $g \cdot L^{-1} \cdot h^{-1}$	[25]
coproduction of astaxanthin with lysine	GRLys1	Astaxanthin: T: 10 $mg \cdot L^{-1}$; C: 0.4 mg g^{-1}; Y= 0.07 $g \cdot g^{-1}$ Lysine: T: 48 $g \cdot L^{-1}$; Y: 0.35 $g \cdot g^{-1}$	[25]
coproduction of decaprenoxanthin with glutamate	MB001	Decaprenoxanthin: T: 8.66 $mg \cdot L^{-1}$; Y= 0.97 $g \cdot g^{-1}$; P: 0.05 $mg \cdot L^{-1} \cdot h^{-1}$ Glutamate: T: 0.02 $g \cdot L^{-1}$; Y: 0.48 $g \cdot g^{-1}$; V: 0.18 $g \cdot L^{-1} \cdot h^{-1}$	[25]
coproduction of decaprenoxanthin with lysine	GRLys1	Decaprenoxanthin: T: 6.10 $mg \cdot L^{-1}$; Y= 0.34 $g \cdot g^{-1}$; P: 0.19 $mg \cdot L^{-1} \cdot h^{-1}$ Lysine: T: 2.79 $g \cdot L^{-1}$; Y: 0.15 $g \cdot g^{-1}$; V: 0.09 $g \cdot L^{-1} \cdot h^{-1}$	[25]
Decaprenoxanthin	MB001	C: 0.4 mg g^{-1} DCW	[102-103]
Glutamate (triggered by ciprofloxycin)	MB001	T: 37 mM; Y: 0.13 g g^{-1}	[100]
lycopene	MB001	C: 0.43 mg g^{-1} DCW	[57,25]
lysine			[99]
ornithine	MB001	Y: 0.52 $g \cdot g^{-1}$	[98]
Oxoglutarate (triggered by ciprofloxycin)	MB001	T: 18 mM	[100]
4-hydroxy-butyrate	MB001	T: 3.3 g g^{-1}	[49]

resveratrol	MB001	T: 158 mg L^{-1}	[101,52]
Proline	MB001	Y: 0.29 $g \cdot g^{-1}$	[98]
Proteins	MB001	Pyruvate kinase: sp.act. 135 U/mg	[107]
Protocatechuate	MB001	T: 2 g g^{-1}	[49]
Putrescine	MB001	Y: 0.17 $g \cdot g^{-1}$	[98]
zeaxanthin	MB001	C: 1.2 mg g^{-1} DCW	[102-103]
β-carotene	MB001	C: 12 mg g^{-1} DCW; V: 3.4 mg L^{-1} h^{-1}	[57,25]

Abbreviations: T: titer or concentration in culture broth, Y: product yield on substrate (unless otherwise indicated glucose was used as substrate); V: volumetric productivity; C: cellular content; CDW: cell dry weight.

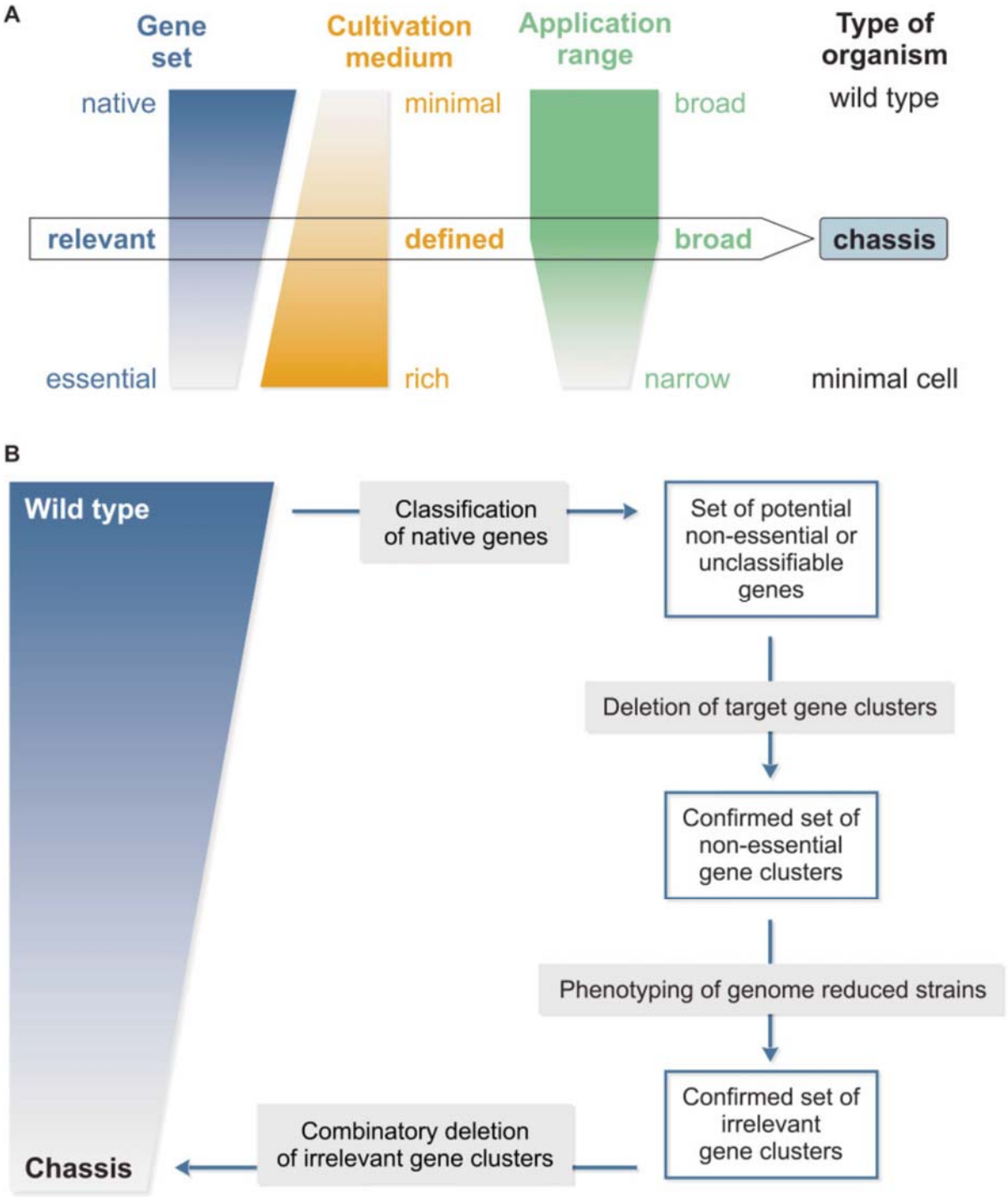

Fig. ##.1. Definitions and workflow for the construction of a chassis organism of *Corynebacterium glutamicum* (Copyright © 2015 Unthan, Baumgart, Radek, Herbst, Siebert, Brühl, Bartsch, Bott, Wiechert, Marin, Hans, Krämer, Seibold, Frunzke, Kalinowski, Rückert, Wendisch, Noack; reproduced from [118]). (A) Definitions considering the interplay of gene set, cultivation medium, and application range for different types of organisms. (B) Scheme of our targeted top-down approach toward a chassis covering only genes that are relevant for

growth on defined medium and maintaining the broad application range of the wild-type organism.

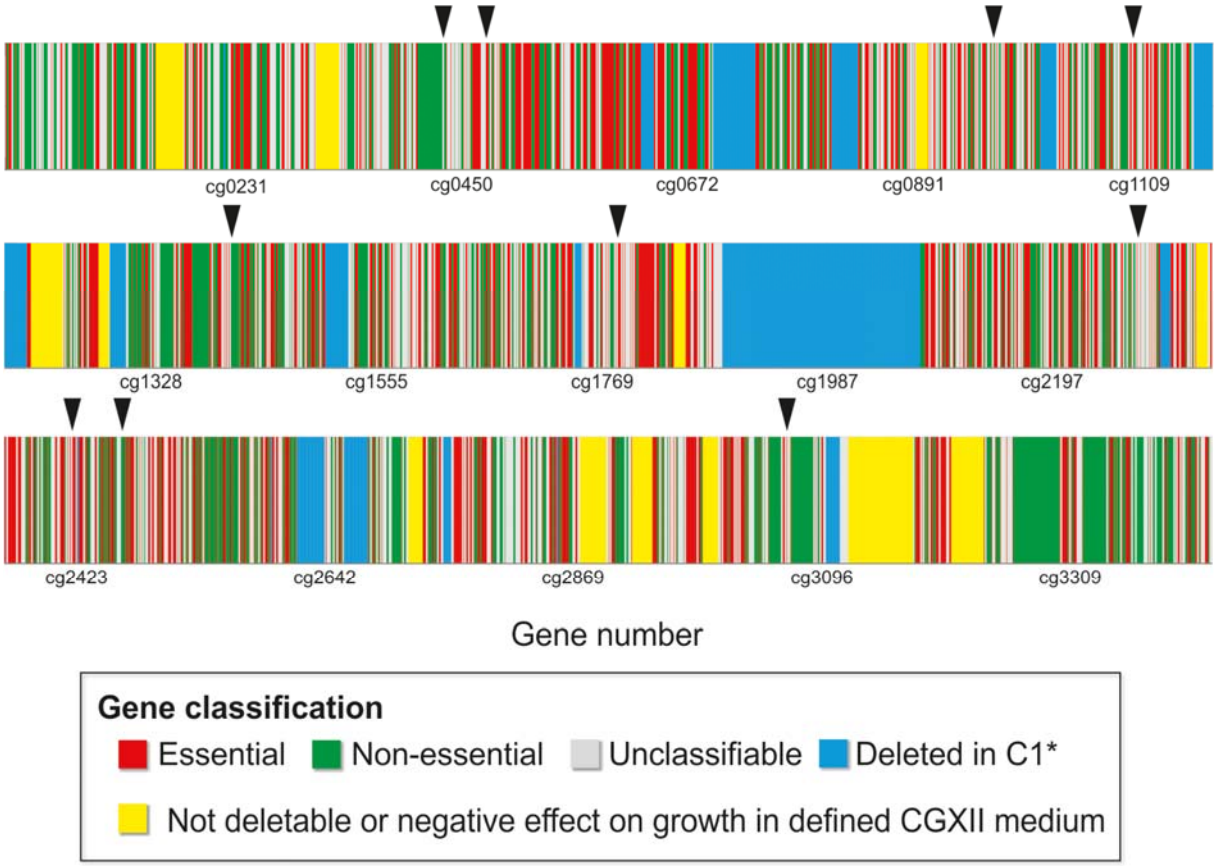

Fig. ##.2. *C. glutamicum* ATCC 13032 genome map with classification results of essential, nonessential and unclassifiable genes. (Copyright © Reprinted with permission from Baumgart M, Unthan S, Kloss R, Radek A, Polen T, Tenhaef N, Muller MF, Kuberl A, Siebert D, Bruhl N, Marin K, Hans S, Kramer R, Bott M, Kalinowski J, Wiechert W, Seibold G, Frunzke J, Ruckert C, Wendisch VF, Noack S (2018) *Corynebacterium glutamicum* Chassis C1*: Building and Testing a Novel Platform Host for Synthetic Biology and Industrial Biotechnology. ACS Synth Biol 7 (1):132-144. Copyright 2018 American Chemical Society. [123]). All clusters deleted in C1* are shown in blue. Clusters that could not be deleted or deletions leading to impaired growth in defined CGXII medium are shown in yellow. Black arrows are pointing toward glycolysis genes *pgi* (cg0973), *pfkA* (cg1409), *fda* (cg3068), *tpi* (cg1789), *gap* (cg1791), *pgk* (cg1790), *gpmA* (cg0482), *eno* (cg1111), *pyk* (cg2291), *aceE* (cg2466), *lpd* (cg0441), and *sucB* (cg2421).

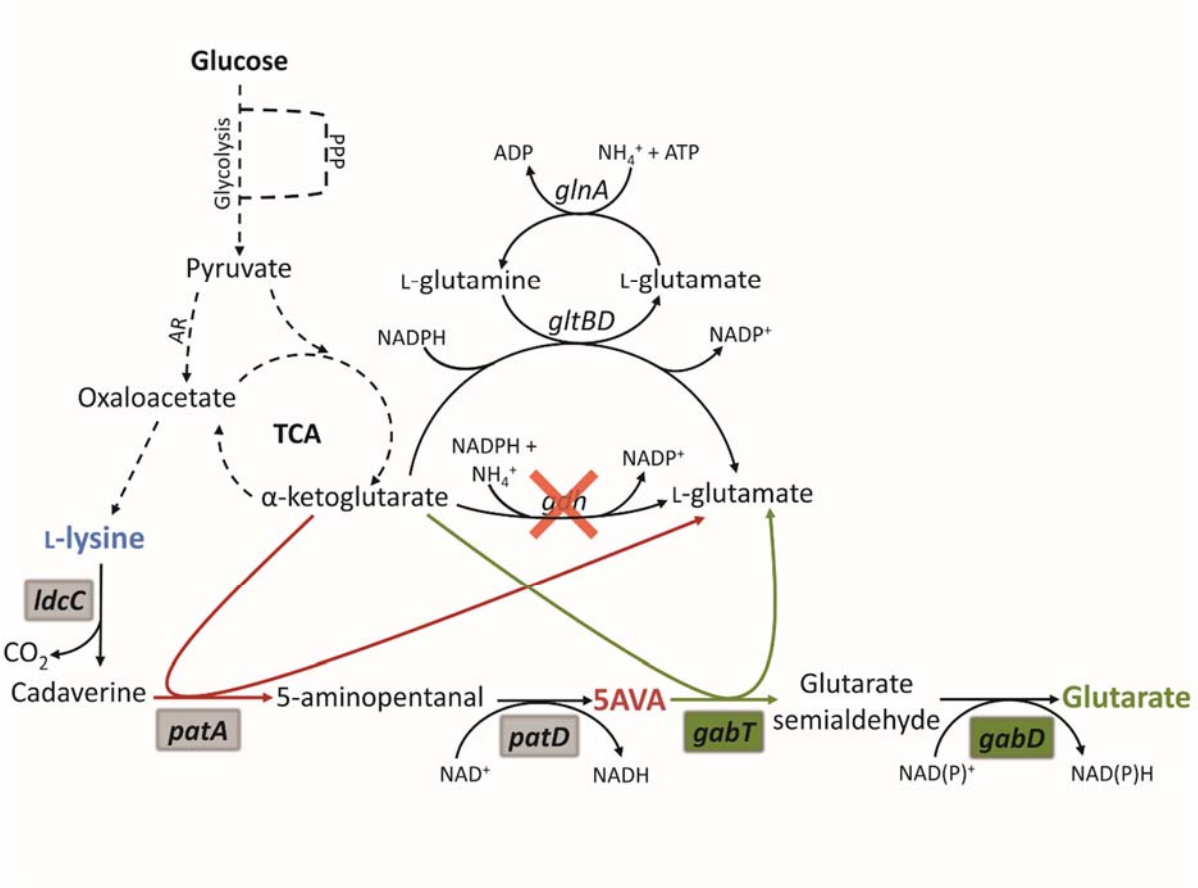

Fig. ##.3 Schematic representation of the metabolic engineering strategy for glutarate production by recombinant *C. glutamicum* (Copyright © 2018 Pérez-García, Jorge, Dreyszas, Risse and Wendisch; reproduced from [43]). The biosynthetic pathway for glutarate production was implemented by heterologous expression in a L-lysine producer and coupled with endogenous L-glutamate synthesis. PPP, pentose phosphate pathway; TCA, tricarboxylic acid cycle; AR, anaplerotic reactions; *glnA*, glutamine synthase gene; *gltBD*, glutamine aminotransferase complex genes; *gdh*, glutamate dehydrogenase; *ldcC*, L-lysine decarboxylase; *patA*, putrescine transaminase; *patD*, γ-aminobutyraldehyde dehydrogenase; *gabT*, GABA/5AVA amino transferase gene; *gabD*, succinate/glutarate-semialdehyde dehydrogenase gene. Magenta arrows depict transamination reaction in the 5AVA pathway. Green arrows depict transamination reaction in the glutarate pathway. Gray shadowed genes

are originally from *E. coli* and were added by heterologous overexpression. Green shadowed genes are originally from *C. glutamicum*, *P. putida, P. syringae,* or *P. stutzeri* and were added by heterologous overexpression.

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