**Engineering *Corynebacterium glutamicum* with a comprehensive genomic
library and phage-based vectors**

Filipe Marques ^{a,b,c,d}, Andriy Luzhetskyy ^{d*} & Marta V. Mendes ^{a,b,c*}

^a Bioengineering and Synthetic Microbiology Group, i3S- Instituto de Investigação e
Inovação em Saúde, Universidade do Porto, 4200-135 Porto, Portugal

^b IBMC – Instituto de Biologia Molecular e Celular, Universidade do Porto, 4200-135
Porto, Portugal.

^c ICBAS – Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, 4050-
313 Porto, Portugal.

^d Pharmazeutische Biotechnologie, Universität des Saarlandes, Campus C2.3, 66123
Saarbrücken, Germany.

*Corresponding authors: AL – a.luzhetskyy@mx.uni-saarland.de; MVM –
mvm@ibmc.up.pt

ABSTRACT

The Gram-positive bacterium *Corynebacterium glutamicum* sustains the industrial production of chiral molecules such as L-amino acids. Through heterologous gene expression, *C. glutamicum* is becoming a sustainable source of small organic molecules and added-value chemicals. The current methods to implement heterologous genes in *C. glutamicum* rely on replicative vectors requiring lasting selection or chromosomal integration using homologous recombination. Here, we present a set of dedicated and transversal tools for genome editing and gene delivery into *C. glutamicum*. We generated a cosmid-based library suitable for efficient double allelic exchange, covering more than 94% of the chromosome with an average 5.1x coverage. We employed the library and an iterative marker excision system to generate the carotenoid-free *C. glutamicum* BT1-C31-Albino (BCA) host, featuring the attachment sites for actinophages ϕ C31 and ϕ BT1 for one-step chromosomal integration. . As a proof-of-principle, we employed a ϕ C31-based integration and a Cre system for the markerless expression of the type III polyketide synthase RppA, and a ϕ BT1-based integration system for the expression of the phosphopantetheinylation-dependent non-ribosomal peptide synthetase BpsA in the *C. glutamicum* BCA host. The developed genomic library and microbial host, and the characterized molecular tools will contribute to study of the physiology and the rise of *C. glutamicum* as leading host for drug discovery.

KEYWORDS

Actinobacteria; Genome editing; Natural products; Heterologous expression.

1. INTRODUCTION

Corynebacterium glutamicum is a generally regarded as safe (GRAS) microorganism that provides a considerable part of the worldwide supply of bulk chemicals namely, amino acids (Kinoshita et al., 1957). Given its favorable physiological properties [reviewed in (Vertes et al., 2013)], *C. glutamicum* has soon become a multifaceted biorefinery for industrial biotechnology, e.g. on the production of organic acids, and pharmaceutical biotechnology, producing recombinant proteins and secondary metabolites (Becker et al., 2018a). The molecular basis of the industrial success of *C. glutamicum* has been unveiled by the analysis of the metabolic flux (Marx et al., 1996) and by the projects of genome and transcriptome sequencing (Bathe et al., 1996; Ikeda and Nakagawa, 2003; Kalinowski et al., 2003; Pfeifer-Sancar et al., 2013; Tauch et al., 2002). Taken together, this knowledge paved the way for the development of genome-scale metabolic maps of *C. glutamicum* to guide systems-based engineering approaches (Kjeldsen and Nielsen, 2009; Shinfuku et al., 2009; Zhang et al., 2017).

The concept of a chassis for industrial biotechnology is centered on genome-minimized strains, whose physiology can be controlled, including the carbon flux, and for which there are well-characterized molecular tools (Vickers et al., 2010). The sequenced strain ATCC 13032 served as the starting point for the development of a chassis based on *C. glutamicum*. First, the removal of prophages (strain MB001) or insertion sequence (IS) elements (strains WJ004/WJ008) led to improvements in the production of recombinant proteins (Baumgart et al., 2013; Choi et al., 2015). Next, 26 clusters of the prophage- and IS element-free strain CR099 were found to be individually irrelevant for growth rate and growth yield in minimal media with glucose as carbon source (Unthan et al.,

2015). Combinatorial knockout of those clusters from the strain CR099 led to the strain C1*, which presents a genome reduction of 13.2%, uncompromised growth rate, and stable genetic traits (Baumgart et al., 2018). All the genetic engineering steps during chassis development were performed by standard double homologous recombination using non-replicative vectors (Schafer et al., 1994) in an essentially error-free manner. The authors did not disclosed the efficiency of the process, but they stated that it took six years from evaluating the essentiality of genes until strain C1* was published (Baumgart et al., 2018).

The available methods for genome engineering in *C. glutamicum* take advantage of the native homologous recombination machinery, with or without help from heterologous recombineering and/or endonuclease systems. Traditionally, genome editing in *C. glutamicum* has been performed using non-replicative vectors as editing templates, and double crossovers have been isolated owing to selection and counter-selection markers (Kim et al., 2011; Ma et al., 2015; Schafer et al., 1994). These techniques rely exclusively on native homologous recombination machinery and are considered precise and virtually error-free. However, in the counter-selection step, cells can revert to the original genotype by excising the suicide vector due to lack of selection for mutants. Recently, strategies using endonucleases have gained momentum in *C. glutamicum* [reviewed in (Becker et al., 2018b)]. On the one hand, homologous recombination templates were coupled to catalytically active endonucleases Cas9 and Cpf1 programmed to introduce double-strand breaks (DSBs) at the target region in the genome of *C. glutamicum* (Cameron Coates et al., 2019; Cho et al., 2017; Jiang et al., 2017; Liu et al., 2017; Peng et al., 2019; Peng et al., 2017; Wang et al., 2018a; Zhang et al., 2019). These techniques impose DSBs that are lethal if left unrepaired; thus, most

surviving clones carry the desired mutation. However, the efficiency of introduction of DSBs is dependent on the design of the sgRNA that guides the endonuclease. Moreover, the expression of endonucleases is deleterious for strains and imposes a great risk of unwanted genome rearrangements due to off-the-target DSBs and unexpected homologous recombination events. On the other hand, catalytically inactive endonucleases engineered with a deaminase domain were programmed to cause base mismatches in the genome of *C. glutamicum*, triggering native repair machinery to act in a predictable manner (Wang et al., 2019; Wang et al., 2018b). Since DSBs are not involved, these techniques are less likely to cause unexpected genome rearrangements. However, the efficiency of base editing is still dependent on the design of the sgRNAs, and there are no universal selection methods for clones carrying mutations. The limitation of the current methods for genome editing in *C. glutamicum* has been addressed with success in other actinobacteria, notably *Streptomyces*. An iterative excision marker system was employed to convert genomic libraries into platforms for genome engineering in *S. albidoflavus* (previously named *S. albus* J1074) and *S. lividans* TK24 (Myronovskyi et al., 2014; Rebets et al., 2018). This method employs the same principle as that of non-replicative vectors but is more efficient due to the larger homology regions and the presence of excisable selection markers in the mutant strains. The biotechnological features and the availability of metabolic engineering methods potentiates *C. glutamicum* as a chassis for the heterologous production of secondary metabolites. Several classes of natural products have been produced in *C. glutamicum*, including polyphenols and terpenoids [recently reviewed in (Kogure and Inui, 2018)], as well as polyketides and non-ribosomal peptides. The only polyketides produced in *C. glutamicum* are flaviolin (3.9 mg/L) encoded by the 1.1-kb *rppA* gene from *Streptomyces*

griseus (Yang et al., 2018), and 6-methylsalicylic acid (41 mg/L) encoded by the 6.4-kb *chlB1* from *Streptomyces antibioticus* (Kallscheuer et al., 2019). The modest titers of most of the published attempts to produce secondary metabolites in *C. glutamicum* highlight the need for sophisticated metabolic analysis and engineering methods. On a positive note, native 4'-phosphopantetheinyl transferases (PPTase) of *C. glutamicum* were able to activate type I polyketide synthases and non-ribosomal peptide synthetases (Kallscheuer et al., 2019). Furthermore, the reported production of 5.4 g L⁻¹ of violacein in an engineered *C. glutamicum* strain (Sun et al., 2016) clearly shows the potential for amino acid-based secondary metabolites in this organism.

The use of *C. glutamicum* as heterologous host to produce complex secondary metabolites requires appropriate vectors and methods to deliver large biosynthetic gene clusters in a straightforward manner. Current methods to integrate heterologous DNA at specific sites into *C. glutamicum* include non-replicative vectors with homologous regions to the chromosome and vectors derived from the integration systems of bacteriophages. Integration based on homologous recombination has been performed using non-replicative vectors with chromosomal homology regions spanning from 300 to 500 bp such as downstream of the *ppc* gene (Vasicová et al., 1998), at the *rrnD* loci (Amador et al., 2000), at repetitive insertion sequences (Correia et al., 1996), between *cg1121* and *cg1122* (Baumgart et al., 2013) or at the *crtEb* locus (Hoffmann et al., 2018). Being technically simple, this technique often presents low integration frequency and requires laborious screening of markerless strains. Alternatively, integration based on site-specific integrases of phages was performed, taking advantage of the native ϕ AAU2 (Marrec et al., 1994), ϕ 16 (Moreau et al., 1999a), ϕ 304L (Moreau et al., 1999b), and β (Oram et al., 2007) attachment sites (*attB*) in *C. glutamicum*; ATCC

13032 and the genome minimized C1* strains retain all the native attachment sites described. However, all methods derived from corynephages rely on the permanent maintenance of recombinant *C. glutamicum* strains on selective antibiotics, restricting their use in large industrial fermentations. The recombination systems of bacteriophages without attachment sites in *C. glutamicum* have also been used for integration of exogenous DNA in engineered strains. The Cre recombinase of P1 phage was employed to catalyze the integration of *lox66*-bearing plasmids into the chromosome of the *C. glutamicum* CRRE strain harboring the recombination site *lox71*, with an efficiency of 3.1×10^4 colony forming units (CFU) per microgram (Suzuki et al., 2007). The integrase of the lactococci phage TP901-1, expressed in *cis*, was shown to promote integration of the *attP*_{TP901-1}-bearing plasmids onto the matching site of the engineered *C. glutamicum* JS34 strain with an efficiency of 1.3×10^3 CFU μg^{-1} (Shen et al., 2017). The authors combined this TP901-1-based system with Cre/*loxP* elements to develop a platform for repetitive and markerless chromosomal integration (Shen et al., 2017).

Herein, we present a general method and tools for genetic engineering of *C. glutamicum* grounded on a genome-wide cosmid library suitable for double allelic exchange and a pre-designed marker excision system. Starting from a pre-optimized chassis, we demonstrated the usefulness of the library by seamlessly replacing the carotenoid biosynthetic gene cluster of *C. glutamicum* by an additional genetic gateway derived from actinobacterial integration systems. The resulting strains was the host for heterologous expression of the polyketide synthase RppA and of the PPTase-dependent non-ribosomal peptide synthetase BpsA, demonstrating the feasibility of producing natural products via phage-based integrative systems in *C. glutamicum*.

2. MATERIALS & METHODS

2.1 Bacterial strains, plasmids, media and growth conditions

All the strains and vectors used in this work are listed in table 1. The *E. coli* strains were routinely cultivated in Lysogeny Broth (LB) with 5 g L⁻¹ NaCl (Lennox, 1955), unless stated otherwise. The *C. glutamicum* strains were routinely cultivated in LB or CGXII defined media (Keilhauer et al., 1993) with 4% (w/v) glucose, 0.2 mg L⁻¹ biotin and 30 mg L⁻¹ protocatechuic acid. When necessary, the following antibiotics were added to the media: ampicillin (Ap, 100 µg mL⁻¹ to *E. coli*), apramycin (Am, 50 µg mL⁻¹ to *E. coli*, 25 µg mL⁻¹ to *C. glutamicum*), kanamycin (Km, 50 µg mL⁻¹ to *E. coli*, 25 µg mL⁻¹ to *C. glutamicum*), hygromycin (Hyg, 80 µg mL⁻¹ to *C. glutamicum*), chloramphenicol (Cm, 12.5 µg mL⁻¹ to *E. coli*, 10 µg mL⁻¹ to *C. glutamicum*). Transformation of *C. glutamicum* was performed by electroporation (Eggeling and Bott, 2005), and selection of transformants was achieved in BHIS medium with half of the usual antibiotic concentration. Integration of plasmids into *C. glutamicum* genome was confirmed by PCR using the appropriate primer pairs. Unless stated otherwise, *E. coli* was grown at 37 °C and *C. glutamicum* was grown at 30 °C.

173 **Table 1.** Strains and vector used in this work used in this study

Bacterial strains and vectors	Description	Source or reference
<i>E. coli</i>		
DH5α	General cloning host	(Hanahan, 1983)
GB05-dir	Host for recombineering procedures	(Fu et al., 2010)
EPI300-T1 ^R	Phage T1-resistant host for generation of cosmid library	Lucigen
<i>C. glutamicum</i>		
CR099attB _{C31}	<i>C. glutamicum</i> CR099 derivative for integration of φ C31-based vectors	Gift of J. Kalinowski (Present address: Center for Biotechnology, Bielefeld University, Bielefeld, Germany) [Based on CR099 strain (Baumgart et al., 2018; Unthan et al., 2015)]
BCA-Am	<i>C. glutamicum</i> CR099attB _{C31} derivative, in which the <i>crt</i> locus (<i>cgp_0717</i> - <i>cgp_0723</i>) has been replaced by <i>saac(3')IV-attB_{BT1}</i> cassette	This work
BCA	<i>C. glutamicum</i> BCA-Am derivative, in which the <i>saac(3')IV</i> marker has been excised.	This work
Plasmids		
pTES	Am ^R ; φ C31-based plasmid for stable and markerless integration via <i>attB_{C31}</i>	(Herrmann et al., 2012)
cos15A-gus	Cm ^R , <i>gusA</i> ; vector for construction of cosmid genome library, suitable for allelic exchange in streptomycetes	Gift of M. Lopatniuk (Present address: Saarland University, Campus C2.3, 66123, Saarbrücken, Germany)
pK19mobsacB	Km ^R , <i>sacB</i> ; plasmid for allelic exchange in <i>C. glutamicum</i>	(Schafer et al., 1994)
cos15A-sacB	cos15A-gus derivative, suitable for allelic exchange in <i>C. glutamicum</i>	This work
patt-saac	Ap ^R ; source of cassette for markerless deletions	(Myronovskyi et al., 2014)
patt-saac-attBT1	patt-saac derivative, source of cassette for markerless deletions with <i>attB_{BT1}</i>	This work
pCG_4_F11	cos15A-sacB derivative containing the <i>crt</i> locus (<i>cgp_0717-0723</i>)	This work

pCG_4_F11_Δ <i>crt::attB_{BT1}-saac(3')IV</i>	pCG_4_F11 derivative for replacement of <i>crt</i> locus by <i>saac(3')IV-attB_{BT1}</i> cassette	This work
pKHInt31	Hyg ^R ; plasmid with temperature-sensitive replicon pSG5 for expression of C31-Int	(Myronovskyi et al., 2014)
pEKEx2	Km ^R ; <i>C. glutamicum</i> / <i>E. coli</i> shuttle vector for inducible gene expression	(Eikmanns et al., 1991)
pUC57-rppA _{Cgl}	Ap ^R ; source of SD_ <i>rppA_{Cgl}</i>	This work
pUC57-rppA _{CglStrept}	Ap ^R ; source of SD_ <i>rppA_{CglStrept}</i>	This work
pEKEx2-rppA _{Cgl}	pEKEx2 derivative for expression of flaviolin (for <i>C. glutamicum</i>)	This work
pEKEx2-rppA _{CglStrept}	pEKEx2 derivative for expression of flaviolin (for <i>C. glutamicum</i> and streptomycetes)	This work
pTES-rppA	pTES derivative for production of flaviolin (<i>C. glutamicum</i> /streptomycetes), via <i>attB_{C31}</i>	This work
pALCRE	Hyg ^R ; plasmid with temperature-sensitive replicon pSG5 for expression of Cre	(Herrmann et al., 2012)
pRT802	Km ^R ; ϕBT1-based integrative plasmid	(Gregory et al., 2003)
pRT801	Am ^R ; ϕBT1-based integrative plasmid	(Gregory et al., 2003)
pMVM3100	pRT801 derivative with insulated multicloning site, constitutive P57 promoter (Siegl et al., 2013) and optimized 5' UTR N12 (Horbal et al., 2018)	M.V. Mendes (unpublished)
pFM69SD	pMVM3100 derivative with insulated multicloning site, constitutive P69 promoter (Zhang et al., 2018) and optimized 5' UTR of pEKEx2-rppA _{Cgl}	This work
pSMART-3B20	Cm ^R ; BAC of the genomic library of <i>S. albidoflavus</i> containing the <i>bpsA</i> gene	Gift of M. Myronovskyi (Present address: Saarland University, Campus C2.3, 66123, Saarbrücken, Germany)
pUC19	Ap ^R ; <i>lacZα</i> , general cloning vector	(Yanisch-Perron et al., 1985)
pUC19-opt-bpsA-H6	Ap ^R ; source of <i>bpsA</i> gene of <i>S. albidoflavus</i> with 5' codon optimization and encoding for a C-terminal His-tag	This work
pFM69SD-bpsA	pFM69SD derivative for expression of indigoidine	This work

Abbreviations: Am^R – apramycin resistance marker; Ap^R – ampicillin resistance marker; *attB_{BT1}* – bacterial attachment site for phage ϕBT1; *attB_{C31}* – bacterial attachment site for phage ϕC31; Cm^R –

chloramphenicol resistance marker; Hyg^R – hygromycin resistance marker; Km^R – kanamycin resistance marker.

174

175 **2.2 Recombinant DNA manipulations**

176 The isolation of plasmid DNA was performed using NZYMiniprep kit following instruction
177 of the manufacturer. The isolation of cosmid DNA was performed using alkaline lysis
178 method (Sambrook and Russell, 2001). The isolation of genomic DNA of *C. glutamicum*
179 was performed according to the salting-out method (Kieser et al., 2000). Purification of
180 enzymatic reactions and DNA fragments from agarose gels was performed using
181 NZYGelpure kit following instruction of the manufacturer. PCR was performed using Q5®
182 High-Fidelity DNA Polymerase (NEB), or GoTaq® G2 Flexi DNA Polymerase (Promega),
183 following manufacturer's instructions. The oligonucleotides used in this study are listed
184 in **table 2**. Restriction enzymes, T4 ligase, alkaline phosphatase (FastAP), T4
185 polynucleotide kinase (T4 PNK) and GeneRuler DNA Ladder Mix were all provided by
186 ThermoFisher Scientific and employed following manufacturer's instructions. Genes
187 were adapted to the codon usages of the specified organism and synthesized by
188 GenScript (Piscataway, NJ, USA). Routine Sanger sequencing was conducted by STAB
189 VIDA (Caparica, Portugal).

190

191 **Table 2. Oligonucleotides and DNA fragments used in this study.**

Primer	Sequence (5' to 3')	Use
pTESmcs_fwd	GCGAGGAAGCGGAAGAG	Sequence pTES-based plasmids and check integration of pTES derivatives at <i>attB</i> _{C31} locus
pTESmcs_rvs	CGATGTAGGAGGGCGTG	
attB-CR99_fwd	CCATCGCAGCCCCGACTTG	Sequence and check integration at <i>attB</i> _{C31} locus
attB-CR99_rvs	AGCAGCCCACCAAACGTC	
sacB_F	CATTTCGGATCCAAATCGCGCGGGTTTG TTAC	Amplify <i>sacB</i> from pK19mobsacB
sacB_R	GAAATTGGATCCATCTCGTGATGGCAG GTTGG	
sacB-seq_F1	AGCGACACTGAATACGG	Check and sequence <i>sacB</i> in cos15A-sacB
sacB-seq_F2	GCATTAGCCGGAGATCC	
sacB-seq_F3	GATGAAACCGCTGATTGC	
sacB-seq_R1	GACATTGGCCTATTGTAAGC	
cosmid-chk-For	GATCTCCATCGACTAAACGT	Sequence <i>PmeI</i> -cloned insert into cos15A-sacB
cosmid-chk-Rev	GTAACTGCGGTCAAGATAT	
IMES_crt-aac_fwd	ACAACGATTGGGAATTTTTCGAGATAA CTGGCCGTGTGATACTCGAATGTCAC TAGGGCGAATTGGC	Create cassette for editing of pCG_4_F11 cosmid
IMES_crt-aac_rvs	GCGTGGGTGGCTAGGCAAGTTACAGAA CTGATCGGATAAAAGCAGAGTTACTGG AGCTGCTTCGTCTAG	
crt_delchk2_fwd	GCGCAATGTCTTCTCCGAC	Sequence <i>crt</i> locus
crt_delchk2_rvs	GCCTACTTGATGAGGCCGAA	
pEKEx2MCS_F	ACTCCCGTTCTGGATAATG	Sequence pEKEx2-based plasmids and produce P _{tac} _SD_ <i>rppA</i> _{CglStrept} fragment
pEKEx2MCS_R	ACTTCTGAGTTCGGCATG	
pRT_ORI_SEQ	GGATTCCCGTTGAGCACCGC	Check integration of pRT801 and pRT802 derivatives at <i>attB</i> _{BT1} locus

P69-RBS_F	TGATTAAAGCTTAAAATTATTTTAAATTT TCCGTTGACAAGACAATGCGCATGAGAT ATAATGGCA	Create cassette with P69 promoter and 5' UTR of <i>rppA</i> _{Cgl}
P69-RBS_R	TGTTATCATATGCTATCTCCTTACTTTTCT GACAGGTACCGCACTATGCCATTATATC TCATGCGC	
bpsA-opt-fwd	TTGGTAGTCGACTGTCAGAAAAGTAAG GAGATAGCATATGTCCACCTCCACCCCA CCTCCAGCAACCCGCCAGCACGCCTCCC TG	Amplify <i>bpsA</i> gene with 5' optimized codons and encoding a 3' His-tag
bpsA-H6-rvs	TAGAAAGGTACCTACTAGTTTAATGGTG ATGGTGATGGTGGGCGAAGAGGTCCAA GGCGCGTA	
bpsA1	AGAAGACGCCGATCAG	Sequence <i>bpsA</i> gene
bpsA2	CGACAACCAGGTCAAAC	
bpsA3	AAGATCCACTTCCTGGG	
bpsA4	CTCGTACTTCTTCGTGG	
bpsA5	TACCCTATGAACCTGCG	

192

193 To produce a suitable genetic backbone for the cosmid library, the **cos15A-sacB cosmid**
194 was constructed. For that, the *sacB* was amplified from pK19mobsacB (Schafer et al.,
195 1994) using the primers sacB_F and sacB_R, digested with *Bam*HI and cloned into the
196 *Bam*HI sites of cos15A-gus (gift of M. Lopatniuk). The orientation of *sacB* was confirmed
197 by DNA restriction and the identity of the gene was confirmed by sequencing.

198 To generate a cassette suitable for iterative marker excision that would leave an *attB*_{BT1}
199 locus as genetic scar, the **patt-saac-attBT1 plasmid** was constructed. For that, a
200 synthetic 152-bp fragment harboring the *attB*_{BT1} locus flanked by terminators was
201 cloned into the *Stu*I site of patt-saac plasmid.

202 To create a platform to delete the main cluster of carotenoid biosynthetic genes in *C.*
203 *glutamicum* (*cgp_0717-0723*), the pCG_4_F11 cosmid from the genomic library of *C.*

204 *glutamicum* CR099attB_{C31} was modified for appropriate allelic exchange. The cassette
205 containing apramycin resistance and the attB_{BT1} sequence was amplified from patt-sacc-
206 attBT1 using the primers IMES_crt-aac_fwd and IMES_crt-aac_rvs. Red/ET-mediated
207 recombination of pCG_4_F11 cosmid with the amplified fragment was performed in
208 GB05-dir cells, following standard protocols (Datsenko and Wanner, 2000). The
209 originated **pCG_4_F11_Δcrt::attB_{BT1}-saac(3')/IV** cosmid was checked by PCR and
210 confirmed by sequencing.

211 To express *rppA* gene in an inducible manner in *C. glutamicum*, the **pEKEx2-rppA_{Cgl}** and
212 **pEKEx2-rppA_{CglStrept}** were constructed. The Shine-Dalgarno (SD) sequence and the *rppA*
213 gene with adapted to the codon usage of *C. glutamicum* (*rppA_{Cgl}*) or to the mixed codon
214 usages of *C. glutamicum* and *Streptomyces* (*rppA_{CglStrept}*) were synthesized and cloned by
215 GenScript to originate the **pUC57-rppA_{Cgl}** and **pUC57-rppA_{CglStrept}** plasmids, respectively.
216 The SD sequence, and *rppA* variants were digested *Pst*I/*Bam*HI from the source plasmids
217 and cloned into the respective sites of pEKEx2. The identity of the generated plasmids
218 was confirmed by sequencing.

219 To express *rppA_{CglStrept}* from an ϕ C31-integrative plasmid, the **pTES-rppA** plasmid was
220 constructed. The P_{tac} promoter, SD sequence and *rppA_{CglStrept}* gene were amplified using
221 the PNK-phosphorylated primers pEKEx2MCS_F and pEKEx2MCS_R. The amplified
222 fragment was blunt cloned into the *Sca*I-digested pTES plasmid to yield pTES-rppA and
223 confirmed by sequencing.

224 To create an ϕ BT1-integrative plasmid with insulated multicloning site, the **pFM69SD**
225 plasmid was constructed. A cassette containing the P69 promoter (Zhang et al., 2018)
226 and the 5' UTR of *rppA_{Cgl}* was created by PCR using the primers P69-RBS_F and P69-

227 RBS_R. The amplified fragment was digested with *HindIII* and *NdeI* and cloned into the
228 respective sites of pMVM3100 to yield pFM69SD. The identity of pFM69SD was
229 confirmed by sequencing.

230 The *bpsA* gene (NCBI accession CP004370.1, positions 6357383 to 6361213) was
231 amplified from pSMART-3B20 using the primers *bpsA-opt-fwd* and *bpsA-H6-rvs*,
232 digested with *SaII* and *KpnI*, and cloned into the respective sites of pUC19 to yield
233 **pUC19-opt-bps-H6**. The optimization of the 5' sequence of *bpsA* gene (included in the
234 primer *bpsA-opt-fwd*) corresponded to the most prevalent codons in *C. glutamicum*
235 ATCC 13032, as described in the Codon Usage Database (Nakamura et al., 2000). The
236 identity of pUC19-opt-bps-H6 was confirmed by sequencing.

237 To express *bpsA* from an ϕ BT1-integrative plasmid, the **pFM69SD-bpsA** plasmid was
238 constructed. The 5'-modified *bpsA* gene (also encoding a C-terminal His-tag) was
239 digested from pUC19-opt-bpsA-H6 plasmid with *NdeI* and *SpeI* and cloned into the
240 respective sites of pFM69SD to yield pFM69SD-bpsA.

241

242 **2.3 Markerless integration of phage-based vectors at ϕ C31 bacterial attachment site**

243 To integrate the ϕ C31-based pTES (Herrmann et al., 2012) and derivative vectors at the
244 ϕ C31 bacterial attachment site (*attB_{C31}*), *C. glutamicum* cells were transformed by
245 electroporation and selected on BHIS agar supplemented with 12.5 μ g.mL⁻¹ apramycin
246 for 48 h. The integration was confirmed PCR using the primers pTESmcs_fwd and attB-
247 CR99_fwd. To excise the backbone of pTES-based plasmids after integration, the
248 relevant *C. glutamicum* strains were transformed with the pALCRE plasmid (Herrmann
249 et al., 2012) and selected on BHIS agar supplemented with 40 μ g.mL⁻¹ hygromycin for 48

h. Several transformants were individually grown in 5 mL LB for 16 h, then plated and grown in LB agar for 16 h. Several clones were picked and grown on Am25Hyg80-, Hyg80-, and LB agar for 16 h. The excision of pTES backbone and the loss of pALCRE plasmid were indicated by sensitivity to apramycin and hygromycin, respectively. The identity of the markerless strains was confirmed by PCR and sequencing using the primer pair attB-CR99_fwd/rvs.

2.4 Generation of the cosmid-based genomic library

The cosmid library of *C. glutamicum* CR099attB_{C31} was designed as a platform to perform two rounds of positive selection of allelic exchange with homologous regions of the chromosome, following the protocol of the MA171E CopyControl™ Fosmid & HTP Fosmid Kit (Lucigen), with minor modifications. The concentration and quality of genomic DNA isolated from *C. glutamicum* CR099attB_{C31} was analyzed by 0.4% (w/v) agarose gel electrophoresis, using GeneRuler High Range DNA Ladder (ThermoFisher Scientific) as reference. Ten micrograms of total genomic DNA were repaired to blunt, 5' phosphorylated sheared DNA fragments, using Lucigen's proprietary End-Repair Enzyme Mix (containing T4 DNA Polymerase and T4 Polynucleotide Kinase) and extending the reaction time to 6 h. The end-repaired DNA fragments were ligated to 0.7 µg of *PmeI*-digested and dephosphorylated cos15A-sacB vector. The ligated DNA was packaged into the phage particles by adding the MaxPlax Packaging Extract to the ligation reaction. Cells for transduction were precultured from a single colony in 10 mL LB supplemented with 10 mM MgSO₄, and 0.2% (w/v) maltose for 16 h, and then sub-cultured by diluting 1:100 of pre-culture in fresh medium, up to an OD₆₀₀ of 0.8.

273 Transduction was performed by mixing 1 volume of packaged phage suspension with 10
274 volumes of cells and incubating at 37 °C for 1 h. The titer of phage particles was
275 determined by transducing the serial dilutions of packaged phages in Phage Dilution
276 Buffer into *E. coli* EPI300-T1^R cells. To prepare the cosmid library, 250 µL of EPI300-T1^R
277 cells were mixed with 25 µL of undiluted packaged phage suspension, and the
278 transduced cells were plated in LB agar supplemented with 12.5 µg of chloramphenicol.
279 After incubation for 20 h, a total of 576 clones of normal morphology were imaged and
280 picked using a QPix2 Automatic Colony Picker and Imager (Genetix) to six 96-well plates
281 containing 250 µL of Cm12.5-LB media. After incubation at 37 °C for 24 h, at 800 rpm,
282 the plates were replicated, and the cell suspensions were diluted in a glycerol solution,
283 to a final concentration of 25% (w/v) glycerol. The sequencing of the genomic library
284 was performed at Genewiz (Takeley, UK), by Sanger sequencing, using the primer
285 cosmid-chk-For. To order the generated cosmid library, the Sanger reads were trimmed
286 and mapped to the reference genome of *C. glutamicum* MB001 (Baumgart et al., 2013),
287 using Geneious Prime[®] 2019.0.3 (Biomatters Ltd). The trimming and mapping
288 parameters were the following: trimming region with more than a 5% chance of an error
289 per base (5' and 3' ends), Geneious mapper with custom sensitivity and fine tuning up
290 to 5 iterations, minimum mapping quality of 20 (imply multiple best matches are
291 mapped to none), gaps not allowed. Only the mapped reads with perfect pairing to the
292 10 nucleotides of cos15A-sacB cloning region upstream the insert were unequivocally
293 considered directional reads and used for calculation of library statistics. To predict the
294 coverage and library statistics, 35-kbp sections were directionally extracted at the
295 starting coordinates of the assigned reads and mapped to the reference genome, using

Geneious Prime® 2019.0.3. Manual curing was performed to assess coverage of the *attB_{C31}* locus and other features.

2.5 Markerless genome edition using modified cosmids

To create a strain unable to produce carotenoids and featuring an *attB_{BT1}* integration site, deletion of the *crt* locus of *C. glutamicum* CR099*attC31* (*cgp_0717-0723*) was performed using a modified cosmid from the generated library and the IMES procedure (Myronovskyi et al., 2014). For that, CR099*attB_{C31}* cells were transformed with pCG_4_F11_Δ*crt::attB_{BT1}-saac(3')*/IV cosmid and selected on BHIS agar supplemented with 12.5 µg.mL⁻¹ apramycin for 48 h. Apramycin-resistant clones were picked and grown on Am25-LB agar plates for 16 h. First crossover events were tested by colony PCR targeting the *sacB* gene of the cosmid, using primers *sacB-seq_F3* and *sacB-seq_R1*, and by the ability to form colonies on Cm10-LB agar in 16 h. Several first crossovers were individually grown in 5 mL LB for 16 h, then plated and grown on LB agar with 10 g.L⁻¹ NaCl and 10 g.L⁻¹ sucrose for 16 h. Fast-growing colonies were picked on Am25- or Cm12.5-LB agar. Second crossover events were indicated by the chloramphenicol-sensitive, apramycin-resistant phenotype. Several second crossovers were picked and grown on Am25-LB agar for 16 h. A second crossover clone (*C. glutamicum* BCA-Am) was cultivated in 5 mL LB for 16 h, and its genomic DNA was isolated. The *crt* locus of the selected clone was amplified from the isolated gDNA using primers *crt_delchk2_fwd* and *crt_delchk2_rvs* and double allelic exchange was confirmed by sequencing. To excise the apramycin resistance marker according to the IMES protocol (Myronovskyi et al., 2014), *C. glutamicum* BCA-Am was transformed with pKHInt31 plasmid for transient

expression of C31-Integrase. The process of strain isolation and plasmid curing proceed was described above for the pALCRE plasmid. The identity of the markerless strains was confirmed by PCR and sequencing using the primers crt_delchk2_fwd and crt_delchk2_rvs. A curated markerless clone dubbed ***C. glutamicum* BCA** was cultivated in 5 mL LB for 16 h, and its genomic DNA was isolated using the GeneJET Genomic DNA Purification Kit (ThermoFisher Scientific), following manufacturer's instructions for Gram-positive bacteria. The isolated DNA from the *C. glutamicum* BCA strain was sent to Novogene UK Company Ltd (Cambridge, UK) for PCR-free library construction (average size of 350 bp) and 150-bp paired-end Illumina (NovaSeq 6000) sequencing (Q30 of 92.58%). De novo assembly and reads mapping were carried out with CLC Genomics Workbench 20 (Qiagen) using as reference the genome of *C. glutamicum* MB001 strain (accession CP005959.1).

2.6 Integration of phage-based vectors at ϕ BT1 bacterial attachment site

To integrate the ϕ BT1-based pRT802 (Gregory et al., 2003) and pFM69SD vectors at the ϕ BT1 bacterial attachment site (*attB_{BT1}*), *C. glutamicum* BCA cells were transformed by electroporation and selected on BHIS agar supplemented with 12.5 $\mu\text{g}.\text{mL}^{-1}$ kanamycin or apramycin, respectively, for 48 h. The integration was confirmed by PCR using the primers pRT_ORI_SEQ and crt_delchk2_rvs.

2.7 Production and quantification of flaviolin and indigoidine

The expression of flaviolin in *C. glutamicum* was based on the replicative pEKEx2-rppA and the ϕ C31 integrative pTES-rppA vectors. The expression of indigoidine in *C.*

342 *glutamicum* was based on the ϕ BT1 integrative pFM69SD-bpsA. The pEKEx2 and pTES,
 343 and pFM69SD vectors served as negative controls for production of flaviolin and
 344 indigoidine, respectively. A single colony was inoculated and cultivated for 6-8 h in 5 mL
 345 LB medium. For pre-culture, 1 mL of this suspension was centrifuged at 5000g for 5 min,
 346 at room temperature, resuspended in 10 mL of CGXII medium and cultivated in a 100-
 347 mL flask for 12-16 h, at 200 rpm. For main culture, 25 mL of fresh CGXII medium were
 348 seeded with pre-culture to an OD600 of 1 (or the equivalent OD800 of 0.72 for
 349 indigoidine) and cultivated in a 250-mL flask for up to 96 h, at 200 rpm. In case of
 350 inducible systems (pEKEx2-based vectors), at 2 h of cultivation, medium was
 351 supplemented with IPTG, to a final concentration of 500 μ M. For cell dry weight (CDW)
 352 measurements, 1 mL of culture broth was collected, centrifuged at 16100g for 10 min,
 353 at room temperature and the pellet was dried at 55 $^{\circ}$ C for 48 h. For UV-Vis analysis, at
 354 defined timepoints, samples of culture broth were transferred to 2-mL tubes and
 355 centrifuged at 16100g for 10 min, at room temperature. Flaviolin was quantified directly
 356 from the supernatant of centrifuged samples. Indigoidine was extracted from the
 357 sediment of 100 μ L of culture broth with 10 volumes of dymethylsulfoxide (DMSO)
 358 (Chatterjee and Brown, 1981) and vigorous vortexing for 2 min, followed by
 359 centrifugation at 16100g for 10 min, at room temperature. The absorbance of flaviolin
 360 and indigoidine extracts were acquired in 1-cm path Optical Glass cuvettes (Hellma), in
 361 a UV Mini 1240 UV/Vis Spectrophotometer (Shimadzu) at 520 nm or 615 nm,
 362 respectively. The absorbance relative to the analyzed pigment (ΔA) was calculated by
 363 subtracting the correspondent absorbance of the negative control. The volumetric
 364 production of flaviolin was calculated using the published extinction coefficient (Krauser
 365 et al., 2012): flaviolin (mg L^{-1}) = $\Delta A_{520\text{nm}} / (1305 \text{ L mol}^{-1} \text{ cm}^{-1} \times 1 \text{ cm}) \times 206.153 \text{ g mol}^{-1} \times$

10³ mg g⁻¹. The volumetric production of indigoidine was calculated using the published extinction coefficient (Yumusak et al., 2019): indigoidine (g L⁻¹) = 10 x ΔA_{615nm} / (1570 L mol⁻¹ cm⁻¹ x 1 cm) x 248.19 g mol⁻¹. The data were parsed using Microsoft Office Excel and analyzed using GraphPad Prism 5 for Windows.

2.8 Nucleotide sequence accession number

All the referenced gene tags correspond to the nomenclature of *C. glutamicum* MB001 (Baumgart et al., 2013), deposited with the NCBI accession CP005959.1. The correspondent *cg* numbers of the mentioned genes can be obtained by replacing the *cgp_* prefix by the *cg* prefix. The sequence of the genome of *C. glutamicum* BCA strain was submitted to NCBI (submissionID: SUB7771938; accession number pending).

3. RESULTS

3.1 Characterization of a markerless integrative system based on actinophage ϕ C31 in *C. glutamicum* CR099attB_{C31}

The *Corynebacterium glutamicum* CR099attB_{C31} host is derived from strain CR099 (Baumgart et al., 2018; Unthan et al., 2015) and harbors an attachment site for the ϕ C31 phage (attB_{C31}) between loci *cgp_0223* and *cgp_0228* (Supp. data 1). The CR099attB_{C31} strain was derived from CR099 strain by double allelic exchange with a pK19mobsacB plasmid carrying the attB_{C31} site of *Streptomyces lividans* TK24 (NCBI accession CP009124.1, coordinates 4286958 - 4286995) (J. Kalinowski, personal communication). To test the functionality of the attB_{C31} site, the strain CR099attB_{C31} was transformed with the ϕ C31-based pTES vector (Herrmann et al., 2012). The pTES vector encodes the phage attachment site for ϕ C31 (attP_{C31}) and the serine recombinase C31-Int that catalyzes the site-directed, directional recombination into attB_{C31} sites (Fig. 1). Integration of pTES into the strain CR099attB_{C31} was verified by the appearance of apramycin-resistant colonies at up to 600 CFU μ g⁻¹ of plasmid. To confirm the site-specific integration of pTES, the genomic DNA of CR099attB_{C31} transformed with pTES was used for Southern blot analysis and plasmid rescue experiments (Supp. methods; Supp. Fig. 1). The results showed that pTES integration occurred at the expected attB_{C31} site. However, the results also suggested that the integration of pTES can be reversed *in vivo*, resulting in episomes. The frequency of pTES self-excision was accessed indirectly by transforming *E. coli* with plasmid DNA isolated from *C. glutamicum* CR099attB_{C31}::pTES (Supp. methods). The results showed that the number of pTES episomes per cell was less than 0.005% that of a pBL1-based replicative vector in *C.*

glutamicum. Several functions of the integrated pTES vector, such as the apramycin resistance marker and C31-Integrase, are unnecessary for lasting expression of the delivered gene of interest (*goi*). To demonstrate the markerless integration of pTES, the Cre/*loxP* system was used to evict the backbone of the pTES vector from the *C. glutamicum* CR099*attB*_{C31}::pTES chromosome (**Fig. 1**), specifically using the pALCRE plasmid (Herrmann et al., 2012). Apramycin-sensitive transformants were obtained with high efficiency (16 sensitive clones out of 16 tested colonies). Three out of four apramycin-sensitive clones were shown to have undergone recombination of the *loxP* sites, resulting in the excision of the 5.5 kb-long backbone of the pTES vector and maintenance of the multi-cloning site. In summary, the *attB*_{C31} locus of the strain CR099*attB*_{C31} was shown to be functional and allowed site-specific and markerless integration of ϕ C31-based plasmids.

Figure 1. Markerless integration of the ϕ C31-based pTES vector into the genome of CR099attB_{C31}. The site-directed integration of the attP_{C31}-harboring pTES vector (Herrmann et al., 2012) into attB_{C31} encoded in the genome of CR099attB_{C31} is catalyzed by C31-Integrase (C31-Int). Integration creates two short scars with low recombination potential, attL_{C31} and attR_{C31}. The gene of interest (goi) is carried in the multicloning site flanked by transcriptional terminators. The backbone elements of the integrated vector (colE1_{oriV}, Am^R, oriT, C31-Int) are flanked by loxP sites and can be excised by expressing Cre recombinase from the curable pALCRE plasmid (Herrmann et al., 2012).

3.2 Generation of an ordered cosmid library of *C. glutamicum* CR099attB_{C31}

To facilitate the genetic manipulation of *C. glutamicum* CR099attB_{C31}, an ordered genomic library was generated. The library was built on the cos15A-sacB cosmid (**Supp. data 2**) that allows λ -mediated transfection into *Escherichia coli* and is suitable for double allelic exchange owing to selection with chloramphenicol and counter-selection

with the levansucrase gene (*sacB*). High-molecular weight genomic DNA of CR099 $attB_{C31}$ was isolated by salting-out method, yielding detectable fragments over 24 kbp only. The isolated genomic DNA of *C. glutamicum* CR099 $attB_{C31}$ was directly end-repaired to blunt, 5'-phosphorilated fragments using an enzyme mix containing T4 DNA polymerase and T4 DNA kinase. The repaired DNA fragments were cloned into the cos15A-*sacB* cosmid and packaged into λ particles. After transduction, 576 *E. coli* clones were selected and placed into 96-well plates (identified as pCG_1-6_A1-H12). From the sequencing of the 5'-end of the inserts, 446 high-quality reads were unambiguously mapped to the *C. glutamicum* MB001 genome (Baumgart et al., 2013) (**Fig. 2, Supp. data 3**), the CR099 ancestor strain whose sequence was publicly available. Of the residual 130 clones that could not be mapped to MB001 genome, in 107 instances failed to one or several of the mapping parameters (namely, 93 instances could not be mapped due to poor base calling quality, 3 instances fell into repetitive regions, 8 instances mapped to the cosmid backbone, and 3 instances mapped to *E. coli* genome or miscellaneous vector regions) and in 23 instances, due to poor base calling quality of the 5' end, reads could not be assigned a direction.

Figure 2. Representation of the coverage of the generated genomic library from *C. glutamicum* CR099attB_{C31}. Position refers to the original numbering of strain MB001. The coverage graph indicates the number of cosmids covering a given region, and the average coverage is indicated in the vertical axis. Hotspots are regions with significant deviation from the average coverage: white strips indicate no-coverage areas, and black strips indicate areas with coverage at least two standard deviations higher than the average. Individual cosmids covering a given region are depicted on the bottom as black horizontal bars, covering 35 kbp-long stretches of the chromosome. Created using Geneious software. attB_{C31} – bacterial attachment site for phage ϕ C31; crt – main cluster of biosynthetic genes of carotenoids in *C. glutamicum*.

Overall, the generated library achieved an average 5.1x coverage of 94.2% of the chromosome of *C. glutamicum* MB001. Notably, the regions not covered by our library were also unrepresented in the cosmid library created for the whole-genome sequencing project of *C. glutamicum* ATCC 13032 (Bathe et al., 1996; Tauch et al., 2002) (**Supp. Fig. 2**). Thorough analysis of the unrepresented genes (**Supp. data 4**) against the PanDaTox gene collection (Kimelman et al., 2012) revealed that seven out of the eight regions without coverage in our library contain homologs reported as unclonable in *E. coli* (**Supp. methods, Supp. data 5**). Access to the bacterial clones of the library is available upon request.

3.3 Generation of a carotenoid-free *C. glutamicum* strain with an additional ϕ BT1 integration site

To demonstrate the potential of the cosmid library in genomic editing procedures, we generated a carotenoid-free *C. glutamicum* strain with an additional attB_{BT1} gateway replacing the main cluster of carotenoid biosynthetic genes (crt locus, cgp_0717-0723) (**Fig. 3**).

473

474 **Figure 3. Markerless replacement of the *crt* locus by the ϕ BT1 attachment site using the developed**
475 **genomic library of *C. glutamicum* CR099attB_{C31}.** The table represents the steps, procedure and time
476 required for markerless genome editing using modified cosmids from the generated library. The panels
477 **(1), (2) and (3)** show the detailed workflow of the markerless genome editing, focusing on the cosmid
478 modification **(1)**, the two consecutive allelic exchanges **(2)** and the marker excision **(3)**. Only the selection
479 markers relevant for each step are shown for clarity. The *pKHInt31* plasmid used for marker excision was
480 developed elsewhere (Myronovskyi et al., 2014). The referenced *cgp_0732* and *cgp_0687* are shown to

481 illustrate the span of the of pCG_4_F11 and pCG_4_F11_Δcrt::attB_{BT1}-saac(3')/IV cosmids. Am^R –
482 apramycin resistance marker; attB_{BT1} – attachment site for the ϕBT1 phage; attB_{C31(CC)} – bacterial
483 attachment site for the ϕC31 phage with central GG core dinucleotides; attP_{C31(GG)} – phage attachment
484 site for the ϕC31 phage with central GG core dinucleotides; C31-Int – integrase of the ϕC31 phage; Cm^R –
485 chloramphenicol resistance marker; crt – main cluster of biosynthetic genes of carotenoids in *C.*
486 *glutamicum*; Hyg^R – hygromycin resistance marker; p15A_{oriV} – plasmid origin of replication for *E. coli*;
487 pSG5_{oriV} – plasmid origin of replication for *C. glutamicum*; RR – attB_{C31(CC)}/attP_{C31(GG)} recombination scar;
488 sacB – sucrose sensitivity marker.

489 The sequence of the introduced attB_{BT1} site corresponds to that of *Streptomyces*
490 *lividans* TK24 (NCBI accession CP009124.1, coordinates 3212045 - 3212117). Initially, the
491 crt locus found in the pCG_4_F11 cosmid was replaced by a cassette generated by PCR
492 harboring the attB_{BT1} sequence and the necessary elements for iterative marker excision
493 (Myronovskyi et al., 2014), in recombineering-proficient *E. coli* strain. In detail, the
494 elements for iterative marker excision were the codon-optimized apramycin resistance
495 marker saac(3')/IV flanked by two engineered attachment sites attB_{C31(CC)} and attP_{C31(GG)}
496 with central GG core dinucleotides, which are orthologous to the original attB_{C31}
497 (Myronovskyi et al., 2014). For comparison purposes, the pK19mobsacB-crt plasmid was
498 created, featuring the same cassette flanked by 432-bp left and 422-bp right homology
499 arms targeting the crt locus (supplemental methods) The CR099attB_{C31} strain was
500 transformed with the pCG_4_F11_Δcrt::attB_{BT1}-saac(3')/IV cosmid or pK19mobsacB-crt
501 plasmid, and the first crossover events were selected by apramycin resistance. The
502 average frequency of first crossover was 9 CFU μg⁻¹ (7 x 10⁻¹¹ CFU per molecule) of
503 pK19mobsacB-crt and 51 CFU μg⁻¹ (2.0 x 10⁻⁹ CFU per molecule) of
504 pCG_4_F11_Δcrt::attB_{BT1}-saac(3')/IV cosmid. To obtain second crossover mutants, two
505 first-crossover clones were grown without antibiotic selection and selected in the

presence of sucrose. The second crossover events were screened by apramycin resistance, and kanamycin or chloramphenicol sensitivity phenotypes, for plasmid or cosmid-based manipulations, respectively. The percentage of second recombination events varied between 0-22% for manipulations using the plasmid and 44-94% using the cosmid. Interestingly, the percentage of events of reversion to the parental phenotype varied between 22-100% for manipulations using the plasmid and 6-56% using the cosmid. The identity of one of the clones that underwent double recombination using the cosmid as recombination template was confirmed by sequencing of the replaced locus and the strain was dubbed *C. glutamicum* BCA-Am. To generate a markerless mutant strain, the apramycin resistance marker was excised from the genome of *C. glutamicum* BCA-Am by recombining the engineered *attB_{C31(CC)}* and *attP_{C31(GG)}* sites flanking the *saac(3')IV* gene. For that, the ϕ C31 integrase was transiently expressed from the pKHInt31 plasmid, and marker-free mutants were screened for apramycin sensitivity. The identity of one apramycin-sensitive clone, named *C. glutamicum* BT1-C31-Albino (BCA), was confirmed by PCR and Sanger sequencing of the *attB_{BT1}* locus (**Supp. data 6**). The engineering process from cosmid modification until the marker-free strain was performed in 14 days, with higher efficiency than conventional plasmid-based methods.

The genome of the *C. glutamicum* BCA strain was re-sequenced and the reads were mapped to the parental strain MB001. In parallel, a *de novo* assembly of the reads was performed originating 28 contigs. The contigs were aligned with the MB001 genome using the Genome Finishing Module of the CLC Genomics Workbench package. The conflicts were curated manually. The genome of the *C. glutamicum* BCA strain was re-sequenced and the reads (a total of 7 668 172 reads) were mapped to the parental strain

530 MB001 (CP005959). In parallel, a *de novo* assembly of the reads was performed
531 originating 28 contigs that were aligned with the MB001 genome using the Genome
532 Finishing Module of the CLC Genomics Workbench package. The conflicts were manually
533 curated comparing the mapping of the reads to the MB001 genome with the contigs
534 from the *de novo* assembly. 98.05% of the reads mapped to the MB001 genome and the
535 average coverage was 374x. The BCA strain has a genome with a length of 3 061 143 bp
536 and compared to MB001 strain it presents 74 nucleotide variants, the replacement of
537 the *crt* cluster (*cgp_0717* to *cgp_0723*) for the *attB_{BT1}*, the replacement of the *cgp_0226*
538 transposase encoding gene for the *attB_{C31}*, the deletion of 8 additional transposase
539 encoding genes (*cgp_4030*, *cgp_0692*, *cgp_1213*, *cgp_2354*, *cgp_2426*, *cgp_2600*,
540 *cgp_2725* and *cgp_2854*), 2 insertions of transposases at the intergenic region between
541 *cgp_1046-cgp_1048* and at the *cgp_3195*. In summary, the genotype of the BCA strain
542 is MB001 $\Delta crt::attB_{BT1} \Delta cgp_0226::attB_{C31} \Delta [cgp_4030; cgp_0692; cgp_1213; cgp_2354;$
543 *cgp_2426; cgp_2600; cgp_2725] cgp_1046-1048::tnp2b cgp_3195::tnp13b*.

544 To assess the carotenoid production of the *C. glutamicum* BCA, the organic
545 extract of the cells was analyzed by spectrophotometry (**Supp. methods; Supp. Fig. 3**).
546 After 24 h of cultivation, the biomass of strain BCA showed less pigmentation than strain
547 CR099*attB_{C31}*. The analysis of the methanolic extracts of the biomass showed that the
548 visible spectrum corresponding to strain BCA lacked the characteristic absorption
549 maxima present in the parental strain CR099*attB_{C31}*.

550 To test the functionality of the newly introduced *attB_{BT1}* locus, the strain BCA was
551 transformed with the well-established ϕ BT1-based pRT802 vector (Gregory et al., 2003).
552 The pRT802 vector encodes the phage attachment site for ϕ BT1 (*attP_{B31}*) and the serine

recombinase BT1-Int that catalyzes the site-directed, directional recombination into the *attB_{BT1}* site. Integration of pRT802 into strain BCA was verified by the appearance of kanamycin-resistant colonies up to 10⁵ CFU µg⁻¹. No colonies were obtained upon transformation of the parental strain CR099*attB_{C31}* with pRT802, which corroborates the absence of an endogenous *attB_{BT1}* locus. To confirm the site-specific integration of pRT802, the genomic DNA of strain BCA transformed with pRT802 was isolated and digested for rescue plasmid experiments (**Supp. methods; Supp. Fig. 4**). The results showed that pRT802 integration occurred at the expected *attB_{BT1}* site. There was no evidence of episomal pRT802 in the assayed clones. The results show that the *attB_{BT1}* locus is functional and represents a unique locus for site-specific integration of ϕ BT1-based vectors into the genome of *C. glutamicum* BCA.

3.4 Production of the polyketide flaviolin in *C. glutamicum* using ϕ C31-based integration

To demonstrate that heterologous gene expression could be achieved using the ϕ C31 attachment site of the *C. glutamicum* strains, a sensitive and practical reporter system based on a polyketide synthase was designed. The candidate gene was *rppA* of *Saccharopolyspora erythraea*, encoding the type III polyketide synthase 1,3,6,8-tetrahydroxynaphthalene (THN) synthase responsible for the production of the diffusible red pigment flaviolin (Cortes et al., 2002). Despite our best efforts, the expression of the native *rppA* gene from *Saccharopolyspora erythraea* was unable to sustain the production of flaviolin in *C. glutamicum*, even though transcription was detected (results not shown). Anticipating problems on the translational level, the *rppA* gene was adapted to the codon usage of *C. glutamicum* (*rppA_{Cgl}*, **Supp. data 7**) and

cloned into the replicative pEKEx2 vector for inducible expression. At 24 h of culture, the broth of the strain CR099*attB*_{C31} harboring pEKEx2-rppA_{Cgl} was orangish, suggesting the production of flaviolin while accumulating the same biomass as that of the control strain. The same behavior was observed in CR099*attB*_{C31} harboring the variant of the *rppA* gene adapted to the mixed codon usage of *C. glutamicum* and *Streptomyces* (*rppA*_{CglStrept}; **Supp. data 8**) via pEKEx2-rppA_{CglStrept}. Noteworthy, both *rppA* variants have codon adaptation indexes (CAI) (Sharp and Li, 1987) over 0.9 and encode no rare codon in *C. glutamicum*. The *rppA*_{CglStrept} gene presents higher %GC content (70.8 vs 61.9), more biased codon usage (27 vs 35 different codons) and sustains 15% lower production of flaviolin (in replicative vector) than the *rppA*_{Cgl} gene. To confirm that the orange coloration was due to the production of flaviolin, the broth of the strain expressing *rppA*_{Cgl} was analyzed by tandem mass spectrometry (MS) coupled to liquid chromatography (**Supp. methods, Supp. Fig. 5**). In positive MS mode, the analysis of the orangish culture revealed a unique peak, eluted at retention time 2.5 min, corresponding to an MS analyte at 207.1 mass-to-charge ratio (m/z), and an MS² fragment at m/z 179, which were confidently assigned to the expected flaviolin molecule.

To study the viability of the ϕ C31 integration system to express heterologous genes in *C. glutamicum*, the *rppA*_{CglStrept} reporter was integrated into the genome of the BCA strain under the control of the Ptac promoter, using the pTES-rppA vector, originating the strain BCA *attB*_{C31}::pTES-rppA (**Fig. 4A and 4B**). The integration of pTES-rppA was confirmed by PCR and the frequency of pTES-rppA episomes per cell (**Supp. methods**) was estimated as less than 0.005% of a pBL1-based replicative vector in *C. glutamicum*. The supernatant of the culture of the strain BCA *attB*_{C31}::pTES-rppA showed

increasing red pigmentation along the growth curve, representing a production of 39.5 mg L⁻¹ of flaviolin at 72 h of culture, without disturbing the biomass accumulation (**Fig. 4C**). To assess the effect of excising the backbone of pTES vector on production levels, the Cre recombinase was employed to generate the markerless strain *BCA attB_{C31}::rppA* (**Fig. 4A and 4B**). This strain maintained the ability to produce flaviolin, up to 9.6 mg L⁻¹ at 72 of culture, although it was reduced to approximately one-quarter that of the strain *BCA attB_{C31}::pTES-rppA* (**Fig. 4D**). The variation of the flaviolin titer may be due the transcriptional readthrough of the promoter of the C31-Integrase, which would cause an overexpression of the *rppA_{CglStrept}* gene before the excision of the pTES-rppA backbone. This hypothesis is supported by the observation that, when the *rppA_{CglStrept}* reporter, under the control of the Ptac promoter, was integrated using the ϕ BT1-based pRT802-rppA vector, the levels of production were similar to that of the strain *BCA attB_{C31}::rppA* (**data not shown**).

The data points and error bars in graph (C), and the values and uncertainty in table (D) represent the average and the standard deviation of two experimental replicates, respectively.

3.5 Production of the non-ribosomal peptide indigoidine in *C. glutamicum* using ϕ BT1-based integration

Indigoidine is an organic molecule that presents an unique combination of two conjugated pyridone rings functionalized with dicarboximide and enamine moieties, and is one of the few blue pigments occurring in Nature (Newsome et al., 2014). The indigoidine biosynthesis consist of the spontaneous condensation of two cyclized and oxidized derivatives of L-glutamine, formed by blue pigment synthetases of the IndC family of non-ribosomal peptide synthetases (NRPS) that require activation by phosphopantetheinylation (Reverchon et al., 2002).

To study the viability of the ϕ BT1 integration system to express heterologous genes in *C. glutamicum*, the cryptic IndC-like NRPS of *Streptomyces albidoflavus* (former *S. albus* J1074) encoded by *bpsA* gene (Olano et al., 2014) was expressed in the engineered strain BCA using a ϕ BT1-based integrative vector. For that purpose, the pFM69SD plasmid (**Supp. data 9**) with an insulated multicloning site, the P69 synthetic promoter for constitutive expression (Zhang et al., 2018) and an Shine-Dalgarno (SD) sequence was built upon the ϕ BT1 integration system of the pRT801/pRT802 vectors (Gregory et al., 2003). A preliminary attempt to produce indigoidine in *C. glutamicum* using the native *bpsA* gene (including native SD) from *S. albidoflavus* was unsuccessful, even though transcription had been detected (data not shown). To overcome eventual issues related to the beginning of translation, the thirty 5' nucleotides of *bpsA* were

652 modified to correspond to the most prevalent codons in *C. glutamicum* ATCC 13032
 653 (Nakamura et al., 2000). The modified *bpsA* gene (**Supp. data 10**) was cloned
 654 downstream the functional SD of the pFM69SD vector to yield the pFM69SD-*bpsA*
 655 construct and integrated at the *attB_{BT1}* locus of the BCA strain (**Fig. 5A**). The
 656 transformants of the strain BCA with pFM69SD-*bpsA*, dubbed BCA *attB_{BT1}::pFM69SD-*
 657 *bpsA*, developed a dark blue pigmentation from 24 h of culture in LB agar plates, that
 658 did not diffuse visibly to the agar even after 1 week (**Fig. 5B**). Upon cultivation in liquid
 659 CGXII defined media, with glucose as carbon source and ammonium sulfate and urea as
 660 nitrogen sources, the culture broth presented a blue pigmentation which mostly
 661 pelleted along with the biomass upon centrifugation. The blue pigment could not be
 662 extracted from the pellet with water nor with methanol, and N,N-dimethylformamide
 663 extracted it only to a small extent. In contrast, the pigment was readily extracted from
 664 the pellet with dimethyl sulfoxide (DMSO) and the extract presented a clear UV-Vis peak
 665 at 614 nm. All the recorded physicochemical properties were in accordance with the
 666 properties of indigoidine (Chatterjee and Brown, 1981; Kuhn et al., 1965) and that
 667 reasoned us to consider that the blue pigment was indigoidine. The monitoring of
 668 indigoidine production (**Fig. 5C**) showed that the greatest accumulation happened
 669 between 48 h and 72 h of cultivation. Quantification by spectrophotometry of the DMSO
 670 extracts, using the published extinction coefficient (Yumusak et al., 2019), resulted in a
 671 volumetric production of indigoidine of $7.3 \pm 0.4 \text{ g L}^{-1}$, at 72 h of cultivation. The specific
 672 production of indigoidine at 72 h of culture was $0.492 \pm 0.006 \text{ g g}^{-1}$, which corresponded
 673 to about 50% of the cell dry weight. Noteworthy, the cell dry weight of the strain BCA
 674 *attB_{BT1}::pFM69SD-bpsA* was 119% that of the control strain BCA *attB_{BT1}::pFM69SD*.
 675 However, since the pigment precipitates along with the biomass, the cell dry weight

includes the mass of indigoidine produced. Therefore, the biomass of cells of the strain BCA *attB_{BT1}::pFM69SD-bpsA* excluding indigoidine was about 70% that of the control strain BCA *attB_{BT1}::pFM69SD*. Considering the chemical formula of indigoidine, C₁₀H₈N₄O₄, the production of the blue pigment at 72 h of culture had theoretically absorbed 22% of the carbon atoms available from glucose and 25% of the nitrogen atoms available from ammonium sulfate and urea. Assuming glucose as the limiting reactant, the titer of indigoidine is in good agreement with the reduced biomass of cells of the strain BCA *attB_{BT1}::pFM69SD-bpsA*, excluding the mass of indigoidine.

Figure 5. Production of indigoidine in *C. glutamicum* using a ϕ BT1-based integration system.

The panel (A) shows a schematic representation of the pFM69SD-bpsA vector employed for ϕ BT1-based integration of the indigoidine biosynthetic gene *bpsA*, and its integration at the bacterial attachment site for phage ϕ BT1 in the chromosome of the BCA strain. The picture (B) shows the phenotype of the control strain BCA *attB_{BT1}::pFM69SD* and the strain expressing *bpsA* gene, BCA *attB_{BT1}::pFM69SD-bpsA*, after 4 days of culture, on LB agar, at 30 °C. The graph (C) represents the cell dry weight (left axis) and the volumetric production of indigoidine extracted with dimethyl sulfoxide from the pellet of culture broth after centrifugation (right axis) of the control strain BCA *attB_{BT1}::pFM69SD* and the strain harboring the pFM69SD-bpsA vector, BCA

694 *attB_{BT1}::pFM69SD-bpsA*. The data points and error bars in graph (C) represent the average and
695 the standard deviation of two biological replicates of the strain BCA *attB_{BT1}::pFM69SD-bpsA*,
696 respectively.

697

698

4. DISCUSSION

The success of synthetic biology-based approaches relies on the development of predictable and efficient chassis as well as stable and robust expression vector tools. (Beites and Mendes, 2015). The industrial workhorse *Corynebacterium glutamicum* has been engineered using top-down approaches towards a general chassis for industrial biotechnology. Here, we demonstrate the utility of a genomic library for chassis engineering by developing a *Corynebacterium*-based chassis for phage-based integration of heterologous genes. The development and characterization of compatible phage-based vectors resulted in the proof-of-principle production of polyketides and non-ribosomal peptides in the generated chassis.

The development of a chassis for synthetic biology application requires genetic editing platforms that encompass comprehensive ranges, traceable methods and targetable tools. The use of programmable endonucleases such as Cas9 and Cpf1 offers great flexibility in genome editing but substantially increases the risk of unwanted off-target effects [reviewed in (Kim et al., 2019)]. In our quest to enhance *C. glutamicum* capabilities as expression chassis, we generated a low-copy number cosmid library of the strain CR099 $\text{att}B_{C31}$ suitable for genome editing by double allelic exchange. The *C. glutamicum* strain CR099 $\text{att}B_{C31}$ is a derivative of the strain CR099 (Baumgart et al., 2018; Unthan et al., 2015) and harbors an attachment site for $\varphi C31$ phage-based DNA integration ($\text{att}B_{C31}$). Apart from the genome reductions, the regions represented in the generated genomic library were coincident with the much larger library of *C. glutamicum* ATCC 13032 prepared in multi-copy vectors (Tauch et al., 2002). Notably, the *relA* gene that regulates the stringent response was absent from both libraries. This

result supports the idea that certain regions of the genome of *C. glutamicum* may be unclonable in *E. coli* even in low copy vectors, due to metabolic disruption. Our library includes all necessary features such as two bacterial selection markers for allelic exchange, a *loxP* site for Cre-mediated excision of genomic regions, and a eukaryotic selection marker for manipulation in yeast. In comparison with conventional plasmid-based allelic exchange platforms, such as pK19mobsacB (Schafer et al., 1994), the use of a cosmid library presents the following advantages: (i) a streamlined procedure to generate allelic exchange platforms, since it relies on pre-designed cassettes and recombineering, and avoids custom primer design and cloning; (ii) higher efficiency of chromosomal integration at first and second crossover steps, with lower occurrence of reversion to parental genotype; (iii) an excisable selection marker, that simplifies phenotypic screening of second crossover events, and allows the selection of knockout mutations that may decrease the fitness of the strain; (iv) and a platform to perform point mutations in essential genes, as exemplified in (Lopatniuk et al., 2019). In summary, the generated library may be advantageous over conventional genome editing methods for the study of the physiology and for metabolic engineering in *C. glutamicum*.

The genomic library developed in this work was employed to further expand the capabilities of *C. glutamicum* as heterologous host, namely by introducing a site for ϕ BT1 phage-based DNA integration. We used the developed library to replace the 7.6-kb *crt* operon encoding the main carotenoid biosynthetic pathway in *C. glutamicum* by a markerless 0.3-kb region harboring the attachment site for ϕ BT1-based vectors (*attB_{BT1}*). The generated strain *C. glutamicum* BCA has an albino phenotype, due to the lack of carotenoids, and features the functional *attB_{C31}* and *attB_{BT1}* sites, for one-step

orthogonal chromosomal integration of phage-based vectors. The high frequency of integration and the swiftness of introducing exogenous DNA using ϕ C31 and ϕ BT1 phage-based vectors can greatly impact on the user-friendliness of *C. glutamicum* BCA as routine heterologous host for biosynthetic pathways, especially for large gene clusters related to specialized metabolites. Furthermore, the lack of endogenous specialized metabolites of *C. glutamicum* BCA is also appealing for the sensitive detection and downstream purification of trace amounts of novel natural products. In conclusion, the development of the strain *C. glutamicum* BCA expands the capacities of the well-established strain ATCC 13032 with novel genetic gateways and turns it into a cutting-edge host for drug discovery.

The standardization of tools and techniques in the synthetic biology field intends transversal and predictable outcomes from genetic circuits from one organism to another. In this work, we aimed to extend the range of applications of tools already developed for other actinobacteria into *C. glutamicum*, specifically the prolific set of tools that has been developed for *Streptomyces* spp. We demonstrated that *C. glutamicum* strains harboring the appropriate attachment sites can integrate ϕ BT1 and ϕ C31-based vectors, originally developed for *Streptomyces* (Gregory et al., 2003; Herrmann et al., 2012), although with different efficiencies. While the ϕ C31-based system integrated at efficiencies one order of magnitude below those of the TP901-1 system (Shen et al., 2017), the ϕ BT1-based system became the most effective system of “non-native” phage-based integration for *C. glutamicum*, surpassing the best Cre-based integrase system (Suzuki et al., 2007) by one order of magnitude. In addition, systems based on Cre and C31-Integrase recombination systems, also originally designed for streptomycetes, have proven to be functional in *C. glutamicum*. The Cre expression

vector pALCRE (Herrmann et al., 2012) was used for the excision of the plasmid backbone after integration, allowing markerless gene expression. On the other hand, the IMES technology (Myronovskyi et al., 2014) was used to generate the markerless strain *C. glutamicum* BCA. Importantly, the excision system can be used iteratively, so cumulative engineering stages can be performed using this method. This strategy can be used to knock-in features or genes into strains or even to deliver promoters, terminators and other short sequences. Overall, a set of molecular tools originally developed for *Streptomyces* were successfully transferred to *C. glutamicum*, bringing closer the prolific sources of novel biosynthetic gene clusters and the debutant heterologous host for specialized metabolites.

Integrative systems are desirable for stable inheritance of the introduced genetic traits. Rare events of instability in the modified strains can be avoided by only expressing the integrase function during introduction of the new traits (Petersen et al., 2013). The chromosomal integration of the ϕ BT1-based vectors (Gregory et al., 2003) caused the disruption of the the promoter region controlling the expression of the vector BT1-Integrase. In accordance, a single rescue pattern was observed for the strains bearing the ϕ BT1-based vectors (**Supp. Fig. 4**). However, in the integration of the ϕ C31-based pTES vector, the promoter region of the C31-Integrase encoding gene is not disrupted after integration. Even though it is described that the C31-Integrase requires an additional phage factor to recombine the *attL_{C31}* and *attR_{C31}* recombination scars (Khaleel et al., 2011), the patterns found by Southern blot and rescue plasmid experiments match exactly an eventual pTES episome excised by *attL_{C31}/attR_{C31}* recombination. However, the frequency of isolation of episomal pTES using a commercial miniprep kit was 0.005% lower than of pBL-1 based plasmid. Furthermore, owing to the *loxP* sites in pTES, the

integrase function can be readily excised using Cre, rendering the genetic traits stable. In summary, the integrative vectors based on ϕ BT1 and ϕ C31 systems tested in this work can assure stable and inheritable integration of exogenous DNA without lasting selection, which is highly desirable for the development of industrial strains.

Flaviolin production was used as a proof-of-principle for the heterologous expression of specialized metabolites in *C. glutamicum* BCA host, namely polyketides, using the ϕ C31-based integration system. The production of flaviolin in *C. glutamicum* had been previously achieved (Yang et al., 2018), albeit only in replicative vectors. We succeeded on the production of flaviolin in *C. glutamicum* BCA host using the ϕ C31-based pTES-rppA platform carrying a codon-optimized version of the heterologous *rppA* gene of *S. erythraea*. The expression of *rppA* using the pTES-rppA vector sustained a flaviolin production of 40 mg L⁻¹, at 72 h of culture, significantly improving the published titer and making room for a more sensitive malonyl-CoA sensor (Yang et al., 2018). The expression of flaviolin using the pTES-rppA also served as a test for the Cre/*loxP* system, originally developed for *Streptomyces* (Herrmann et al., 2012) for the development of markerless heterologous expression platforms. Unexpectedly, upon excision of the backbone of pTES-rppA vector, the titer of flaviolin dropped considerably relative to the previous situation. Interestingly, the titer of flaviolin obtained by the ϕ BT1-based integration of pRT802-rppA, carrying the same promoter and *rppA* variant, was similar to that after the excision of pTES-rppA backbone. From these results, we reasoned that the observed effect was likely caused by overexpression of *rppA* in the strain BCA *attB*_{C31}::pTES-rppA, due to the readthrough of the promoter of C31-Integrase. In spite of the presence of a *fd* transcriptional terminator between *rppA* and the integrase gene, transcription can only be partially blocked by the intrinsic terminator, up to 85% in this

particular case (Bilyk et al., 2017). To prevent the influence of *C31-Int* promoter in the pTES vector, it is, therefore, advisable to clone the genes of interest in the opposite direction. Of note, the highly efficient Cre/*loxP* system in combination with φ C31-based pTES vector (Herrmann et al., 2012) provide an universal platform for markerless heterologous expression in the *C. glutamicum* BCA host and an alternative to conventional non-replicative vectors, especially for delivery of large gene clusters or genes that decrease cell fitness. In summary, the pTES-rppA platform for production of flaviolin clearly demonstrated the effectiveness of the φ C31 phage-based system for heterologous expression in *C. glutamicum* BCA host and showcased the possibility to obtain markerless producers using site-specific recombinases.

Indigoidine production was used as a proof-of-principle for the heterologous expression of specialized metabolites in *C. glutamicum* BCA host, namely non-ribosomal peptides, using the φ BT1-based integration system. Indigoidine occurs naturally in many organisms, some of which phytopathogens (Reverchon et al., 2002), and several attempts to produce it in heterologous host have been reported. In *Streptomyces coelicolor* CH999, an indigoidine titer of 0.5935 g L⁻¹ was reported (Yu et al., 2013). After several rounds of optimization, the production of indigoidine in *E. coli* strains reached 8.8 g L⁻¹ (Xu et al., 2015; Yu et al., 2013). The fungi that served as hosts for indigoidine were *Saccharomyces cerevisiae* and *Rhodospiridium toruloides*, and titers in these hosts ranged from 0.210 g L⁻¹ up to 86 g L⁻¹ in glucose-based fed-batch cultivations (Wehrs et al., 2019a; Wehrs et al., 2018; Wehrs et al., 2019b). Taking advantage of well-established capacity of *C. glutamicum* to produce compounds based on amino acids, we sought to redirect L-glutamine to the production of the blue dye indigoidine. We succeeded on the production of indigoidine in *C. glutamicum* BCA host using the developed φ BT1-based

pFM69SD-*bpsA* platform carrying the a slightly modified version of the heterologous *bpsA* gene of *Streptomyces albidoflavus*. Notably, the titer of indigoidine produced by *C. glutamicum* BCA strain harboring a single copy *bpsA* gene in standard cultivation conditions is comparable to that of the above described heterologous hosts. Importantly, the production of indigoidine in *C. glutamicum* confirmed that native PPTases are able to activate non-ribosomal peptide synthases *in vivo* (Kallscheuer et al., 2019).

The phage-based integrative vectors and the proof-of-principle biosynthetic enzymes employed in this work relied heavily on GC-rich heterologous genes, which imposed significant challenges to the translation machinery of *C. glutamicum*. Our attempt to express the *rppA* and *bpsA* genes with native codon usage and 5' UTRs resulted in no production of flaviolin nor indigoidine, respectively, although transcription was detected in both cases (data not shown). Even though *rppA* gene of *S. erythraea* presented a global CAI of 0.8, it contained fifteen rare codons, and started with the GTG codon, a known factor for diminished translation in *C. glutamicum* (Becker et al., 2010). Thus, we developed *rppA*-based reporters for usage in *C. glutamicum* by employing variants of the *rppA* gene from *S. erythraea* optimized according to the coding preferences of *C. glutamicum* and/or *Streptomyces*. The integration of the codon-optimized *rppA*_{CglStrept} in *C. glutamicum* BCA led to the production of flaviolin at significantly better yields than those previously reported for the expression of *rppA* from *S. griseus* modified with an N-terminal hexa-histidine tag in *C. glutamicum* ATCC 13032 (Yang et al., 2018), even though the copy number was about 30-fold higher in the latter. Our results using two differently codon-optimized *rppA* genes (*rppA*_{Cgl} and *rppA*_{CglStrept}) also showed that a codon usage biased to *Streptomyces* and higher GC-content reduce

translation in *C. glutamicum*. However, the major barrier to heterologous expression seems to reside in improper 5' UTRs and unfit starting codons. For instance, a simple upgrade of SD and of the first ten codons (old CAI: 0.76; new CAI: 0.96) was enough to convert the inert *bpsA* gene (overall CAI: 0.76) into a gene able to sustain high titer of indigoidine production in *C. glutamicum*. Likewise, the lower frequency of integration of the ϕ C31 phage-based vectors when comparing to the ϕ BT1 phage-based vectors could also be explained by an impairment on the beginning of translation of the respective integrases. In fact, despite the similar overall CAI of both genes (0.7), the adaptation index of the first ten codon of the *C31-int* (0.7) is higher than *BT1-Int* (0.5), and the former includes two rare codons. These examples showcase the challenges that can arise when expressing codon-biased, GC-rich genes from *Streptomyces* in the *C. glutamicum* host and pinpoint the 5'-UTR and the initial codons as interesting targets for metabolic engineering.

Overall, we developed a comprehensive platform for efficient genome editing of *C. glutamicum* based on a cosmid library . Using this platform, we generated the *C. glutamicum* BCA host, featuring the attachment sites for ϕ C31 and ϕ BT1 phage-based vectors. We confirmed the efficacy of the iterative marker excision system, the ϕ C31 and ϕ BT1 phage-based vectors and other tools originally developed for other actinobacteria. We improved the output of a described malonyl-CoA sensor derived from the polyketide synthase RppA and first demonstrated that the phosphopantetheinylation-dependent biosynthesis of non-ribosomal peptides is feasible in *C. glutamicum*. The microbial chassis and molecular toolbox characterized in this work are prepared to boost drug discovery efforts with forefront capabilities for heterologous expression and effective platforms for further metabolic engineering.

DECLARATIONS OF INTEREST

None

ACKNOWLEDGEMENTS

The authors are grateful to Nikolas Eckert and Liliya Horbal for the help on producing the cosmid library, and to Anja Paluszczak for the help on HPLC-MS analysis. The authors would like to acknowledge Jörn Kalinowski and Christian Rückert for providing *C. glutamicum* strain CR099attB_{C31}, Giacomo Giacomelli for providing the pK19mobsacB and the pEKEx2 vectors, Maria Lopatniuk for providing cos15A-gus vector, and Birgit Rosenkränzer and Maksym Myronovskyi for providing pTES, pALCRE, pSMART-3B20 and the IMES plasmids.

FUNDING

This work was supported by National Funds through FCT – Fundação para a Ciência e a Tecnologia, I.P., under the project ERA-IB-2/0001/2015; fellowships SFRH/BD/131939/2016 (FM) and SFRH/BPD/95683/2013 (MVM) and the FCT contract DL57/2016/CP1355/CT0023. The work was also supported by FEDER - Fundo Europeu de Desenvolvimento Regional through the COMPETE 2020 - Operacional Programme for Competitiveness and Internationalisation (POCI), Portugal 2020, and by Portuguese funds through FCT - Fundação para a Ciência e a Tecnologia/Ministério da Ciência, Tecnologia e Ensino Superior, and "Institute for Research and Innovation in Health Sciences" (POCI-01-0145-FEDER-007274), by the project NORTE-01-0145-FEDER-000012, Structured Programme on Bioengineering Therapies for Infectious Diseases and Tissue Regeneration, supported by Norte Portugal Regional Operational Programme

912 (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the
913 European Regional Development Fund (ERDF). This work was also supported by the DFG
914 grant LU1524/4-1. The funders of the study had no role in study design, data collection,
915 data analysis, data interpretation, writing of the report or decision to submit the article
916 for publication.

917

918 REFERENCES

- 919 Amador, E., Martin, J. F., Castro, J. M., 2000. A *Brevibacterium lactofermentum* 16S rRNA gene
920 used as target site for homologous recombination. *FEMS Microbiol Lett.* 185, 199-204.
- 921 Bathe, B., Kalinowski, J., Puhler, A., 1996. A physical and genetic map of the *Corynebacterium*
922 *glutamicum* ATCC 13032 chromosome. *Mol Gen Genet.* 252, 255-65.
- 923 Baumgart, M., Unthan, S., Kloss, R., Radek, A., Polen, T., Tenhaef, N., Muller, M. F., Kuberl, A.,
924 Siebert, D., Bruhl, N., Marin, K., Hans, S., Kramer, R., Bott, M., Kalinowski, J., Wiechert,
925 W., Seibold, G., Frunzke, J., Ruckert, C., Wendisch, V. F., Noack, S., 2018.
926 *Corynebacterium glutamicum* Chassis C1*: Building and Testing a Novel Platform Host
927 for Synthetic Biology and Industrial Biotechnology. *ACS Synth Biol.* 7, 132-144.
- 928 Baumgart, M., Unthan, S., Ruckert, C., Sivalingam, J., Grunberger, A., Kalinowski, J., Bott, M.,
929 Noack, S., Frunzke, J., 2013. Construction of a prophage-free variant of *Corynebacterium*
930 *glutamicum* ATCC 13032 for use as a platform strain for basic research and industrial
931 biotechnology. *Appl Environ Microbiol.* 79, 6006-15.
- 932 Becker, J., Buschke, N., Bucker, R., Wittmann, C., 2010. Systems level engineering of
933 *Corynebacterium glutamicum* - Reprogramming translational efficiency for superior
934 production. *Engineering in Life Sciences.* 10, 430-438.
- 935 Becker, J., Rohles, C. M., Wittmann, C., 2018a. Metabolically engineered *Corynebacterium*
936 *glutamicum* for bio-based production of chemicals, fuels, materials, and healthcare
937 products. *Metab. Eng.* 50, 122-141.
- 938 Becker, J., Rohles, C. M., Wittmann, C., 2018b. Metabolically engineered *Corynebacterium*
939 *glutamicum* for bio-based production of chemicals, fuels, materials, and healthcare
940 products. *Metab Eng.* 50, 122-141.
- 941 Becker, J., Zelder, O., Hafner, S., Schroder, H., Wittmann, C., 2011. From zero to hero--design-
942 based systems metabolic engineering of *Corynebacterium glutamicum* for L-lysine
943 production. *Metab Eng.* 13, 159-68.
- 944 Beites, T., Mendes, M. V., 2015. Chassis optimization as a cornerstone for the application of
945 synthetic biology based strategies in microbial secondary metabolism. *Front Microbiol.*
946 6, 906.
- 947 Bilyk, B., Horbal, L., Luzhetskyy, A., 2017. Chromosomal position effect influences the
948 heterologous expression of genes and biosynthetic gene clusters in *Streptomyces albus*
949 J1074. *Microb. Cell. Fact.* 16, 5.
- 950 Cameron Coates, R., Blaskowski, S., Szyjka, S., van Rossum, H. M., Vallandingham, J., Patel, K.,
951 Serber, Z., Dean, J., 2019. Systematic investigation of CRISPR-Cas9 configurations for
952 flexible and efficient genome editing in *Corynebacterium glutamicum* NRRL-B11474. *J*
953 *Ind Microbiol Biotechnol.* 46, 187-201.
- 954 Chatterjee, A. K., Brown, M. A., 1981. Chromosomal location of a gene (idg) that specifies
955 production of the blue pigment indigoidine in *Erwinia chrysanthemi*. *Current*
956 *Microbiology.* 6, 269-273.
- 957 Cho, J. S., Choi, K. R., Prabowo, C. P. S., Shin, J. H., Yang, D., Jang, J., Lee, S. Y., 2017. CRISPR/Cas9-
958 coupled recombineering for metabolic engineering of *Corynebacterium glutamicum*.
959 *Metab Eng.* 42, 157-167.
- 960 Choi, J. W., Yim, S. S., Kim, M. J., Jeong, K. J., 2015. Enhanced production of recombinant proteins
961 with *Corynebacterium glutamicum* by deletion of insertion sequences (IS elements).
962 *Microb Cell Fact.* 14, 207.
- 963 Correia, A., Martin, J. F., Castro, J. M., 1996. Targeted integration of foreign genes into repetitive
964 sequences of the *Brevibacterium lactofermentum* chromosome. *FEMS Microbiol. Lett.*
965 142, 259-264.

- Cortes, J., Velasco, J., Foster, G., Blackaby, A. P., Rudd, B. A., Wilkinson, B., 2002. Identification and cloning of a type III polyketide synthase required for diffusible pigment biosynthesis in *Saccharopolyspora erythraea*. *Mol Microbiol.* 44, 1213-24.
- Datsenko, K. A., Wanner, B. L., 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A.* 97, 6640-5.
- Eggeling, L., Bott, M., 2005. *Handbook of corynebacterium glutamicum*. Taylor & Francis, Boca Raton.
- Eikmanns, B. J., Kleinertz, E., Liebl, W., Sahm, H., 1991. A family of *Corynebacterium glutamicum*/*Escherichia coli* shuttle vectors for cloning, controlled gene expression, and promoter probing. *Gene.* 102, 93-98.
- Fu, J., Teucher, M., Anastassiadis, K., Skarnes, W., Stewart, A. F., 2010. A Recombineering Pipeline to Make Conditional Targeting Constructs. *Methods in Enzymology, Vol 477: Guide to Techniques in Mouse Development, Part B: Mouse Molecular Genetics, Second Edition.* 477, 125-144.
- Gregory, M. A., Till, R., Smith, M. C., 2003. Integration site for *Streptomyces* phage ϕ BT1 and development of site-specific integrating vectors. *J Bacteriol.* 185, 5320-3.
- Hanahan, D., 1983. Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol.* 166, 557-80.
- Herrmann, S., Siegl, T., Luzhetskaya, M., Petzke, L., Jilg, C., Welle, E., Erb, A., Leadlay, P. F., Bechthold, A., Luzhetskyy, A., 2012. Site-specific recombination strategies for engineering actinomycete genomes. *Appl Environ Microbiol.* 78, 1804-12.
- Hoffmann, S. L., Jungmann, L., Schiefelbein, S., Peyriga, L., Cahoreau, E., Portais, J.-C., Becker, J., Wittmann, C., 2018. Lysine production from the sugar alcohol mannitol: Design of the cell factory *Corynebacterium glutamicum* SEA-3 through integrated analysis and engineering of metabolic pathway fluxes. *Metab. Eng.* 47, 475-487.
- Horbal, L., Siegl, T., Luzhetskyy, A., 2018. A set of synthetic versatile genetic control elements for the efficient expression of genes in Actinobacteria. *Sci Rep.* 8, 491.
- Ikeda, M., Nakagawa, S., 2003. The *Corynebacterium glutamicum* genome: features and impacts on biotechnological processes. *Appl Microbiol Biotechnol.* 62, 99-109.
- Jiang, Y., Qian, F., Yang, J., Liu, Y., Dong, F., Xu, C., Sun, B., Chen, B., Xu, X., Li, Y., Wang, R., Yang, S., 2017. CRISPR-Cpf1 assisted genome editing of *Corynebacterium glutamicum*. *Nat Commun.* 8, 15179.
- Kalinowski, J., Bathe, B., Bartels, D., Bischoff, N., Bott, M., Burkovski, A., Dusch, N., Eggeling, L., Eikmanns, B. J., Gaigalat, L., Goesmann, A., Hartmann, M., Huthmacher, K., Kramer, R., Linke, B., McHardy, A. C., Meyer, F., Mockel, B., Pfefferle, W., Puhler, A., Rey, D. A., Ruckert, C., Rupp, O., Sahm, H., Wendisch, V. F., Wiegrabe, I., Tauch, A., 2003. The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. *J Biotechnol.* 104, 5-25.
- Kallscheuer, N., Kage, H., Milke, L., Nett, M., Marienhagen, J., 2019. Microbial synthesis of the type I polyketide 6-methylsalicylate with *Corynebacterium glutamicum*. *Appl Microbiol Biotechnol.* 103, 9619-9631.
- Keilhauer, C., Eggeling, L., Sahm, H., 1993. Isoleucine synthesis in *Corynebacterium glutamicum*: molecular analysis of the *ilvB-ilvN-ilvC* operon. *J Bacteriol.* 175, 5595-603.
- Khaleel, T., Younger, E., McEwan, A. R., Varghese, A. S., Smith, M. C., 2011. A phage protein that binds ϕ C31 integrase to switch its directionality. *Mol Microbiol.* 80, 1450-63.
- Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F., Hopwood, D. A., John Innes, F., 2000. *Practical Streptomyces genetics*. John Innes Foundation, Norwich.
- Kim, D., Luk, K., Wolfe, S. A., Kim, J. S., 2019. Evaluating and Enhancing Target Specificity of Gene-Editing Nucleases and Deaminases. *Annu Rev Biochem.* 88, 191-220.

1016 Kim, I. K., Jeong, W. K., Lim, S. H., Hwang, I. K., Kim, Y. H., 2011. The small ribosomal protein S12P
1017 gene rpsL as an efficient positive selection marker in allelic exchange mutation systems
1018 for *Corynebacterium glutamicum*. *J Microbiol Methods*. 84, 128-30.

1019 Kimelman, A., Levy, A., Sberro, H., Kidron, S., Leavitt, A., Amitai, G., Yoder-Himes, D. R., Wurtzel,
1020 O., Zhu, Y., Rubin, E. M., Sorek, R., 2012. A vast collection of microbial genes that are
1021 toxic to bacteria. *Genome Res*. 22, 802-9.

1022 Kinoshita, S., Udaka, S., SHIMONO, M., 1957. Studies on the amino acid fermentation. *The*
1023 *Journal of general and applied microbiology*. 3, 193-205.

1024 Kjeldsen, K. R., Nielsen, J., 2009. In silico genome-scale reconstruction and validation of the
1025 *Corynebacterium glutamicum* metabolic network. *Biotechnol Bioeng*. 102, 583-97.

1026 Kogure, T., Inui, M., 2018. Recent advances in metabolic engineering of *Corynebacterium*
1027 *glutamicum* for bioproduction of value-added aromatic chemicals and natural products.
1028 *Appl Microbiol Biotechnol*. 102, 8685-8705.

1029 Krauser, S., Kiefer, P., Heinzle, E., 2012. Multienzyme Whole-Cell In Situ Biocatalysis for the
1030 Production of Flaviolin in Permeabilized Cells of *Escherichia coli*. *ChemCatChem*. 4, 786-
1031 788.

1032 Kuhn, R., Starr, M. P., Kuhn, D. A., Bauer, H., Knackmuss, H. J., 1965. Indigoidine and Other
1033 Bacterial Pigments Related to 3,3'-Bipyridyl. *Arch Mikrobiol*. 51, 71-84.

1034 Lennox, E. S., 1955. Transduction of linked genetic characters of the host by bacteriophage P1.
1035 *Virology*. 1, 190-206.

1036 Liu, J., Wang, Y., Lu, Y., Zheng, P., Sun, J., Ma, Y., 2017. Development of a CRISPR/Cas9 genome
1037 editing toolbox for *Corynebacterium glutamicum*. *Microb Cell Fact*. 16, 205.

1038 Lopatniuk, M., Myronovskiy, M., Nottebrock, A., Busche, T., Kalinowski, J., Ostash, B., Fedorenko,
1039 V., Luzhetskyy, A., 2019. Effect of "ribosome engineering" on the transcription level and
1040 production of *S. albus* indigenous secondary metabolites. *Appl. Microbiol. Biotechnol*.
1041 103, 7097-7110.

1042 Ma, W., Wang, X., Mao, Y., Wang, Z., Chen, T., Zhao, X., 2015. Development of a markerless gene
1043 replacement system in *Corynebacterium glutamicum* using upp as a counter-selection
1044 marker. *Biotechnol Lett*. 37, 609-17.

1045 Marrec, C. L., Michotey, V., Blanco, C., Trautwetter, A., 1994. aU2, a temperate bacteriophage
1046 specific for '*Arthrobacter aureus*', whose integrative functions work in other
1047 corynebacteria. *Microbiology*. 140, 3071-3077.

1048 Marx, A., de Graaf, A. A., Wiechert, W., Eggeling, L., Sahm, H., 1996. Determination of the fluxes
1049 in the central metabolism of *Corynebacterium glutamicum* by nuclear magnetic
1050 resonance spectroscopy combined with metabolite balancing. *Biotechnol. Bioeng*. 49,
1051 111-129.

1052 Moreau, S., Blanco, C., Trautwetter, A., 1999a. Site-specific integration of corynephage phi16:
1053 construction of an integration vector. *Microbiology*. 145 (Pt 3), 539-548.

1054 Moreau, S., Le Marrec, C., Blanco, C., Trautwetter, A., 1999b. Analysis of the integration
1055 functions of phi304L: an integrase module among corynephages. *Virology*. 255, 150-9.

1056 Myronovskiy, M., Rosenkranzer, B., Luzhetskyy, A., 2014. Iterative marker excision system. *Appl*
1057 *Microbiol Biotechnol*. 98, 4557-70.

1058 Nakamura, Y., Gojobori, T., Ikemura, T., 2000. Codon usage tabulated from international DNA
1059 sequence databases: status for the year 2000. *Nucleic Acids Res*. 28, 292.

1060 Newsome, A. G., Culver, C. A., van Breemen, R. B., 2014. Nature's palette: the search for natural
1061 blue colorants. *Journal of agricultural and food chemistry*. 62, 6498-511.

1062 Olano, C., Garcia, I., Gonzalez, A., Rodriguez, M., Rozas, D., Rubio, J., Sanchez-Hidalgo, M., Brana,
1063 A. F., Mendez, C., Salas, J. A., 2014. Activation and identification of five clusters for
1064 secondary metabolites in *Streptomyces albus* J1074. *Microbial biotechnology*. 7, 242-
1065 56.

- Oram, M., Woolston, J. E., Jacobson, A. D., Holmes, R. K., Oram, D. M., 2007. Bacteriophage-based vectors for site-specific insertion of DNA in the chromosome of *Corynebacteria*. *Gene*. 391, 53-62.
- Peng, F., Liu, X., Wang, X., Chen, J., Liu, M., Yang, Y., Bai, Z., 2019. Triple deletion of *clpC*, *porB*, and *mepA* enhances production of small ubiquitin-like modifier-N-terminal pro-brain natriuretic peptide in *Corynebacterium glutamicum*. *J Ind Microbiol Biotechnol*. 46, 67-79.
- Peng, F., Wang, X., Sun, Y., Dong, G., Yang, Y., Liu, X., Bai, Z., 2017. Efficient gene editing in *Corynebacterium glutamicum* using the CRISPR/Cas9 system. *Microb Cell Fact*. 16, 201.
- Petersen, K. V., Martinussen, J., Jensen, P. R., Solem, C., 2013. Repetitive, marker-free, site-specific integration as a novel tool for multiple chromosomal integration of DNA. *Appl Environ Microbiol*. 79, 3563-9.
- Pfeifer-Sancar, K., Mentz, A., Rückert, C., Kalinowski, J., 2013. Comprehensive analysis of the *Corynebacterium glutamicum* transcriptome using an improved RNAseq technique. *BMC Genomics*. 14, 888.
- Rebets, Y., Tsohis, K. C., Guethmundsdottir, E. E., Koepff, J., Wawiernia, B., Busche, T., Bleidt, A., Horbal, L., Myronovskyi, M., Ahmed, Y., Wiechert, W., Rückert, C., Hamed, M. B., Bilyk, B., Anne, J., Friethjonsson, O., Kalinowski, J., Oldiges, M., Economou, A., Luzhetskyy, A., 2018. Characterization of Sigma Factor Genes in *Streptomyces lividans* TK24 Using a Genomic Library-Based Approach for Multiple Gene Deletions. *Front Microbiol*. 9, 3033.
- Reverchon, S., Rouanet, C., Expert, D., Nasser, W., 2002. Characterization of indigoidine biosynthetic genes in *Erwinia chrysanthemi* and role of this blue pigment in pathogenicity. *J Bacteriol*. 184, 654-65.
- Sambrook, J., Russell, D. W., 2001. *Molecular cloning : a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Schafer, A., Tauch, A., Jager, W., Kalinowski, J., Thierbach, G., Puhler, A., 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene*. 145, 69-73.
- Sharp, P. M., Li, W.-H., 1987. The codon adaptation index-a measure of directional synonymous codon usage bias, and its potential applications. *Nucleic Acids Research*. 15, 1281-1295.
- Shen, J., Chen, J., Jensen, P. R., Solem, C., 2017. A novel genetic tool for metabolic optimization of *Corynebacterium glutamicum*: efficient and repetitive chromosomal integration of synthetic promoter-driven expression libraries. *Appl Microbiol Biotechnol*. 101, 4737-4746.
- Shinfuku, Y., Sorpitiporn, N., Sono, M., Furusawa, C., Hirasawa, T., Shimizu, H., 2009. Development and experimental verification of a genome-scale metabolic model for *Corynebacterium glutamicum*. *Microb Cell Fact*. 8, 43.
- Siegl, T., Tokovenko, B., Myronovskyi, M., Luzhetskyy, A., 2013. Design, construction and characterisation of a synthetic promoter library for fine-tuned gene expression in actinomycetes. *Metab Eng*. 19, 98-106.
- Sun, H., Zhao, D., Xiong, B., Zhang, C., Bi, C., 2016. Engineering *Corynebacterium glutamicum* for violacein hyper production. *Microb Cell Fact*. 15, 148.
- Suzuki, N., Inui, M., Yukawa, H., 2007. Site-directed integration system using a combination of mutant *lox* sites for *Corynebacterium glutamicum*. *Appl Microbiol Biotechnol*. 77, 871-8.
- Tauch, A., Homann, I., Mormann, S., Rüberg, S., Billault, A., Bathe, B., Brand, S., Brockmann-Gretza, O., Rückert, C., Schischka, N., Wrenger, C., Hoheisel, J., Möckel, B., Huthmacher, K., Pfefferle, W., Pühler, A., Kalinowski, J., 2002. Strategy to sequence the genome of *Corynebacterium glutamicum* ATCC 13032: use of a cosmid and a bacterial artificial chromosome library. *Journal of Biotechnology*. 95, 25-38.

1117 Unthan, S., Baumgart, M., Radek, A., Herbst, M., Siebert, D., Bruhl, N., Bartsch, A., Bott, M.,
 1118 Wiechert, W., Marin, K., Hans, S., Kramer, R., Seibold, G., Frunzke, J., Kalinowski, J.,
 1119 Ruckert, C., Wendisch, V. F., Noack, S., 2015. Chassis organism from *Corynebacterium*
 1120 *glutamicum*--a top-down approach to identify and delete irrelevant gene clusters.
 1121 *Biotechnol J.* 10, 290-301.
 1122 Vasicová, P., Abbrámová, Z., Nesvera, J., Pátek, M., Sahm, H., Eikmanns, B., 1998. Integrative and
 1123 autonomously replicating vectors for analysis of promoters in *Corynebacterium*
 1124 *glutamicum*. *Biotechnology Techniques.* 12, 743-746.
 1125 Vertes, A. A., Inui, M., Yukawa, H., 2013. The biotechnological potential of *Corynebacterium*
 1126 *glutamicum*, from Umami to Chemurgy. *Corynebacterium glutamicum*. Springer, pp. 1-
 1127 49.
 1128 Vickers, C. E., Blank, L. M., Krömer, J. O., 2010. Grand challenge commentary: Chassis cells for
 1129 industrial biochemical production. *Nat Chem Biol.* 6, 875-7.
 1130 Wang, B., Hu, Q., Zhang, Y., Shi, R., Chai, X., Liu, Z., Shang, X., Zhang, Y., Wen, T., 2018a. A RecET-
 1131 assisted CRISPR-Cas9 genome editing in *Corynebacterium glutamicum*. *Microb Cell Fact.*
 1132 17, 63.
 1133 Wang, Y., Liu, Y., Li, J., Yang, Y., Ni, X., Cheng, H., Huang, T., Guo, Y., Ma, H., Zheng, P., Wang, M.,
 1134 Sun, J., Ma, Y., 2019. Expanding targeting scope, editing window, and base transition
 1135 capability of base editing in *Corynebacterium glutamicum*. *Biotechnol Bioeng.* 116,
 1136 3016-3029.
 1137 Wang, Y., Liu, Y., Liu, J., Guo, Y., Fan, L., Ni, X., Zheng, X., Wang, M., Zheng, P., Sun, J., Ma, Y.,
 1138 2018b. MACBETH: Multiplex automated *Corynebacterium glutamicum* base editing
 1139 method. *Metab Eng.* 47, 200-210.
 1140 Wehrs, M., Gladden, J. M., Liu, Y., Platz, L., Prah, J.-P., Moon, J., Papa, G., Sundstrom, E.,
 1141 Geiselman, G. M., Tanjore, D., Keasling, J. D., Pray, T. R., Simmons, B. A., Mukhopadhyay,
 1142 A., 2019a. Sustainable bioproduction of the blue pigment indigoidine: Expanding the
 1143 range of heterologous products in *R. toruloides* to include non-ribosomal peptides.
 1144 *Green Chemistry.* 21, 3394-3406.
 1145 Wehrs, M., Prah, J. P., Moon, J., Li, Y., Tanjore, D., Keasling, J. D., Pray, T., Mukhopadhyay, A.,
 1146 2018. Production efficiency of the bacterial non-ribosomal peptide indigoidine relies on
 1147 the respiratory metabolic state in *S. cerevisiae*. *Microb Cell Fact.* 17, 193.
 1148 Wehrs, M., Prah, J. P., Moon, J., Li, Y., Tanjore, D., Keasling, J. D., Pray, T., Mukhopadhyay, A.,
 1149 2019b. Correction to: Production efficiency of the bacterial non-ribosomal peptide
 1150 indigoidine relies on the respiratory metabolic state in *S. cerevisiae*. *Microb Cell Fact.*
 1151 18, 218.
 1152 Wittmann, C., De Graaf, A., 2005. 12 Metabolic Flux Analysis in *Corynebacterium glutamicum*.
 1153 *Handbook of Corynebacterium glutamicum.* 277.
 1154 Xu, F., Gage, D., Zhan, J., 2015. Efficient production of indigoidine in *Escherichia coli*. *J Ind*
 1155 *Microbiol Biotechnol.* 42, 1149-55.
 1156 Yang, D., Kim, W. J., Yoo, S. M., Choi, J. H., Ha, S. H., Lee, M. H., Lee, S. Y., 2018. Repurposing type
 1157 III polyketide synthase as a malonyl-CoA biosensor for metabolic engineering in bacteria.
 1158 *Proc Natl Acad Sci U S A.* 115, 9835-9844.
 1159 Yanisch-Perron, C., Vieira, J., Messing, J., 1985. Improved M13 phage cloning vectors and host
 1160 strains: nucleotide sequences of the M13mpl8 and pUC19 vectors. *Gene.* 33, 103-119.
 1161 Yu, D., Xu, F., Valiente, J., Wang, S., Zhan, J., 2013. An indigoidine biosynthetic gene cluster from
 1162 *Streptomyces chromofuscus* ATCC 49982 contains an unusual IndB homologue. *J Ind*
 1163 *Microbiol Biotechnol.* 40, 159-68.
 1164 Yumusak, C., Prochazkova, A. J., Apaydin, D. H., Seelajaroen, H., Sariciftci, N. S., Weiter, M.,
 1165 Krajcovic, J., Qin, Y., Zhang, W., Zhan, J., Kovalenko, A., 2019. Indigoidine –
 1166 Biosynthesized organic semiconductor. *Dyes and Pigments.* 171, 107768.
 1167 Zhang, J., Yang, F., Yang, Y., Jiang, Y., Huo, Y. X., 2019. Optimizing a CRISPR-Cpf1-based genome
 1168 engineering system for *Corynebacterium glutamicum*. *Microb Cell Fact.* 18, 60.

1169 Zhang, S., Liu, D., Mao, Z., Mao, Y., Ma, H., Chen, T., Zhao, X., Wang, Z., 2018. Model-based
 1170 reconstruction of synthetic promoter library in *Corynebacterium glutamicum*.
 1171 *Biotechnol Lett.* 40, 819-827.

1172 Zhang, Y., Cai, J., Shang, X., Wang, B., Liu, S., Chai, X., Tan, T., Zhang, Y., Wen, T., 2017. A new
 1173 genome-scale metabolic model of *Corynebacterium glutamicum* and its application.
 1174 *Biotechnology for biofuels.* 10, 169.

1175