Article

Improved Plasmid-Based Inducible and Constitutive Gene Expression in Corynebacterium glutamicum

Nadja A. Henke, Irene Krahn and Volker F. Wendisch *

Abstract: Corynebacterium glutamicum has been safely used in white biotechnology for the last 60 years and the portfolio of new pathways and products is increasing rapidly. Hence, expression vectors play a central role in discovering endogenous gene functions and in establishing heterologous gene expression. In this work, new expression vectors were designed based on two strategies: (i) a library screening of constitutive native and synthetic promoters and (ii) an increase of the plasmid copy number. Both strategies were combined and resulted in a very strong expression and overproduction of the fluorescence protein GfpUV. As a second test case, the improved vector for constitutive expression was used to overexpress the endogenous xylulokinase gene xylB in a synthetic operon with xylose isomerase gene xylA from Xanthomonas campestris. The xylose isomerase activity in crude extracts was increased by about three-fold as compared to that of the parental vector. In terms of application, the improved vector for constitutive xylA and xylB expression was used for production of the N-methylated amino acid sarcosine from monomethylamine, acetate, and xylose. As a consequence, the volumetric productivity of sarcosine production was 50% higher as compared to that of the strain carrying the parental vector.

Keywords: expression vector; Corynebacterium glutamicum; overexpression; promoter; origin of replication

1. Introduction

C. glutamicum was discovered in the 1960s as a natural L-glutamate producer [1]. Since then, both its genetic toolbox [2] and its number of heterologous pathways [3,4] have been extended. On the one side, production of value-added compounds such as amino acids [5,6], organic acids [7,8], and terpenoids [9,10] has been established. Recently, the production of sarcosine (N-methylglycine) was enabled by overexpression of the imine reductase DpkA from Pseudomonas putida in a glyoxylate-overproducing C. glutamicum strain by providing monomethylamine as the methyl-donor [11]. On the other side, several approaches were followed in order to establish a flexible feedstock concept that allows C. glutamicum production strains to grow/produce on the basis of a variety of non-food competitive substrates such as industrial or agricultural/aquatic side streams [12]. The access to glycerol, the stoichiometric byproduct of biodiesel production, was enabled and applied to various production strains [13,14]. Moreover, recent attempts have aimed to establish the methylophyrophy in C. glutamicum for methanol utilization [15]. Besides the sugar polymers starch [16] and cellulose [17], the pentose sugars xylose and arabinose [18,19] that derive from hemicellulose can be used as alternative substrates for a variety of high-value products including the fragrance compound patchoulol [20] and the potential antipsychotic compound sarcosine [11].

Many of these production strain-engineering efforts rely on gene expression vectors, which represent a powerful tool not only for metabolic engineering, but also for in depth
analysis of basic metabolic principles in *C. glutamicum* that facilitate the development of new metabolic engineering strategies. For *C. glutamicum*, a wide range of different expression vectors have been used in research: inducible expression vectors, constitutive expression vectors [21], dual-expression vectors [22,23], promoter-probe vectors [24], and suicide-vectors for homologous recombination [25]. Most of the expression vectors used today for *C. glutamicum* are based on the origins of replication pBL1, pGA1, pCG1, and pHM1519 that derive from natural plasmids of corynebacteria [26].

Among many others, pVWEx1 and pECXT99A represent well-established expression vectors for *C. glutamicum* that use the Ptac/Ptrc promoter and carry a lac gene allowing for IPTG-inducible gene expression [27,28]. These IPTG-inducible expression systems make use of the Lac repressor from *E. coli* since *C. glutamicum* lacks a homolog and they provide lac operator(s) [29]. Replication of pVWEx1 in *C. glutamicum* relies on the pHM1519 replicon. Recently, it was shown that a mutation in the initiator protein RepA improved the plasmid copy number of pHM1519 origin to around 800 copies per cell [30].

The promoter structure and consensus sequence have been intensively examined [31]. In addition, the assignment of sigma factors of the RNA polymerase to the respective promoters [32] paved the way to the identification of their regulatory networks [33–35]. The promoters that are depending on the house-keeping sigma factor SigA or the alternative sigma factor SigB are suitable for application in metabolic engineering [35,36]. Therefore, the choice of interesting promoters as genetic elements in vector design also relies on the respective sigma factor that facilitates promoter recognition. Libraries of natural and synthetic promoters have been screened and identified, e.g., the synthetic promoter H36 was described to be 16-fold stronger than the Ptrc promoter [37] and a synthetic promoter comprising the −10 consensus sequence TAnnnT from *C. glutamicum* and the −35 motif TTGACA supported very high transcription [38].

In this work, we assessed the improvement of plasmid copy number and the choice of strong promoters alone or in combination for plasmid-borne target gene expression. In addition to scoring fluorescent reporter gene expression, we applied the gained insight to improve production of the N-methylated amino acid sarcosine from monomethylamine, acetate, and xylose.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

Strains and plasmids used in this study are listed in Table 1. Newly constructed vectors were evaluated to be functional in *C. glutamicum* WT. Chemicals were delivered by Carl Roth (Karlsruhe, Germany) if not stated differently. Precultures of *C. glutamicum* strains were grown in complex medium Luria broth (LB) (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl) or brain heart infusion (BHI) (37 g/L) supplemented with 50 mL 2% glucose overnight in 500 mL baffled flasks. Main cultures were grown in CGXII minimal medium (20 g/L (NH₄)₂SO₄, 5 g/L urea, 1 g/L KH₂PO₄, 1 g/L K₂HPO₄, 42 g/L MOPS, 0.25 g/L MgSO₄ × 7 H₂O, 10 mg/L CaCl₂, 10 mg/L FeSO₄ × 7H₂O, 10 mg/L MnSO₄ × H₂O, 1 mg/L ZnSO₄ × 7H₂O, 0.2 mg/L CuSO₄, 0.02 mg/L NiCl₂ × 6H₂O, 0.2 mg/L biotin (Sigma-Aldrich, Taufkirchen, Germany), and 30 mg/L proteocatechuate (VWR, Darmstadt, Germany)) supplemented with the named carbon source after washing in TN-buffer (10 mM Tric-HCl (pH 6.3), 10 mM NaCl). Sarcosine production was performed with 12 g/L xylose and 20 g/L potassium acetate as carbon sources and a reduced concentration of nitrogen (2 g/L (NH₄)₂SO₄ and 0.5 g/L urea) in the presence of 3.1 g/L monomethylamine (MMA) (TCI, Eschborn, Germany). Main cultures were inoculated to an initial OD₆₀₀ nm of 1 using a Shimadzu UV-1202 spectrophotometer (Duisburg, Germany). To achieve the high aeration required for aerobic cultures of *C. glutamicum*, cultivations were performed in 50 mL of culture medium in 500 mL baffled shake flasks at 120 rpm, or alternatively in 1 mL culture medium in the Biolector® flowerplate system (m2p-labs GmbH, Baesweiler, Germany) at 1100 rpm at 30 °C. *E. coli* DH5α cells were cultivated at 37 °C in LB medium. Tetracycline,
kanamycin, and spectinomycin (VWR, Darmstadt, Germany) were added if appropriate in concentrations of 5, 25, and 100 µg mL⁻¹.

Table 1. Strains and plasmids used in this study.

| Strain | Characteristics of strains and plasmids | Reference |
|--------|----------------------------------------|-----------|
| WT     | Wild type, ATCC 13032                   | [39]      |
| SAR3   | WT ΔaceB iacT (pVWE1-dpkA_RBSopt) (pECXT99A-xylAB) | [11]      |
| SAR3*  | WT ΔaceB iacT (pVWE1-dpkA_RBSopt) (pECXT-Psyn-xylAB) | This work |
| E. coli DH5α | F-thi-1 endA1 hsdR17(r-, m-) sapE44 ΔlacU169 (o080lacZAM15) recA1 gyrA96 | [40]      |
| pEKE3  | Spec<sup>+</sup>, P<sub>int/lac</sub>, pBL1 oriV<sub>CP</sub>, C. glutamicum/E. coli expression shuttle vector | [41]      |
| pEKE3<sup>-gfp</sup>UV | pEKE3 derivative for inducible expression of gfpUV from P<sub>intc</sub> promoter |         |
| pECXT99A<sup>-gfp</sup>UV | Tet<sup>+</sup>, P<sub>int/lac</sub>, pGA1 oriV<sub>CP</sub>, C. glutamicum/E. coli expression shuttle vector | [2]       |
| pECXT99A<sup>-gfp</sup>UV | pECXT99A derivative for inducible expression of gfpUV from P<sub>intc</sub> promoter | This work |
| pECXT<sup>-gfp</sup>xylAB | pECXT99A derivative for inducible expression of xylA from Xanthomonas campestris and xylB from C. glutamicum from P<sub>intc</sub> promoter | [43]      |
| pECXT-Pgk | pECXT99A derivative for constitutive expression from C. glutamicum pgk promoter | This work |
| pECXT-PiicC | pECXT99A derivative for constitutive expression from C. glutamicum ilvC promoter | This work |
| pECXT-PssdA | pECXT99A derivative for constitutive expression from C. glutamicum sodA promoter | This work |
| pECXT-PgapA | pECXT99A derivative for constitutive expression from C. glutamicum gapA promoter | This work |
| pECXT-Ptuf | pECXT99A derivative for constitutive expression from C. glutamicum tuf promoter | This work |
| pECXT-PHI36 | pECXT99A derivative for constitutive expression from synthetic PH36 promoter | This work |
| pECXT-P45 | pECXT99A derivative for constitutive expression from synthetic P45 promoter | This work |
| pECXT-Psyn | pECXT99A derivative for constitutive expression from synthetic Psyn promoter | This work |
| pECXT-P<sup>-gfp</sup>-gfpUV | pECXT99A derivative for constitutive expression of gfpUV from C. glutamicum pgk promoter | This work |
| pECXT-PiicC-<sup>-gfp</sup>UV | pECXT99A derivative for constitutive expression of gfpUV from C. glutamicum ilvC promoter | This work |
| pECXT-PssdA-<sup>-gfp</sup>UV | pECXT99A derivative for constitutive expression of gfpUV from C. glutamicum sodA promoter | This work |
| pECXT-PgapA-<sup>-gfp</sup>UV | pECXT99A derivative for constitutive expression of gfpUV from C. glutamicum gapA promoter | This work |
| pECXT-Ptuf-<sup>-gfp</sup>UV | pECXT99A derivative for constitutive expression of gfpUV from C. glutamicum tuf promoter | This work |
| pECXT-PHI36-<sup>-gfp</sup>UV | pECXT99A derivative for constitutive expression of gfpUV from synthetic PH36 promoter | This work |
| pECXT-P45-<sup>-gfp</sup>UV | pECXT99A derivative for constitutive expression of gfpUV from synthetic P45 promoter | This work |
| pECXT-Psyn-<sup>-gfp</sup>UV | pECXT99A derivative for constitutive expression of gfpUV from synthetic Psyn promoter | This work |
| pECXT-Psyn-xylAB | pECXT99A derivative for constitutive expression of xylA from Xanthomonas campestris and xylB from C. glutamicum from synthetic Psyn promoter | This work |
| pVWE1  | Km<sup>+</sup>, P<sub>int/lac</sub>, pHM1519 oriV<sub>CP</sub>, C. glutamicum/E. coli expression shuttle vector | [28]      |
| pVWE4  | pVWE1 derivative with mutation repA | This work |
| pVWE6  | pVWE4 derivative with Psyn promoter and lac operator for IPTG inducible expression | This work |
| pVWE1-<sup>-gfp</sup>UV | pVWE1 derivative for IPTG-inducible expression of gfpUV from P<sub>intc</sub> promoter | [42]      |
| pVWE1-<sup>-dpkA_RBSopt</sup> | pVWE1 derivative for IPTG-inducible expression of dpkA from P. putida KT2440 and change of its start codon GTG to ATG and an RBS optimised for C. glutamicum | [11]      |
| pVWE4-<sup>-gfp</sup>UV | pVWE4 derivative for IPTG-inducible expression of gfpUV from P<sub>intc</sub> promoter | This work |
| pVWE6-<sup>-gfp</sup>UV | pVWE6 derivative for IPTG-inducible expression of gfpUV from P<sub>intc</sub> promoter | This work |
2.2. Construction of New Expression Vectors

The new expression plasmids were constructed in E. coli DH5α. First, target promoters or genes were amplified by a high-fidelity PCR (All-in HiFi, highQu, Kraichtal, Germany) and cloned into digested or backbone-amplified expression vectors by Gibson-Assembly [44]. The oligonucleotides are listed in Table 2 and were delivered by Metabion (Planegg/Steinkirchen, Germany). The PCR amplificates were purified with a PCR and gel extraction kit (Macherey-Nagel, Düren, Germany). E. coli DH5α-competent cells were prepared with CaCl₂ and transformed via heat shock. Recombinant clones were screened by colony-PCR and plasmids were isolated with a miniprep kit (GeneJET, Thermo Fisher Scientific, Schwerte, Germany). New expression vectors were confirmed by sequencing. C. glutamicum cells were transformed by electroporation as described elsewhere [45].

Table 2. Oligonucleotides used in this study.

| Oligonucleotide | Target | Sequence (5' → 3') |
|-----------------|--------|--------------------|
| HN12            | Ptuf-fw| CTGTGCCTACTTACACCGCATTTCATTCAGGCTATGCTGTCAGTAGGC |
| HN13            | Ptuf-rv| CCGGTACCAGCGGCTTAATCCATGTGACTGAAATCTGAGGGCAACG |
| HN14            | PgapA-fw| CTGTGCCTACTTACACCGCATTTCATTCAGGCTATGCTGTCAGTAGGC |
| HN15            | PgapA-rv| CCGGTACCAGCGGCTTAATCCATGTGACTGAAATCTGAGGGCAACG |
| HN16            | PilvC-fw| CTGTGCCTACTTACACCGCATTTCATTCAGGCTATGCTGTCAGTAGGC |
| HN17            | PilvC-rv| CCGGTACCAGCGGCTTAATCCATGTGACTGAAATCTGAGGGCAACG |
| HN30            | PsodA-fw| CTGTGCCTACTTACACCGCATTTCATTCAGGCTATGCTGTCAGTAGGC |
| HN31            | PsodA-rv| CCGGTACCAGCGGCTTAATCCATGTGACTGAAATCTGAGGGCAACG |
| HN97            | Ppgk-fw| CTGTGCCTACTTACACCGCATTTCATTCAGGCTATGCTGTCAGTAGGC |
| HN98            | Ppgk-rv| CCGGTACCAGCGGCTTAATCCATGTGACTGAAATCTGAGGGCAACG |
| HA02            | P45-fw| CTGTGCCTACTTACACCGCATTTCATTCAGGCTATGCTGTCAGTAGGC |
| HA03            | P45-rv| CCGGTACCAGCGGCTTAATCCATGTGACTGAAATCTGAGGGCAACG |
| HA04            | PH36-fw| CTGTGCCTACTTACACCGCATTTCATTCAGGCTATGCTGTCAGTAGGC |
| HA05            | PH36-rv| CCGGTACCAGCGGCTTAATCCATGTGACTGAAATCTGAGGGCAACG |
| HA06            | Psyn-fw| GGCACCTGATGCGGTTATTCTTCTCCATACACCGCATTTCATTCAGGCTATGCTGTCAGTAGGC |
| HA07            | Psyn-rv| CTGCAGGTCACTCTAGAGGATCCCAGTTAGCCATCGAATTCCATGAGCCCATCAATTTCATTCAGGCTATGCTGTCAGTAGGC |
| HA36            | repA-fw| AAAATCGCTTGACATTTGCAGGTTG |
| HA37            | repA-rv| CTTTACCTTCCTACAGTGTGCCTGAC |
| HA40            | repA-seq| TGCTGGTAGACAGAGCCAG |
| N101            | pVWEx4-fw| ATGCATGCCGCTTGATGCCATGACATTTGTACGATTCAGGCTATGCTGTCAGTAGGC |
| N102            | pVWEx4-rv| CGGCCCTGATGCGGTTATTCTTCTCCATACACCGCATTTCATTCAGGCTATGCTGTCAGTAGGC |
| N103            | xylAB-fw| ATGGAATTTGACCTTACACCGCATTTCATTCAGGCTATGCTGTCAGTAGGC |
| N104            | xylAB-rv| ATGCATGCCGCTTGATGCCATGACATTTGTACGATTCAGGCTATGCTGTCAGTAGGC |
| N105            | Psyn-fw2| ATGCATGCCGCTTGATGCCATGACATTTGTACGATTCAGGCTATGCTGTCAGTAGGC |
| N106            | Psyn-rv2| ATGCATGCCGCTTGATGCCATGACATTTGTACGATTCAGGCTATGCTGTCAGTAGGC |
| N107            | gfpUV-fw| ATGCCGCTGATGCGGTTATTCTTCTCCATACACCGCATTTCATTCAGGCTATGCTGTCAGTAGGC |
| N108            | gfpUV-rv| ATGCCGCTGATGCGGTTATTCTTCTCCATACACCGCATTTCATTCAGGCTATGCTGTCAGTAGGC |

2.3. Cloning of pECXT99A and pVWEx1-Based Expression Vectors

For construction of the pECXT99A-based expression vectors, pECXT99A was digested with NcoI and NdeI (Thermo Scientific Fisher, Schwerte, Germany) and dephosphorylated (Antarctic phosphatase, New England Biolabs, Frankfurt, Germany). Target promoters were amplified using the oligonucleotides as follows: Ptuf (cg0587): HN12 + HN13; PgapA (cg1791): HN14 + HN15; PilvC (cg1437): HN16 + HN17; PsodA...
was determined with Bradford Reagent (Sigma) for 9 min. After ultracentrifugation (1 h, 45,000×g) with an amplitude of 60% and a pulsing cycle of 0.5 s., the protein concentration was determined from a blue solid solution (Sigma). GAGGGCACTAATTTTGCTAAAGTAAGTGACGAAGAAGTTC was synthesized or inserted by oligonucleotides in the case of P45. All constructs were checked by sequencing. Newly constructed expression vectors were digested with BamHI and the fluorescence reporter gene gfpUV (HN49 + HN50) was cloned via Gibson-Assembly.

For construction of the pVWEx4 expression vectors, a site-directed mutagenesis in the repA gene was performed on pVWEx1 for an increased plasmid copy number. Site-directed mutagenesis was conducted via plasmid backbone amplification (HA36 + HA37) with Pfu Turbo DNA Polymerase. The vector pVWEx6 was constructed via digestion of pVWEx4 with KpnI and MauBI and insertion of the Psyn promoter including a lac operator motif (N105 + N106).

2.4. Fluorescence Analysis

GfpUV fluorescence was measured on a FACS GalliosTM (Beckmann Coulter GmbH, Krefeld, Germany) with 405 nm excitation from a blue solid-state laser. Forward-scatter characteristics (FSC) and side-scatter characteristics (SSC) were monitored as small- and large-angle scatters of the 405 nm laser. Fluorescence was detected using a band-pass filter (500/50 nm). The produced GfpUV protein possesses characteristic emission at 509 nm with an excitation wavelength of 385 nm. C. glutamicum WT harboring the newly constructed plasmids or the control plasmid were harvested in stationary growth, washed once in phosphate-buffered saline, and the optical density was adjusted to OD600nm ~0.1. C. glutamicum WT was used to determine autofluorescence. Median fluorescence intensities of 20,000 cells were calculated from each culture.

2.5. SDS-PAGE

C. glutamicum WT cells carrying the newly constructed plasmids were grown at 30 °C in 1 mL in Biolector® flowerplate system (m2p-labs GmbH, Baesweiler, Germany). Cells were harvested by centrifugation and stored at ~20 °C. For cell extract preparation, thawed cells were re-suspended in KPI buffer (100 mM disodium hydrogenophosphate and 100 mM sodium dihydrogenphosphate with pH 6.9). Cells were disrupted by ultrasonification using Hielscher UP200S2 (Teltow, Germany) with an amplitude of 60% and a pulsing cycle of 0.5 s. for 9 min. After ultracentrifugation (1 h, 45,000×g, 4 °C) the protein concentration was determined with Bradford Reagent (Sigma-Aldrich, Germany). Then, a 10 µg protein sample was mixed with Lämmli-Buffer and loaded on an SDS-PAGE, comprising a 4% stacking gel and 10% running gel. Gels were initially run at 50 V and then at 100 V. Protein gels were stained in 0.1% Coomassie blue solution (30% methanol; 10% acetic acid).

2.6. Enzyme Assay for Xylose Isomerase XylA

Cells of the strains WT (pECXT99A-xylAB) and WT (pECXT_Psyn-xylAB) were inoculated from a fresh LB overnight culture into a LB main culture with antibiotics and IPTG (1 mM) if applicable. Cultures were inoculated with an initial OD600 nm of 0.6 and grown until OD600 nm was 4. All cells from each culture were harvested at 4 °C and stored at ~20 °C till further use. Cells were washed in Tris-HCl Buffer (100 mM pH 7.5) and resuspended in 2 mL buffer prior to ultrasonification using Hielscher UP200S2 (Teltow, Germany) with an amplitude of 60% and a pulsing cycle of 0.5 s. for 9 min. After ultracentrifugation (1 h, 45,000×g, 4 °C) the protein concentration was determined with Bradford Reagent (Sigma-Aldrich, Germany). Enzymatic activity of
XylA was measured from the raw extract in a total volume of 1 mL at 30 °C: 100 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 0.17 mM NADH, and 1 U sorbitol dehydrogenase. Then, 200 μL D-xylene (2.5 M) was added and absorption was measured for an additional 3 min using a Shimadzu UV-1650 PC photometer (Shimadzu, Duisburg, Germany). The assay was performed with three appropriate protein concentrations each, applying 5/10/15 μL of crude extracts from strains WT (pECXT99A-xyl/AB) and WT (pECXT_Psyn-xyl/AB) with protein concentrations of 5.8 mg/mL and 1.72 mg/mL, respectively. Specific activities were calculated as μmol min⁻¹ (mg protein)⁻¹, defined as one unit (U/mg).

2.7. Sarcosine Quantification

Sarcosine quantification was performed from the clear supernatant of culture samples. Samples were derivatized with 9-fluorenylmethyl chlorocarbonate (FMOC) as described elsewhere [46]. Identification and separation was performed on a reversed phase HPLC. The column system consisted of a precolumn (LiChrospher 100 RP18 EC-5, 40 × 4 mm) and a main column (LiChrospher 100 RP18 EC-5, 125 × 4 mm) (CS Chromatographie Service GmbH, Langerwehe, Germany). Sodium acetate (50 mM pH 4.2) (A) and acetonitrile (B) were used as the mobile phases. A gradient with a flow rate of 1.2 mL min⁻¹ was used as follows: 0 min 38% B; 10 min 38% B; 17 min 57% B; 19 min 76% B; 20 min 76% B, and 23 min 38% B. Detection of the fluorescent derivatives was performed by a fluorescence detector (FLD G1321A, 1200 series, Agilent Technologies, Ratingen, Germany) with an excitation wavelength of 263 nm and emission wavelength of 310 nm. Calibration was conducted with a sarcosine standard (Sigma-Aldrich, Steinheim, Germany). L-proline was used as an internal standard.

3. Results

3.1. Screening of Strong Constitutive Promoters in the pECXT99A-Based Vector System

A selection of native and synthetic promoters was cloned in the pECXT99A-backbone (lacking the lacI gene to allow for constitutive expression) in order to test the different promoter strengths using the promoterless reporter gene gfpUV. The promoters from the endogenous genes tuf (cg0587), ilvC (cg1437), sodA (cg3237), gapA (cg1791), and pgk (cg1790) were amplified from the genome of C. glutamicum WT. The synthetic promoters PH36 and Psyn were generated by gene synthesis, whereas P45 was generated by oligonucleotide insertion. GfpUV reporter fluorescence in C. glutamicum WT carrying the plasmids was monitored 18 h after inoculation in CGXII with 4% glucose without any inducer. The strain carrying the parental plasmid pECXT99A-gfpUV (with lacIq) was chosen as a reference (cultured in the presence of 1 mM IPTG), as this vector served as the starting point for the cloning of the promoter library. All tested promoters showed activity and, thus, allowed for constitutive gene expression without the need to add an inducer to the medium (Figure 1). The synthetic promoter PH36 (norm. MFI: 2.7 ± 13%) and the endogenous promoters Ppgk (norm. MFI: 4.0 ± 12%) and PIlvC (norm. MFI: 7.0 ± 5%) showed significant expression in comparison to that of the autofluorescence (norm. MFI: 1.0 ± 10%). However, the expression was relatively low in comparison to that of the other vectors. The endogenous promoters PsodA (norm. MFI: 10.8 ± 1%), PgapA (norm. MFI: 19 ± 9%), and Ptuf (norm. MFI: 50 ± 15%) showed increasing expression levels with the latter one showing comparable expression values to that of the synthetic P45 (norm. MFI: 56 ± 7%). Thus, gfpUV expression from the constitutive P45 was as strong as IPTG-induced gfpUV expression from Ptrc in pECXT99A (with lacIq and Pptrc; norm. MFI: 56 ± 6%). Notably, gfpUV expression from the synthetic promoter Psyn exceeded this level by about a factor of five (norm. MFI: 286 ± 4%). Thus, the newly constructed pECXT_Psyn showed the highest gfpUV expression of the compared vectors (Figure 1).
Figure 1. Fluorescence reporter assay of a promoter library in the pECXT99A plasmid background in C. glutamicum WT. Median GfpUV fluorescence intensities were normalized to an autofluorescent control. Mean values and standard deviations of biological triplicates are shown. Measurements were performed 18 h after inoculation in CGXII medium with 4% glucose. Endogenous promoters: P_{pgk}, P_{ilvC}, P_{sodA}, P_{gapA}, and P_{tuf}. Synthetic promoters: PH36, P45, and Psyn. Reference: the IPTG-inducible promoter P_{trc} induced with 1 mM IPTG.

3.2. Improving Plasmid-Borne Inducible Gene Expression in C. glutamicum by Combination of a Stronger Promoter with Increased Plasmid Copy Number

First, the properties of the commonly used IPTG-inducible expression vectors pEKEx3, pVWEx1, and pECXT99A were compared with regard to gfpUV gene expression by FACS analysis (Figure 2) and protein abundance by SDS-PAGE (Figure 3). Induction factors were determined from reporter fluorescence measured in the absence and presence of 1 mM IPTG. With vector pEKEx3-gfpUV, a maximal norm. MFI of 64 ± 13% was reached, but expression was leaky, thus, an induction factor of only 6 resulted (Figure 2). The vector pECXT99A-gfpUV supported a maximal norm. MFI of 48 ± 3% with an induction factor of 47. Expression from pVWEx1-gfpUV without IPTG was as tight as that observed for pECXT99A-gfpUV. With higher maximal norm. MFI of 99 ± 3% an induction factor of 76 was observed with pVWEx1-gfpUV. SDS-PAGE revealed lower GfpUV protein abundance for pEKEx3-gfpUV as compared to that of pECXT99A-gfpUV, which was highest among the three vectors for pVWEx1-gfpUV (Figure 3).
Figure 2. Fluorescence reporter assay of conventional and newly constructed IPTG-inducible expression vectors in *C. glutamicum* WT. Vector pECXT99A-gfpUV expressed gfpUV from *Ptrc*, while *P tac* was used for vectors pEKEx3-gfpUV, pVWEx1-gfpUV, pVWEx4-gfpUV, and pVWEx6-gfpUV. Median GfpUV fluorescence intensities were normalized to an autofluorescent control. Mean values and standard deviations of biological triplicates are shown. Measurements were performed 20 h after inoculation in CGXII medium with 4% glucose. Newly constructed vectors: pVWEx4 and pVWEx6. Reference vectors: pEKEx3, pECXT99A, and pVWEx1. Concentration of the inductor IPTG is given in mM.

Next, we sought to improve inducible gene expression vector pVWEx1, which possesses the origin of replication pHM1519. It has been shown that pHM1519-based vectors are maintained at plasmid copy numbers of approximately 140 in *C. glutamicum* cells [47]. Importantly, it has recently been shown that the copy number of vectors with a pHM1519 replicon can be increased about 5-fold to about 800 [30]. This finding prompted us to increase the plasmid copy number of pVWEx1. Therefore, the *repA* gene encoding an initiator protein for the pHM1519 replicon was mutated (base transition from G to A at nucleotide 1286) to exchange Gly at position 429 of RepA protein by Glu via site-directed mutagenesis, which was designated as *copA1* [30]. The resulting vector based on pVWEx1 with the mutation RepA<sup>G429D</sup> was named pVWEx4. FACS analysis revealed an approximately 1.5-fold increased IPTG-induced gfpUV gene expression using vector pVWEx4-gfpUV as compared to that using pVWEx1-gfpUV (norm. MFI of 145 ± 7% as compared to 99 ± 3%). Since expression without IPTG was almost as tight for pVWEx4 as for pVWEx1, the induction factor with pVWEx4 was higher than that with pVWEx1 (121 as compared to 76). Thus, introduction of the *copA1* mutation into the *repA* gene increased the plasmid copy number as expected, and allowed for a very strong and more than 100-fold inducible target gene expression.
Third, since P_{trc} was weaker than P_{syn} (Figure 1), we hypothesized that replacing P_{trc} in plasmid pVWEx4 by the stronger P_{syn}, while maintaining the lacIq and lac operator sequences for IPTG-inducible gene expression, would lead to increased target gene expression that could be modulated by titrating IPTG concentrations. The resulting vector was named pVWEx6. This vector allowed for an approximately 50-fold induction with 1 mM IPTG, leading to a maximal norm. MFI of 410 ± 1% (Figure 2 and Table 3).

Moreover, we sought to find a lower IPTG concentration for pVWEx6 that would achieve comparable expression as that of the fully induced pVWEx1, pEXCT99A, and/or pEKEx3. Our choice of 0.025 mM and 0.050 mM IPTG as intermediate IPTG concentrations was guided by prior experience with vectors pVWEx1 and pEKEx3 for expression of genes for membrane proteins or transporter proteins [48–50]. Overexpression of genes for membrane proteins or transporter proteins proved difficult as too high levels reduced growth, probably by altering membrane integrity. However, with 0.025 mM or 0.050 mM IPTG as the intermediate IPTG concentrations, functional overexpression of genes coding for membrane proteins or transporter proteins was achieved without observed growth impairment [48–50]. Here, intermediate GfpUV fluorescence was observed with 0.025 and 0.05 mM IPTG. SDS-PAGE analysis of GfpUV reporter protein abundance (Figure 3) confirmed the FACS results of very strong expression (Figure 2). In fact, the IPTG-induction factor was as high as those obtained with pVWEx1 and pECXT99A, while the fully induced expression strength supported by pVWEx6 was one magnitude higher than those achieved with pEKEx3, pECXT99A, and pVWEx1.
Table 3. Comparison of C. glutamicum expression vectors. *: high-copy number variant of the pHM1519 replicon.

| Plasmid        | pEKEx3 | pVWEx1 | pVWEx4 | pVWEx6 | pECXT99A | pECXT_Psyn |
|----------------|--------|--------|--------|--------|----------|------------|
| Expression     | Inducible | Inducible | Inducible | Inducible | Constitutive |            |
| Repressor gene | lacI    | lacI    | lacI    | lacI    | lacI     |            |
| Promoter       | Plac    | Plac    | Plac    | Psyn    | PTrc     | Psyn       |
| Induction factor | 6      | 76     | 121    | 51      | 40       | non        |
| Maximal expression | 64    | 99     | 145    | 410     | 47       | 140        |
| Origin for Cg  | pBL1    | pHM1519* | pHM1519* | pHM1519* | pGA1 mini | pGA1 mini  |
| Origin for Ec  | ColE1 ori | ColE1 ori | ColE1 ori | ColE1 ori | pMB1     | pMB1       |
| Resistance     | Spec    | Kan    | Kan    | Kan    | pMB1     | Tet        |

3.3. Fast Production of Sarcosine from Xylose by Application of the Newly Constructed pECXT_Psyn

As an application test, sarcosine production by *C. glutamicum* was chosen (Figure 4A). *C. glutamicum* was engineered to produce the N-methylated amino acid sarcosine in the enzyme reaction catalyzed by the *Pseudomonas putida* KT2440 imine reductase DpkA using glyoxylate as the 2-oxo acid substrate and monomethylamine as the N-alkyl donor [11]. To enable xylose utilization, the WT carrying malate synthase gene deletion ΔaceB, a start codon exchange from ATG to GTG for isocitrate dehydrogenase gene icd and the vector pVWEx1-dpkA_RBSopt for IPTG-inducible expression of dpkA, was transformed with pECXT99A-xylAB as the second vector. Notably, the resulting *C. glutamicum* strain SAR3 produced sarcosine with higher yield coefficients than that with glucose, however, the volumetric productivity was low due to slow growth with xylose [11]. To test if stronger expression of xylAB using the newly constructed vector pECXT99A_Psyn-xylAB accelerates xylose-based sarcosine production, strain SAR3* that carried pECXT99A_Psyn-xylAB instead of pECXT99A-xylAB was constructed (Figure 4A).

First, to score xylose catabolism an enzyme assay of the heterologous xylose isomerase XylA was performed. To this end, crude extracts of *C. glutamicum* WT carrying either pECXT99A_Psyn-xylAB or pECXT99A-xylAB were prepared and assayed spectrophotometrically for XylA activity (Figure 4B). A more than 3-fold increase in the specific XylA activity indicated that pECXT99A_Psyn-xylAB provided higher xylAB expression than pECXT99A-xylAB (Figure 4B). Next, sarcosine production by strains SAR3 and SAR3* was compared in media containing monomethylamine, potassium acetate, and xylose. When grown in the presence of acetate, *C. glutamicum* is known to carry a high flux from acetyl-CoA into the TCA cycle and to glyoxylate [7,51] (Figure 4A). This is due to transcriptional regulation of the isocitrate lyase gene aceA (and malate synthase gene aceB that is deleted in SAR3) by the transcriptional activator RamA and the transcriptional repressor RamB [52,53]. Xylose served as the primary carbon and energy source for growth under these conditions. Growth of SAR3* was 1.3-fold faster than growth of SAR3 (growth rates of 0.13 ± 0.01 h⁻¹ as compared to 0.10 ± 0.01 h⁻¹, respectively). Importantly, after 20 h, sarcosine accumulated to titers of 1.0 ± 0.08 g L⁻¹ for SAR3 and 1.5 ± 0.13 g L⁻¹ for SAR3* (Figure 4C). As a consequence, the volumetric productivity was improved by 50% (0.077 g L⁻¹ h⁻¹ for SAR3*) as compared to SAR3 (0.051 g L⁻¹ h⁻¹). Thus, pECXT99A_Psyn-xylAB proved useful to improve the xylose-based sarcosine production by recombinant *C. glutamicum*. 
Microorganisms 2021, 9, 204

Figure 4. Schematic representation of sarcosine production by recombinant C. glutamicum (A). Xylose isomerase (XylA) activities in crude extracts measured in triplicates (B), and sarcosine titers produced by C. glutamicum strains SAR3 and SAR3* measured 20 h after inoculation from triplicates (C). XylA: xylose isomerase; XylB: xylulose kinase; PPP: pentose phosphate pathway; GAP: glyceraldehyde 3-phosphate; AceB: malate synthase; AceA: isocitrate lyase; DpkA: imine reductase; Icd: isocitrate dehydrogenase; MMA: monomethylamine.

4. Discussion

Expression vectors play a crucial role in basic research to investigate endogenous metabolism and to establish microbial bio-production by metabolic engineering. However, the development and/or optimization of expression systems is rarely the focus of scientific work. In this study, we focused on the optimization of the two well-established expression vectors pECXT99A and pVWEx1 by application of a strong promoter and by an increased plasmid copy number, respectively. Due to different C. glutamicum origins of replication and antibiotic resistance markers (Table 3), these vectors can coexist: pEKEx3 plus pECXT99A or pECXT_Psyn plus pVWEx1 or pVWEx4 or pVWEx6.

The expression level of a vector system is dependent on a wide range of aspects that affect either transcription or translation of an overexpressed gene or the protein stability itself. For example, plasmid-driven gene expression relies on the number of gene copies (plasmid-copy number) and the promoter strength. Both aspects were part of this work. In addition, the translational efficiency of target genes may be optimized by changing the translational start codon [54] or by changing ribosome binding sites as a trade-off between an optimal RBS (consensus C. glutamicum: 5′-GAAAGGAGG-3′) and the prevention of secondary mRNA structures [55–57]. Independent of vector characteristics, the target gene (and gene product) may be optimized. Protein stability can be improved either by adding tags, by construction of fusion enzymes [58,59], or by truncations to improve protein solubility [60,61] while taking into account dimerization domains, catalytic centers, and cofactor binding sites [62]. If their termini are freely accessible, membrane proteins may be fused as shown in the fusion of the cytosolic C terminus of CrtZ to the cytosolic N terminus of CrtW, which resulted in improved astaxanthin production by C. glutamicum [10]. The improved vectors developed here (pVWEx4, pVWEx6, and pECXT99A_Psyn) add to the toolbox available to microbiologists studying the physiology of C. glutamicum as well as for metabolic engineering in strain and process development. Moreover, the expression characteristics obtained in comparative fluorescence reporter gene expression experiments will guide the choice of vectors according to the task of interest.
To compare plasmid-borne target gene expression, the endogenous promoters of the genes pgk, ilvC, sodA, gapA, and tuf were chosen in this study. The promoters of the glycolytic genes pgk [63] and gapA [64], encoding phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase, respectively, as well as of the superoxide dismutase gene sodA [65], the ketol-acid reductoisomerase gene ilvC [65], and the transcription elongation factor gene tuf [66] have already been described as strong constitutive promoters. In a different vector background, it was shown that PilvC and PsodA showed strong expression in a GFP reporter assay with comparable expression levels as that of Ptlac in the case of PilvC and around a 2.5-fold higher expression in the case of PsodA [65]. In the study presented here, expression levels of both promoters were lower than that of Ptrc. As discussed above, the promoter is one of several factors influencing reporter gene expression, making comparisons of reporter gene expression using different vector backbones, ribosome binding sites, etc. difficult. Nevertheless, the previously described vectors along with those described here allow for low, but constitutive expression and are of interest for the production of membrane proteins, transporter proteins, or enzymes that lead to the formation of toxic byproducts and for balancing of engineered pathways [48].

To maximize target gene expression, the design and usage of synthetic promoters for genetic engineering in C. glutamicum is on the rise, as these usually short promoters can achieve high transcription levels [37,38]. Moreover, the utilization of artificial promoters allows researchers to circumvent the challenges of regulatory interference in metabolic engineering approaches for overproduction. The PH36 promoter was identified by Yim et al. 2013, where it showed a 60-fold higher expression level in the pCES plasmid background in comparison to that of the Ptrc promoter [37]. In contrast, here, PH36 showed the weakest expression in the fluorescence assay. The synthetic Psyn was designed and identified as it showed high promoter activity in a β-galactosidase assay [38]. Although this result is difficult to compare with the fluorescence assay used here, we have shown that the short synthetic promoter Psyn (5’-TTGACATTAATT-GAATCTGTGTATAATGTTAATGC-3’) enabled tunable and strong expression in combination with the lac operator sequence in cis (5’-CTGGAATTGTGAGCGGATAACAATTC-3’) and lacIq in trans. The newly constructed pVWEx6 vector showed a gene expression strength that is one magnitude higher than that of other commonly used expression vectors. Thus, the application of pVWEx6 can reduce the costs for IPTG, as 25 µM IPTG lead to comparable expression patterns as that of pVWEx1, pECXT99A, and pEKEx3 induced with 1 mM IPTG. Potentially, leakiness of these vectors can be further minimized as shown very recently for pEKEx2 by restoring lacIq gene sequences and by avoiding duplicate DNA sequences [67].

As an application case, we transferred the findings about elevated gene expression to fermentative sarcosine production with C. glutamicum. Strain SAR3* was based on the newly designed pECXT_Psyn for expression of xylose utilization gene xylAB and it showed a volumetric productivity for sarcosine with 0.077 g L⁻¹ h⁻¹ as compared to that of the reference strain (0.051 g L⁻¹ h⁻¹) [11]. Our previous work on sarcosine production [11] identified slow growth with xylose as the limitation of the volumetric productivity for sarcosine. Here, we have shown that accelerating growth with xylose improved the volumetric productivity of SAR3* by 50% as compared to that of SAR3. We deliberately did not use pVWEx4 or pVWEx6 for dpkA expression here, since N-methylation of glyoxylate to yield sarcosine as catalyzed by DpkA does not limit sarcosine production. This was deduced from our previous work [68], where pVWEx1-dpkA supported a volumetric productivity of up to 0.35 g L⁻¹ h⁻¹ for N-methylation of pyruvate to N-methylalanine. Since it was shown that doubling of the MMA concentration as well as optimization of the carbon source composition (5 g/L xylose and 30 g/L acetate) improved the sarcosine production by SAR3 about 3-fold, combination of the newly constructed strain SAR3* with those culture conditions might further improve sarcosine production with C. glutamicum.
Author Contributions: N.A.H. and V.F.W. designed the experiments. N.A.H. and I.K. planned and performed the experiments. N.A.H., I.K., and V.F.W. analyzed the data. N.A.H. and V.F.W. drafted the manuscript. V.F.W. coordinated the study and finalized the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the European Regional Development Fund (ERDF) and the Ministry of Economic Affairs, Innovation, Digitalization and Energy of the State of North Rhine-Westphalia, grant number EFRE-0400184 (Bimmer). Support for the Article Processing Charge by the Deutsche Forschungsgemeinschaft and the Open Access Publication Fund of Bielefeld University is acknowledged.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in article.

Acknowledgments: We thank Anna Hamker for assistance during plasmid construction and Petra Peters-Wendisch for scientific discussion.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References
1. Kinoshita, S.; Udaka, S.; Shimono, M. Studies on the amino acid fermentation. Production of L-glutamic acid by various microorganisms. J. Gen. Appl. Microbiol. 1957, 3, 193–205, doi:10.2323/jgam.3.193.
2. Kirchner, O.; Tauch, A. Tools for genetic engineering in the amino acid-producing bacterium Corynebacterium glutamicum. J. Biotecnol. 2003, 104, 287–299.
3. Becker, J.; Rohles, C.M.; Wittmann, C. Metabolically engineered Corynebacterium glutamicum for bio-based production of chemicals, fuels, materials, and healthcare products. Metab. Eng. 2018, 50, 122–141, doi:10.1016/j.men.2018.07.008.
4. Heider, S.A.; Wendisch, V.F. Engineering microbial cell factories: Metabolic engineering of Corynebacterium glutamicum with a focus on non-natural products. Biotechnol. J. 2015, 10, 1170–1184, doi:10.1002/biot.201400590.
5. Wendisch, V.F. Metabolic engineering advances and prospects for amino acid production. Metab. Eng. 2020, 58, 17–34, doi:10.1016/j.men.2019.03.008.
6. Pérez-García, F.; Peters-Wendisch, P.; Wendisch, V.F. Engineering Corynebacterium glutamicum for fast production of L-lysine and L-picolinic acid. Appl. Microbiol. Biotechnol. 2016, 100, 8075–8090, doi:10.1007/s00253-016-7682-6.
7. Zahoor, A.; Otten, A.; Wendisch, V.F. Metabolic engineering of Corynebacterium glutamicum for glycolate production. J. Biotechnol. 2014, 192, 366–375, doi:10.1016/j.jbiotec.2013.12.020.
8. Wieschalka, S.; Blombach, B.; Bott, M.; Eikmanns, B.J. Bio-based production of organic acids with Corynebacterium glutamicum. Microb. Biotechnol. 2013, 6, 87–102, doi:10.1111/j.1751-7915.2012.12013.
9. Frohwitter, J.; Heider, S.A.; Peters-Wendisch, P.; Beekwilder, J.; Wendisch, V.F. Production of the sesquiterpene (+)-valencene by metabolically engineered Corynebacterium glutamicum. J. Biotechnol. 2014, 191, 205–213, doi:10.1016/j.jbiotec.2014.05.032.
10. Henke, N.A.; Wendisch, V.F. Improved Astaxanthin Production with Corynebacterium glutamicum by Application of a Membrane Fusion Protein. Mar. Drugs 2019, 17, 621, doi:10.3390/md17110621.
11. Mindt, M.; Heuser, M.; Wendisch, V.F. Xylose as preferred substrate for sarcosine production by recombinant Corynebacterium glutamicum. Bioreour. Technol. 2019, 281, 135–142, doi:10.1016/j.biortech.2019.02.084.
12. Wendisch, V.F.; Brito, L.F.; Gil Lopez, M.; Hennig, G.; Pfeifenschneider, J.; Sgobba, E.; Veldmann, K.H. The flexible feedstock concept in Industrial Biotechnology: Metabolic engineering of Escherichia coli, Corynebacterium glutamicum, Pseudomonas, Bacillus and yeast strains for access to alternative carbon sources. J. Biotechnol. 2016, 234, 139–157, doi:10.1016/j.jbiotec.2016.07.022.
13. Rittmann, D.; Lindner, S.N.; Wendisch, V.F. Engineering of a glycerol utilization pathway for amino acid production by Corynebacterium glutamicum. Appl. Environ. Microbiol. 2008, 74, 6216–6222, doi:10.1128/AEM.00963-08.
14. Meiswinkel, T.M.; Rittmann, D.; Lindner, S.N.; Wendisch, V.F. Crude glycerol-based production of amino acids and putrescine by Corynebacterium glutamicum. Bioreour. Technol. 2013, 145, 254–258, doi:10.1016/j.biortech.2013.02.053.
15. Hennig, G.; Haupka, C.; Brito, L.F.; Rückert, C.; Cahoreau, E.; Heux, S.; Wendisch, V.F. Methanol-Essential Growth of Corynebacterium glutamicum: Adaptive Laboratory Evolution Overcomes Limitation due to Methanethiol Assimilation Pathway. Int. J. Mol. Sci. 2020, 21, 3617, doi:10.3390/ijms21103617.
16. Seibold, G.; Aachter, M.; Berens, S.; Kalinowski, J.; Eikmanns, B.J. Utilization of soluble starch by a recombinant Corynebacterium glutamicum strain: Growth and lysine production. J. Biotechnol. 2006, 124, 381–391, doi:10.1016/j.jbiotec.2005.12.027.
17. Anusree, M.; Wendisch, V.F.; Nampoothiri, K.M. Co-expression of endoglucanase and β-glucosidase in Corynebacterium glutamicum DM1729 towards direct lysine fermentation from cellulose. Bioreour. Technol. 2016, 213, 239–244, doi:10.1016/j.biortech.2016.03.019.
43. Gopinath, V.; Meiswinkel, T.M.; Wendisch, V.F.; Nampoothiri, K.M. Amino acid production from rice straw and wheat bran hydrolysates by recombinant pentose-utilizing Corynebacterium glutamicum. Appl. Microbiol. Biotechnol. 2011, 92, 985–996, doi:10.1007/s00253-011-3478-x.

42. Gopinath, V.; Murali, A.; Dhar, K.S.; Nampoothiri, K.M. Corynebacterium glutamicum as a potent biocatalyst for the bioconversion of pentose sugars to value-added products. Appl. Microbiol. Biotechnol. 2012, 93, 95–106, doi:10.1007/s00253-011-3686-4.

41. Henke, N.A.; Wichmann, J.; Baier, T.; Frohwitter, J.; Lauersen, K.J.; Risse, J.M.; Peters-Wendisch, P.; Kruse, O.; Wendisch, V.F. Patchouli Production with Metabolically Engineered Corynebacterium glutamicum. Genes (Basel) 2018, 9, 219, doi:10.3390 GENES9040219.

40. Henke, N.A.; Heider, S.A.; Peters-Wendisch, P.; Wendisch, V.F. Production of the Marine Carotenoid Astaxanthin by Metabolically Engineered Corynebacterium glutamicum. Mar. Drugs 2014, 12, 144, doi:10.3390/md14070124.

39. Gauttam, R.; Desiderato, C.; Jung, L.; Shah, A.; Eikmanns, B.J. A step forward: Compatible and dual-inducible expression vectors for gene co-expression in Corynebacterium glutamicum. Plasmid 2019, 101, 20–27, doi:10.1016/j.plasmid.2018.12.004.

38. Goldbeck, O.; Seibold, G.M. Construction of pOGOduet—An inducible, bicistronic vector for synthesis of recombinant proteins in Corynebacterium glutamicum. Plasmid 2019, 95, 11–15, doi:10.1016/j.plasmid.2018.01.001.

37. Knoppova, M.; Phensaijai, M.; Vesely, M.; Zemanova, M.; Nesvera, J.; Patek, M. Plasmid vectors for testing in vivo promoter activities in Corynebacterium glutamicum and Rhodococcus erythropolis. Curr. Microbiol. 2007, 55, 234–239.

36. Schäfer, A.; Tauch, A.; Jäger, W.; Kalinowski, J.; Thierbach, G.; Puhler, A. 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 1994, 145, 69–73.

35. Eggeling, L.; Bott, M. (Eds.) Handbook of Corynebacterium Glutamicum; CRC Press Taylor & Francis Group: Boca Raton, FL, USA, 2005.

34. Tauch, A.; Kirchner, O.; Löffler, B.; Gotter, S.; Puhler, A.; Kalinowski, J. Efficient electrotansformation of Corynebacterium diptheriae with a mini-replicon derived from the Corynebacterium glutamicum plasmid pGAI. Curr. Microbiol. 2002, 45, 362–367.

33. Peters-Wendisch, P.G.; Schiel, B.; Wendisch, V.F.; Katsoulidis, E.; Mockel, B.; Sahm, H.; Eikmanns, B.J. Pyruvate carboxylase is a major bottleneck for glutamate and lysine production by Corynebacterium glutamicum. J. Mol. Microbiol. Biotechnol. 2001, 3, 295–300.

32. Oehler, S.; Amouyal, M.; Kolkhof, P.; von Willecken-Bergmann, B.; Müller-Hill, B. Quality and position of the three lac operators of E. coli define efficiency of repression. EMBO J. 1994, 13, 3348–3355.

31. Hashiro, S.; Yasueda, H. Plasmid copy number mutation in repA gene encoding RepA replication initiator of cryptic plasmid pHM1519 in Corynebacterium glutamicum. Biosci. Biotechnol. Biochem. 2018, 82, 2212–2224, doi:10.1080/09168451.2018.1508986.

30. Patek, M.; Eikmanns, B.J.; Patek, J.; Sahm, H. Promoters from Corynebacterium glutamicum: Cloning, molecular analysis and search for a consensus motif. Microbiol. 1996, 142, 1297–1309.

29. Dostalova, H.; Holatko, J.; Busche, T.; Rucka, L.; Rapoport, A.; Halada, P.; Nesvera, J.; Kalinowski, J.; Patek, M. Assignment of sigma factors of RNA polymerase to promoters in Corynebacterium glutamicum. AMB Express 2017, 7, 133, doi:10.1186/s13567-017-0436-8.

28. Taniguchi, H.; Busche, T.; Patschkowski, T.; Niehaus, K.; Patek, M.; Kalinowski, J.; Wendisch, V.F. Physiological roles of sigma factor SigD in Corynebacterium glutamicum. BMC Microbiol. 2017, 17, 158, doi:10.1186/s12866-017-1067-6.

27. Busche, T.; Silar, R.; Plémanová, M.; Pátek, M.; Kalinowski, J. Transcriptional regulation of the operon encoding stress-responsive ECF sigma factor SigH and its anti-sigma factor RshA, and control of its regulatory network in Corynebacterium glutamicum. BMC Genom. 2012, 13, 445, doi:10.1186/1471-2164-13-445.

26. Patek, M.; Nesvera, J. Sigma factors and promoters in Corynebacterium glutamicum. J. Biotechnol. 2011, 154, 101–113, doi:10.1016/j.jbiotec.2011.01.017.

25. Patek, M.; Holatko, J.; Busche, T.; Kalinowski, J.; Nesvera, J. Corynebacterium glutamicum promoters: A practical approach. Microbiol. 2013, 6, 103–117, doi:10.1111/1117-7915.12019.

24. Yim, S.S.; An, S.J.; Kang, M.; Lee, J.; Jeong, K.J. Isolation of fully synthetic promoters for high-level gene expression in Corynebacterium glutamicum. Biotechnol. Bioeng. 2013, 110, 2959–2969, doi:10.1002/bit.24954.

23. Ryttter, J.V.; Helmark, S.; Chen, J.; Lezyk, M.J.; Solem, C.; Jensen, P.R. Synthetic promoter libraries for Corynebacterium glutamicum. Appl. Microbiol. Biotechnol. 2014, 98, 2617–2623, doi:10.1007/s00253-013-5481-x.

22. Abe, S.; Takayama, K.; Kinoshita, S. Taxonomical studies on glutamic acid producing bacteria. J. Gen. Appl. Microbiol. 1967, 13, 279–301.

21. Hanahan, D. Studies on transformation of Escherichia coli with plasmids. J. Mol. Biol. 1983, 166, 557–580.

20. Stansen, C.; Uy, D.; Delaunay, S.; Eggeling, L.; Goergen, J.L.; Wendisch, V.F. Characterization of a Corynebacterium glutamicum lactate utilization operon induced during temperature-triggered glutamate production. Appl. Environ. Microbiol. 2005, 71, 5920–5928.

19. Gobba, E.; Stumpf, A.K.; Vortmann, M.; Jagmann, N.; Krehenbrink, M.; Dirks-Hofmeister, M.E.; Moerschbacher, B.; Philipp, B.; Wendisch, V.F. Synthetic Escherichia coli–Corynebacterium glutamicum consortia for l-lysine production from starch and sucrose. Biosourc. Technol. 2018, 260, 302–310, doi:10.1016/j.biotech.2018.03.113.

18. Veldmann, K.H.; Dachwitz, S.; Risse, J.M.; Lee, J.H.; Sewald, N.; Wendisch, V.F. Bromination of L-tryptophan in a Fermentative Process With Corynebacterium glutamicum. Front. Bioeng. Biotechnol. 2019, 7, 219, doi:10.3389/fbioe.2019.00219.
Microorganisms 2021, 9, 204

44. Gibson, D.G.; Young, L.; Chuang, R.Y.; Venter, J.C.; Hutchison, C.A., 3rd; Smith, H.O. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat. Methods 2009, 6, 343–345, doi:10.1038/nmeth.1318.

45. van der Rest, M.E.; Lange, C.; Molenaar, D. A heat shock following electroporation induces highly efficient transformation of Corynebacterium glutamicum with xenogenic plasmid DNA. Appl. Microbiol. Biot. 1999, 52, 541–545.

46. Mindt, M.; Walter, T.; Risse, J.M.; Wendisch, V.F. Fermentative Production of N-Methylglutamate From Glycerol by Recombinant Pseudomonas putida. Front. Bioeng. Biotechnol. 2018, 6, 159, doi:10.3389/fbioe.2018.00159.

47. Miwa, K.; Matsu, H.; Terabe, M.; Nakamori, S.; Sano, K.; Momose, H. Cryptic Plasmids in Glutamic Acid-producing Bacteria. Agricultural Biol. Chem. 1984, 48, 2901–2903, doi:10.1006/abch.1984.10866615.

48. Youn, J.W.; Jolkver, E.; Kramer, R.; Marin, K.; Wendisch, V.F. Characterization of the dicarboxylate transporter DctA in Corynebacterium glutamicum. J. Bacteriol. 2009, 191, 5480–5488, doi:10.1128/JB.00640-09.

49. Taniguchi, H.; Wendisch, V.F. Exploring the role of sigma factor gene expression on production by Corynebacterium glutamicum: Sigma factor H and FMN as example. Front. Microbiol. 2015, 6, 740, doi:10.3389/fmicb.2015.00740.

50. Binder, D.; Frohwitter, J.; Mehr, R.; Bier, C.; Grunberger, A.; Loschke, A.; Peters-Wendisch, P.; Kohlheyer, D.; Pietruszka, J.; Frunze, J.; et al. Light-Controlled Cell Factories: Employing Photocaged Isopropyl-beta-d-Thiogalactopyranoside for Light-Mediated Optimization of lac Promoter-Based Gene Expression and (+)-Valencene Biosynthesis in Corynebacterium glutamicum. Appl. Environ. Microbiol. 2016, 82, 6411–6419, doi:10.1128/aem.01457-16.

51. Wendisch, V.F.; Spies, M.; Reinscheid, D.J.; Schnicke, S.; Sahm, H.; Eikmanns, B.J. Regulation of acetate metabolism in Corynebacterium glutamicum: Transcriptional control of the isocitrate lyase and malate synthase gene cluster. Arch. Microbiol. 1997, 168, 262–269.

52. Cramer, A.; Gerstmeir, R.; Schaffer, S.; Bott, M.; Eikmanns, B.J. Identification of RamA, a novel LuxR-type transcriptional regulator of genes involved in acetate metabolism of Corynebacterium glutamicum. J. Bacteriol. 2006, 188, 2554–2567.

53. Auchtner, M.; Cramer, A.; Huser, A.; Ruckert, C.; Emer, D.; Schwarz, P.; Arndt, A.; Lange, C.; Kalinowski, J.; Wendisch, V.F.; et al. RamA and RamB are global transcriptional regulators in Corynebacterium glutamicum and control genes for enzymes of the central metabolism. J. Biotechnol. 2011, 154, 126–139, doi:10.1016/j.jbiotec.2010.07.001.

54. Jensen, J.V.; Wendisch, V.F. Ornithine cyclodeaminase-based proline production by Corynebacterium glutamicum. Microb. Cell Fact. 2013, 12, 63, doi:10.1186/1475-2859-12-63.

55. Pfeifer-Sancar, K.; Mentz, A.; Ruckert, C.; Kalinowski, J. Comprehensive analysis of the Corynebacterium glutamicum transcriptome using an improved RNAseq technique. BMC Genom. 2013, 14, 888, doi:10.1186/1471-2164-14-888.

56. Espah Borujeni, A.; Cetnar, D.; Farasat, I.; Smith, A.; Lundgren, N.; Salis, H.M. Precise quantification of translation inhibition by mRNA structures that overlap with the ribosomal footprint in N-terminal coding sequences. Nucleic Acids Res. 2017, 45, 5437–5448, doi:10.1093/nar/gks061.

57. Salis, H.M.; Mirsky, E.A.; Voigt, C.A. Automated design of synthetic ribosome binding sites to control protein expression. Nat. Biotechnol. 2009, 27, 946–950, doi:10.1038/nbt.1568.

58. Kosobokova, E.N.; Skrypnik, K.A.; Kosorukov, V.S. Overview of Fusion Tags for Recombinant Proteins. Biochemistry (Mosc) 2016, 81, 187–200, doi:10.1134/s0006297916030019.

59. Widakowich, G.; Zhang, C.; Harris, S.; Mitri, K.; Powers, G.; Troung, K.S.; Hearn, M.T. Effects of IMAC specific peptide tags on the stability of recombinant green fluorescent protein. Biotechnol. Progr. 2011, 27, 1048–1053, doi:10.1002/bipro.546.

60. Cooper, C.D.O.; Marsden, B.D. N- and C-Terminal Truncations to Enhance Protein Solubility and Crystallization: Predicting Protein Domain Boundaries with Bioinformatics Tools. In Heterologous Gene Expression in E.coli: Methods and Protocols, Burgess-Brown, N.A., Ed.; Springer: New York, NY, USA, 2017; doi:10.1007/978-1-493-94603-9_2pp., 11–31.

61. Speck, J.; Hecky, J.; Tam, H.K.; Arndt, K.M.; Einsle, O.; Müller, K.M. Exploring the molecular linkage of protein stability traits for enzyme optimization by iterative truncation and evolution. Biochemistry 2012, 51, 4850–4867, doi:10.1021/bi2018783.

62. Sinha, R.; Shukla, P. Current Trends in Protein Engineering: Updates and Progress. Curr. Protein Pept. Sci. 2019, 20, 398–407, doi:10.2174/138920372066618119120120.

63. Han, S.O.; Inui, M.; Yawaka, H. Expression of Corynebacterium glutamicum glycolytic genes varies with carbon source and growth phase. Microbiology 2007, 153, 2190–2202.

64. Wang, Y.; Liu, J.; Ni, X.; Lei, Y.; Zheng, P.; Diao, A. Screening efficient constitutive promoters in Corynebacterium glutamicum based on time-series transcriptome analysis. Sheng Wu Gong Cheng Xue Bao 2018, 34, 1760–1771, doi:10.13345/j.cjb.180041.

65. Lee, J. Development and characterization of expression vectors for Corynebacterium glutamicum. J. Microbiol. Biotechnol. 2014, 24, 70–79, doi:10.4014/jmb.1310.10032.

66. Kind, S.; Jeong, W.K.; Schroder, H.; Wittmann, C. Systems-wide metabolic pathway engineering in Corynebacterium glutamicum for bio-based production of diaminopentane. Metab. Eng. 2010, doi:10.1016/j.ymben.2010.03.005.

67. Bakkes, P.J.; Ramp, P.; Bida, A.; Dohmen-Olma, D.; Bott, M.; Freudl, R. Improved pEKE2-derived expression vectors for tightly controlled production of recombinant proteins in Corynebacterium glutamicum. Plasmid 2020, 112, 102540, doi:10.1016/j.plasmid.2020.102540.

68. Mindt, M.; Risse, J.M.; Gruß, H.; Sewald, N.; Eikmanns, B.J.; Wendisch, V.F. One-step process for production of N-methylated amino acids from sugars and methylamine using recombinant Corynebacterium glutamicum as biocatalyst. Sci. Rep. 2018, 8, 12895, doi:10.1038/s41598-018-31309-5.