Fermentative N-Methylanthranilate Production by Engineered Corynebacterium glutamicum

Tatjana Walter 1, Nour Al Medani 1, Arthur Burgardt 1, Katarina Cankar 2, Lenny Ferrer 1, Anastasia Kerbs 1, Jin-Ho Lee 3, Melanie Mindt 1,2, Joe Max Risse 4 and Volker F. Wendisch 1,*

1 Genetics of Prokaryotes, Faculty of Biology and CeBiTec, Bielefeld University, 33615 Bielefeld, Germany; t.walter@uni-bielefeld.de (T.W.); mohamad.al@uni-bielefeld.de (N.A.M.); arthur.burgardt@uni-bielefeld.de (A.B.); lferrer@cebitec.uni-bielefeld.de (L.F.); anastasia.kerbs@uni-bielefeld.de (A.K.); melanie.mindt@wur.nl (M.M.)

2 BU Bioscience, Wageningen University & Research, 6700AA Wageningen, The Netherlands; katarina.cankar@wur.nl

3 Major in Food Science & Biotechnology, School of Food Biotechnology & Nutrition, Kyungsung University, Busan 48434, Korea; jhlee83@ks.ac.kr

4 Fermentation Technology, Technical Faculty and CeBiTec, Bielefeld University, 33615 Bielefeld, Germany; jrisse@uni-bielefeld.de

* Correspondence: volker.wendisch@uni-bielefeld.de

Received: 22 May 2020; Accepted: 5 June 2020; Published: 8 June 2020

Abstract: The N-functionalized amino acid N-methylanthranilate is an important precursor for bioactive compounds such as anticancer acridone alkaloids, the antinociceptive alkaloid O-isopropyl N-methylanthranilate, the flavor compound O-methyl-N-methylanthranilate, and as a building block for peptide-based drugs. Current chemical and biocatalytic synthetic routes to N-alkylated amino acids are often unprofitable and restricted to low yields or high costs through cofactor regeneration systems. Amino acid fermentation processes using the Gram-positive bacterium Corynebacterium glutamicum are operated industrially at the million tons per annum scale. Fermentative processes using C. glutamicum for N-alkylated amino acids based on an imine reductase have been developed, while N-alkylation of the aromatic amino acid anthranilate with S-adenosyl methionine as methyl-donor has not been described for this bacterium. After metabolic engineering for enhanced supply of anthranilate by channeling carbon flux into the shikimate pathway, preventing by-product formation and enhancing sugar uptake, heterologous expression of the gene anmt encoding anthranilate N-methyltransferase from Ruta graveolens resulted in production of N-methylanthranilate (NMA), which accumulated in the culture medium. Increased SAM regeneration by coexpression of the homologous adenosylhomocysteinase gene sahH improved N-methylanthranilate production. In a test bioreactor culture, the metabolically engineered C. glutamicum C1* strain produced NMA to a final titer of 0.5 g·L⁻¹ with a volumetric productivity of 0.01 g·L⁻¹·h⁻¹ and a yield of 4.8 mg·g⁻¹ glucose.

Keywords: N-functionalized amines; N-methylanthranilate; Corynebacterium glutamicum; metabolic engineering; sustainable production of quinoline precursors; acridone; quinazoline alkaloid drugs

1. Introduction

N-Functionalization of natural products as well as fine and bulk chemicals includes N-hydroxylation, N-acetylation, N-phosphorylation, or N-alkylation. These amine and amino acid modifications are found in all domains of life, and they fulfill various physiological roles such as resistance of bacteria to the antibiotic rifampicin by its N-hydroxylation [1], biosynthesis of the hormone melatonin via N-acetylated serotonin in plants and mammals [2], or assimilation of methylamine as carbon and energy source in methylotrophic bacteria [3].
The biotechnological and chemical interest in N-functionalized amines, especially in N-alkylated amino acids, has increased recently because of their beneficial impact as building blocks when incorporated into peptide-based drugs. Better membrane permeability, increased stability against proteases, stabilization of discrete confirmations, prevention of peptide aggregation by reduced formation of hydrogen bonds, or increased receptor subtype selectivity were shown for peptide-based drugs as consequence of amino acid N-alkylation [4]. For example, N-methylation of the N-CoA peptide bonds of transition state mimetics developed to inhibit malarial protease, which is required for infecting erythrocytes, improved their lipophilicity and stability against proteolysis, thus enhancing activity against Plasmodium parasites [5]. Free N-alkylated amines such as the N-ethylated glutamine derivative L-theanine, which prominently occurs in green tea, or O-methyl-N-methylanthranilate of grapes are flavoring compounds with applications in the food, cosmetics, flavor, and fragrances industries.

Chemical synthesis of free N-alkylated amino acids is well studied, and various routes are known, such as by nucleophilic substitution of α-bromo acids, N-methylation of sulfonamides, carbamates or amides, reduction of Schiff bases generated with an amino acid and formaldehyde or other aldehydes, by direct alkylation of protected amino acids or by ring-opening of 5-oxazolidinones [6–9]. However, these processes are often limited by low product yields, over-methylation, toxic reagents, or their incomplete enantiopurity [10,11]. Recently, enzyme catalysis routes with N-methyltransferases, dehydrogenases, ketimine reductases, or imine reductases that depend on cofactor regeneration systems have been described [12]. Fermentation processes using simple mineral salts media have been developed for three different routes for de novo production of N-alkylated amino acids. Two metabolic engineering strategies for reductive alkylation of 2-oxo acids with monomethylamine that either make use of a C1-assimilation pathway present in methylotrophic bacteria [13] or of the imine reductase DpkA [14] have been established. S-Adenosyl-L-methionine (SAM)-dependent methylation of aromatic amino acids by N-methyltransferases has also been described [15].

N-methylanthranilate (NMA) is an intermediate of the acridone alkaloid biosynthesis in plants. The SAM-dependent transfer of a methyl group to anthranilate initiates the biosynthesis of NMA-dependent biosynthesis of N-methylated acridone alkaloids and avenacin in plants [16,17]. Until now only one N-methyltransferase enzyme ANMT was characterized from the common rue, Ruta graveolens L., which accumulates N-methylated acridones exclusively. This enzyme shows narrow specificity for anthranilate, not accepting methylated catechol, salicylate, caffeate, 3- and 4-hydroxybenzoate, and anthraniloyl-CoA as substrates [16]. The acridone alkaloids and avenacin pathways diverge after SAM-dependent N-methylation of acridone anthranilate with regard to activation for transfer to the respective alkaloid intermediate. An ATP-dependent transfer of CoA is postulated for the acridone alkaloid biosynthesis [18], while UDP glucose-dependent O-glycosylation was shown as second step of the avenacin biosynthesis [17]. Acridone alkaloids and avenacin are known as bioactive compounds with cytotoxic, anticancer, antimicrobial, or antiparasitic properties and are, therefore, used for pharmaceutical and therapeutic purposes. Several N-methylated acridones, namely citrusamine, evoxanthine, arborinine, or normelicopine, were identified in diverse plants [19]. Arborinine, as an example, was found in ethyl acetate extracts from Glycosmis parva, it showed anticancer activity against human cervical cancer cells since activation of caspase-dependent apoptosis without inducing the DNA damage response was observed [20]. N-methylanthranilate also serves as precursor for the flavoring agent O-methyl-N-methylanthranilate, which has an orange blossom and grape-like odor, the antinociceptive alkaloid O-isopropyl-N-methylanthranilate, or the anti-inflammatory active compound O-propyl-N-methylanthranilate [21–23].

Safe production of amino acids for the food and feed industry has been established at the annual million-ton scale for decades with Corynebacterium glutamicum as the dominant production host [24]. C. glutamicum grows on simple mineral salts media and can utilize various sugars [25,26], acids such as citrate [27], and alcohols such as ethanol [28]. A well-established toolbox enabled metabolic engineered-based approaches for production of diverse value-added compounds. Besides the production of proteinogenic amino acids, also a broad range of non-proteinogenic amino acid
products like γ-aminobutyrate [29], 5-aminovalerate [30,31], pipecolic acid [32,33], N-methylated amino acids like N-methylalanine (NMeAla) [34] and sarcosine [35], aromatic compounds like 4-hydroxybenzoate [36,37] or protocatechuic acid [38], and functionalized aromatics like 7-chloro- or 7-bromo-tryptophan [39,40] and O-methylanthranilate [41] have been demonstrated.

Here, we describe fermentative N-methylanthranilate production by metabolic engineering of genome-reduced chassis strain *C. glutamicum* C1*, a robust basic strain for synthetic biology and industrial biotechnology [42]. Fermentative NMA production from glucose involved SAM-dependent ANMT from *R. gravelolens* combined with metabolic engineering for efficient supply of the precursor anthranilate (Figure 1).

**Figure 1.** Schematic representation of N-methylanthranilate (NMA) biosynthesis (A) embedded into aromatic amino acid metabolism of engineered *C. glutamicum* (B). Continuous arrows indicate single reactions, dashed arrows indicate multiple reactions. Green arrows and gene names indicate genome-based overexpression, yellow arrows and gene names indicate vector-based expression, crossed arrows and red gene names indicate gene deletion. (A) N-methylation of anthranilate by N-methylanthranilate transferase (ANMT) from *R. gravelolens* under consumption of S-adenosylmethionine (SAM). The SAM regeneration cycle is depicted with overexpression of *sahH*,...
2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

All bacterial strains used are listed in Table 1. *Escherichia coli* DH5α [43] was used for plasmid construction. *C. glutamicum* C1* was used as host organism for shikimate, anthranilate, and NMA production. Pre-cultures of *E. coli* and *C. glutamicum* were performed in lysogeny broth (LB) and brain heart infusion (BHI) medium at 37 or 30 °C in baffled shake flasks on a rotary shaker (160 rpm or 120 rpm). Cultures were inoculated freshly from LB agar plates. When necessary, spectinomycin (100 µg·mL⁻¹) and kanamycin (25 µg·mL⁻¹) were added to the medium. For induction of gene expression from vectors pEKEx3 and pGold, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the medium. For the performance of growth or production experiments of *C. glutamicum*, pre-cultures were inoculated as described above. After cell harvesting (3200× g, 7 min), cells were washed with TN-buffer pH 6.3 (50 mM Tris-HCL, 50 mM NaCl) and inoculated to an optical density at 600 nm (OD₆₀₀) of 0.25 g cell dry weight per liter.

### Table 1. Bacterial strains used in this study.

| Strains                     | Description                                      | Source                        |
|-----------------------------|--------------------------------------------------|-------------------------------|
| *Cornebacterium glutamicum* |                                                   |                               |
| WT                          | *C. glutamicum* wild-type strain ATCC13032        | ATCC                          |
| C1*                         | Genome-reduced chassis strain derived from [42]   |                               |
| ARO01                       | ΔsdhC::P₆lac-aroG <sub>D146N</sub> mutant of C1*  | This work                    |
| ARO02                       | ΔldhA mutant of ARO01                            | This work                    |
| ARO03                       | ΔsugR mutant of ARO02                            | This work                    |
| ARO04                       | ΔaroR::P₆lac-aroF mutant of ARO03                 | This work                    |
| ARO05                       | ΔpsuABCD::P₆thi-psuC mutant of ARO04              | This work                    |
| ARO06                       | Δppc::P₆thi-aroB mutant of ARO05                  | This work                    |
| ARO07                       | ΔP₆thi-aroQ mutant of ARO06                       | This work                    |
| ARO08                       | ΔsugR::P₆lac-sugR mutant of ARO08                 | This work                    |
| NMA01                       | ARO09 carrying pEKEx3 and pGold                  | This work                    |
| NMA02                       | ARO09 carrying pEKEx3 and pGold-sahH              | This work                    |
| NMA03                       | ARO09 carrying pEKEx3-trpEFBB and pGold          | This work                    |
| NMA04                       | ARO09 carrying pEKEx3-trpEFBB and pGold-sahH     | This work                    |
| NMA05                       | ARO09 carrying pEKEx3-trpEFBB and pGold-sahH     | This work                    |
| *Escherichia coli*          |                                                   |                               |
| S17-1                       | recA pro hsdR RP4-2-Tc::Mu-Km::Tn7               | [45]                          |
| DH5α                        | F-thi-1 endA1 hsdrl7(r-, m-)-supE44 lacL169 (Φ80lacZ1M15) recA1 gyrA96 | [43]                          |
Evaluation of the effects of anthranilate and NMA on *C. glutamicum* growth was performed in the microboreactor system BioBector (m2p-labs; Aachen, Germany). Pre-cultures were grown in BHI-rich medium overnight and transferred to second pre-culture of CGXII minimal medium with 40 g·L\(^{-1}\) glucose until the early exponential phase before inoculating to the main medium of CGXII minimal medium and 40 g·L\(^{-1}\) glucose with addition of varying anthranilate (solved in water) and NMA (solved in methanol) concentrations. Each condition with NMA contained 1.65 M methanol. Growth experiments in the BioBector were carried out using 48-well flower plates (MTP-48-B; m2p-labs) with a filling volume of 1 mL, at 30 °C, and 1200 rpm shaking frequency. Humidity was kept constant at 85%, and online biomass measurements of scattered light were monitored with backscatter gain of 20.

### 2.2. Fed-Batch Cultivation

Fed-Batch fermentation of *C. glutamicum* NMA105 was performed in an initial volume of 2 L in a bioreactor (3.7 L KLF, Bioengineering AG, 8636 Wald, Switzerland) at 30 °C, 0.2 bar overpressure, and an aeration rate of 2 NL·min\(^{-1}\). We did not perform off-gas analysis. To maintain relative dissolved oxygen saturation at 30%, stirrer speed was controlled during growth. The pH was maintained at pH 7.0 due to controlled addition of KOH (4 M) and phosphoric acid (10% (w/w)). To avoid foaming, the antifoam Sruktol® J647 was added manually when necessary. Feeding with 400 g·L\(^{-1}\) glucose and 150 g·L\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\) (total volume: 500 mL) was activated when the relative dissolved oxygen saturation (rDOS) signal rose above 60% and stopped when rDOS fell below 60%. Samples were taken automatically every 4 h during the whole cultivation and cooled down to 4 °C until further use. *C. glutamicum* NMA105 cells were transferred from a first pre-culture grown in LB in shake flasks to a second pre-culture in standard CGXII (pH 7.0) medium with 40 g·L\(^{-1}\) glucose (without IPTG) and the required antibiotics. For the bioreactor culture, standard CGXII medium without addition of 3-(N-morpholino)propanesulfonic acid (MOPS) and antibiotics was used. The fermenter was inoculated with the second pre-culture to an OD of 1.5 and immediately induced with 1 mM of IPTG.

### 2.3. Molecular Genetic Techniques and Strain Construction

Standard molecular genetic techniques were performed as described [46]. Competent *E. coli* DH5α [43] was performed with the RbCl method and transformed by heat shock [46]. Transformation of *C. glutamicum* was performed by electroporation [44]. The gene *trpEFBR* was amplified using specific primers (Table 2) with ALLin™ HiFi DNA Polymerase (highQu GmbH, Kraichtal, Germany). The PCR products were assembled with *Bam*HI restricted pEKEx3 via Gibson Assembly [44].

For heterologous expression of the *N*-methylanthranilate transferase gene, firstly, the pEC-XK99E vector was modified to be suitable for Golden Gate based modular assembly of multiple genes simultaneously. To this end, the three *Bsa*I sites present in the vector located in the *rrnB* terminator, the vector backbone, and the *repA* ORF were removed. Next, a linker containing two *Bsa*I sites (CAGATGAGACCGCATGCTGCAAGGTCTCAGTAT) was added to the MCS between *EcoRI* and *SacI* restriction sites. The resulting vector was named pGold (GenBank: MT521917). The coding sequence (CDS) of the plant gene *anmt* (GenBank: DQ884932.1) encoding the *N*-methylanthranilate transferase of *Ruta graveolens* was codon-harmonized to the natural codon frequency of *C. glutamicum* ATCC13032 with the codon usage table of kazusa database [47] and synthesized with Golden Gate assembly compatible flanking regions including recognition site for the restriction enzyme type 2 *Bsa*I and pGold complementary sequences and an optimized RBS [48,49] (Supplementary Data Table S1). The gene *anmt* was amplified using specific primers (Table 2) with ALLin™ HiFi DNA Polymerase according to the manufacturer (highQu GmbH, Kraichtal, Germany). The PCR products were assembled with digested pGold-*anmt* with *Bam*HI via Gibson Assembly [44].
Table 2. Oligonucleotides used in this study.

| Name          | Oligonucleotide Sequence (5' to 3')                                      |
|---------------|-----------------------------------------------------------------------|
| vdh-conf-fw   | GACCTCTAGGCGACGCAGTG                                                  |
| vdh-conf-rv   | CTGTTCACGGCTATTAGGC                                                   |
| ldha-conf-fw  | TGAAGCACCACGATTTCCAGGT                                               |
| ldha-conf-rv  | CCATGTACCAGATGGATGTA                                                  |
| sugR-conf-fw  | CGAgATGCCTGCTTTGTTTTGAG                                               |
| sugR-conf-rv  | GTTATCGCTGGTGGGAAGGT                                                  |
| US-aroR-fw    | CCTGCAAGTCGACCTACTGAGAGGCAAAACTTGCAAGTTAG                              |
| US-aroR-rv    | CGGACGTCTCGCTTGGAGTTGAAATTTAACACAGCTTTC                               |
| PlvC-aroR-fw  | GAAAGTGTTTAAAGGTTGAGATGGAGGAGAAATCTCGACTCTTG                         |
| PlvC-aroR-rv  | GAAAGGACCTCTTTTCTATGTCGGTTATGCGTATGCTGATGAGGAGAAATCTCGACTCTTG         |
| DS-aroR-fw    | ATACCAATCAATACCTAAGACGTAAGGAGATTTTTATCAGAGCTTATTTTTAAGCTTTTT          |
| DS-aroR-rv    | GAATTCGCTGCAACGCTCCAGGACACACTTTACTTTAGTTTTTTATCAGAGCTTATTTTT          |
| arnR-conf-fw  | GGAAGGCTCGTGGACACTCGTACTAGGTGGGATATAGCCTTACATGCTTGTGAGGAGAGAGATTTTT |
| arnR-conf-rv  | TTCTGCTGCATGTTAGGCTATATGACTTGAGGCTATATGACTTGAGGCTATATGACTTGAGGAGAG |
| US-qsuA-fw    | CCTGCAAGTCGACCTACTGAGAGGCAAAACTTGCAAGTTAG                              |
| US-qsuA-rv    | CGGACGTCTCGCTTGGAGTTGAAATTTAACACAGCTTTC                               |
| Ptuq-qsu-fw   | GAAAGAGTTTGATCAGGCTATAGGTGGGAGTGCTATGCTGTTGAGGCTATAGGTGGGAGTGCTATG |
| Ptuq-qsu-rv   | CGTCTGCTGCATGTTAGGCTATATGACTTGAGGCTATATGACTTGAGGCTATATGACTTGAGGAG |
| US-ppc-fw     | GCTGCAAGTCGACCTACTGAGAGGCAAAACTTGCAAGTTAG                              |
| US-ppc-rv     | GAATTCGCTGCAACGCTCCAGGACACACTTTACTTTAGTTTTTTATCAGAGCTTATTTTT          |
| Ptuq-ppc-fw   | GAAAGGACCTCTTTTCTATGTCGGTTATGCGTATGCTGATGAGGAGAAATCTCGACTCTTG         |
| Ptuq-ppc-rv   | GAAAGGACCTCTTTTCTATGTCGGTTATGCGTATGCTGATGAGGAGAAATCTCGACTCTTG         |
| US-tkt-fw     | CCTGCAAGTCGACCTACTGAGAGGCAAAACTTGCAAGTTAG                              |
| US-tkt-rv     | CGGACGTCTCGCTTGGAGTTGAAATTTAACACAGCTTTC                               |
| Ptuq-tkt-fw   | GAAAGGACCTCTTTTCTATGTCGGTTATGCGTATGCTGATGAGGAGAAATCTCGACTCTTG         |
| Ptuq-tkt-rv   | GAAAGGACCTCTTTTCTATGTCGGTTATGCGTATGCTGATGAGGAGAAATCTCGACTCTTG         |
| US-iolR-fw    | CCTGCAAGTCGACCTACTGAGAGGCAAAACTTGCAAGTTAG                              |
| US-iolR-rv    | CGGACGTCTCGCTTGGAGTTGAAATTTAACACAGCTTTC                               |
| DS-iolR-fw    | CAGGAGGACATACAAAGAAGGGACCTCTTTCTACTGAGGACACACTTTACTTTAGTTTTTTATCAGAG |
| DS-iolR-rv    | CGGACGTCTCGCTTGGAGTTGAAATTTAACACAGCTTTC                               |
| US-aroE-fw    | CCTGCAAGTCGACCTACTGAGAGGCAAAACTTGCAAGTTAG                              |
| US-aroE-rv    | CGGACGTCTCGCTTGGAGTTGAAATTTAACACAGCTTTC                               |
| Ptuq-aroE-fw  | GAAAGGACCTCTTTTCTATGTCGGTTATGCGTATGCTGATGAGGAGAAATCTCGACTCTTG         |
| Ptuq-aroE-rv  | GAAAGGACCTCTTTTCTATGTCGGTTATGCGTATGCTGATGAGGAGAAATCTCGACTCTTG         |
| US-sugR-fw    | CCTGCAAGTCGACCTACTGAGAGGCAAAACTTGCAAGTTAG                              |
| US-sugR-rv    | CGGACGTCTCGCTTGGAGTTGAAATTTAACACAGCTTTC                               |
| Ptuq-sugR-fw  | GAAAGGACCTCTTTTCTATGTCGGTTATGCGTATGCTGATGAGGAGAAATCTCGACTCTTG         |
| Ptuq-sugR-rv  | GAAAGGACCTCTTTTCTATGTCGGTTATGCGTATGCTGATGAGGAGAAATCTCGACTCTTG         |
| US-y3trp-fw   | CCTGCAAGTCGACCTACTGAGAGGCAAAACTTGCAAGTTAG                              |
| US-y3trp-rv   | CGGACGTCTCGCTTGGAGTTGAAATTTAACACAGCTTTC                               |
| DS-y3trp-fw   | CCTGCAAGTCGACCTACTGAGAGGCAAAACTTGCAAGTTAG                              |
| DS-y3trp-rv   | CGGACGTCTCGCTTGGAGTTGAAATTTAACACAGCTTTC                               |
| US-ppc-fw     | GCTGCAAGTCGACCTACTGAGAGGCAAAACTTGCAAGTTAG                              |
| US-ppc-rv     | GAATTCGCTGCAACGCTCCAGGACACACTTTACTTTAGTTTTTTATCAGAGCTTATTTTT          |
| Ptuq-ppc-fw   | GAAAGGACCTCTTTTCTATGTCGGTTATGCGTATGCTGATGAGGAGAAATCTCGACTCTTG         |
| Ptuq-ppc-rv   | GAAAGGACCTCTTTTCTATGTCGGTTATGCGTATGCTGATGAGGAGAAATCTCGACTCTTG         |
| US-tkt-fw     | CCTGCAAGTCGACCTACTGAGAGGCAAAACTTGCAAGTTAG                              |
| US-tkt-rv     | CGGACGTCTCGCTTGGAGTTGAAATTTAACACAGCTTTC                               |
| Ptuq-tkt-fw   | GAAAGGACCTCTTTTCTATGTCGGTTATGCGTATGCTGATGAGGAGAAATCTCGACTCTTG         |
| Ptuq-tkt-rv   | GAAAGGACCTCTTTTCTATGTCGGTTATGCGTATGCTGATGAGGAGAAATCTCGACTCTTG         |
| US-iolR-fw    | CCTGCAAGTCGACCTACTGAGAGGCAAAACTTGCAAGTTAG                              |
| US-iolR-rv    | CGGACGTCTCGCTTGGAGTTGAAATTTAACACAGCTTTC                               |
| DS-iolR-fw    | CAGGAGGACATACAAAGAAGGGACCTCTTTCTACTGAGGACACACTTTACTTTAGTTTTTTATCAGAG |
| DS-iolR-rv    | CGGACGTCTCGCTTGGAGTTGAAATTTAACACAGCTTTC                               |
| US-sugRfw     | GACATAGAGGACACCAAGGCG                                                  |
| US-sugR-rv    | GACATAGAGGACACCAAGGCG                                                  |
| pGANMT-sahH-fw| ATGCACTGGTCAGGTCTGCAAGATGGAGGAGACGATATGCAAGATGGAGGAGTCTCAATCACGTCAC   |
| pGANMT-sahH-rv| ATGCACTGGTCAGGTCTGCAAGATGGAGGAGACGATATGCAAGATGGAGGAGTCTCAATCACGTCAC   |

Ribosomal binding sites are in bold, and binding regions of Gibson oligonucleotides are underlined.

Chromosomal gene deletions and replacements in C1*-derived strains were performed by two-step homologous recombination [44] using the suicide vector pK19mobsacB [50]. The genomic regions flanking the respective gene for homologous recombination were amplified from C. glutamicum WT as described elsewhere [51] using the respective Primer pairs containing artificial RBS ([48,49], Table 2).
The purified PCR products were assembled and simultaneously cloned into restricted pK19mobsacB by Gibson Assembly resulting in the plasmids listed in Table 3. Transfer of the suicide vectors was carried out by trans-conjugation using *E. coli* S17 as donor strain [33]. For the first recombination event, integration of the vector in one of the targeted flanking regions was selected via kanamycin resistance. The resulting clones showed sucrose sensitivity due to the levansucrase gene sacB. Suicide vector excision was selected by sucrose resistance. Gene deletions or replacements were verified by PCR and sequencing with respective primers (Table 2).

### Table 3. List of plasmids used in this study.

| Plasmids          | Description                                                                                     | Source |
|-------------------|-------------------------------------------------------------------------------------------------|--------|
| pK19mobsacB       | Km<sup>R</sup>, *E. coli*/*C. glutamicum* shuttle vector for construction of insertion and deletion mutants in *C. glutamicum* (pK19 oriV<sub>Ec</sub> sacB lacZ<sub>A</sub>) | [50]   |
| pK19-Δdhc::P<sub>Ec</sub>-aroG<sup>D146N</sup> | pK19mobsacB with a construct for replacement of *vdh* (cg2953) by *aroG<sup>D146N</sup>* from *E. coli* under control of *C. glutamicum* promoter P<sub>Ec</sub> | [36]   |
| pK19-ΔdhA         | pK19mobsacB with a construct for deletion of *ldh* (cg3219)                                    | [52]   |
| pK19-ΔsugR        | pK19mobsacB with a construct for deletion of *sugR* (cg2115)                                   | [53]   |
| pK19-∆aroR::P<sub>Ec</sub> | pK19mobsacB with a construct for replacement of *aroR* and the native promoter of *aroF* by *C. glutamicum* promoter P<sub>Ec</sub> and an artificial RBS | This work |
| pK19-ΔqsuABCD::P<sub>Ec</sub>-qsuC                | pK19mobsacB with a construct for replacement of *qsuABCD* (cg0501-cg0504) by *qsuC* (cg0503) with an artificial RBS under control of *C. glutamicum* promoter P<sub>Ec</sub> | This work |
| pK19-Δppc::P<sub>Ec</sub>-aroB                   | pK19mobsacB with a construct for replacement of *ppc* (cg1787) by *aroB* (cg1827) with an artificial RBS under control of *C. glutamicum* promoter P<sub>Ec</sub> | This work |
| pK19-ΔP<sub>Ec</sub>l::P<sub>Ec</sub>         | pK19mobsacB with a construct for replacement of the *tkt* (cg1774) promoter by *C. glutamicum* promoter P<sub>Ec</sub> and artificial RBS | This work |
| pK19-ΔiolR::P<sub>Ec</sub>-aroE                   | pK19mobsacB with a construct for replacement of *iolR* (cg0196) by *aroE* (cg1835) with an artificial RBS under control of *C. glutamicum* promoter P<sub>Ec</sub> | This work |
| pK19-AsugR::sugR         | pK19mobsacB with a construct for reintegration of *sugR* (cg2115) into its native locus          | This work |
| pEKEx3             | Spec<sup>R</sup>, P<sub>Ec</sub>lac<sup>P</sup>, pBL1 oriV<sub>Ec</sub>, *C. glutamicum*/*E. coli* expression shuttle vector | [54]   |
| pEKEx3-3-trpEF<sub>Bi</sub>     | Spec<sup>R</sup>, pEKEx3 overexpressing *trpES<sup>Z</sup>F<sup>R</sup>* from *E. coli* K12 containing an artificial RBS | This work |
| pEC-XK99E          | Km<sup>R</sup>, P<sub>Ec</sub>lac<sup>P</sup>, pGAI oriV<sub>Ec</sub>, *C. glutamicum*/*E. coli* expression shuttle vector | [55]   |
| pGold              | Km<sup>R</sup>, P<sub>Ec</sub>lac<sup>P</sup>, pGAI oriV<sub>Ec</sub>, *C. glutamicum*/*E. coli* expression shuttle vector with *8sd* recognition site for Golden Gate assembly | This work |
| pGold-anmt         | Km<sup>R</sup>, pGold overexpressing codon harmonized *anmt* from *Ruta graveolens* with an artificial RBS | This work |
| pGold-anmt-sahH    | Km<sup>R</sup>, pGold overexpressing a synthetic operon with codon harmonized *anmt* from *R. graveolens* with an artificial RBS and *sahH* from *C. glutamicum* with an artificial RBS | This work |

### 2.4. Quantification of Amino Acids and Organic Acids

Extracellular amino acids and carbohydrates were quantified by high-performance liquid chromatography (HPLC) (1200 series, Agilent Technologies Deutschland GmbH, Böblingen, Germany). The culture supernatants were collected at different time points and centrifuged (20,200× *g*) for HPLC analysis.

For the detection of α-ketoglutarate (*α*-KG), trehalose, and lactate, an amino exchange column (Aminex, 300 mm × 8 mm, 10 μm particle size, 25 Å pore diameter, CS Chromatographie Service GmbH, 52379 Langerwehe, Germany) was used. The measurements were performed under isocratic conditions for 17 min at 60 °C with 5 mM sulfuric acid and a flow rate of 0.8 mL·min<sup>−1</sup>. The detection
was carried out with a Diode Array Detector (DAD, 1200 series, Agilent Technologies, Santa Clara, CA 95051, USA) at 210 nm.

Separation of shikimate, anthranilate, and NMA was performed with a pre-column (LiChrospher 100 RP18 EC-5µ (40 × 4 mm), CS Chromatographie Service GmbH, Langerwehe, Germany) and a main column (LiChrospher 100 RP18 EC-5µ (125 × 4 mm), CS Chromatographie Service GmbH). A mobile phase of buffer A (0.1% trifluoroacetic acid dissolved in water) and buffer B (acetonitrile) was used with a flow rate of 1 mL·min⁻¹. The following gradient was applied: 0–1 min 10% B; 1–10 min a linear gradient of B from 10% to 70%; 10–12 min 70% B; 12–14 min a linear gradient of B from 70% to 10%; 14–18 min 10% B [41]. The injection volume was 20 µL, and detection was performed with DAD at 210, 280, and 330 nm.

3. Results

3.1. Corynebacterium glutamicum as Suitable Host for NMA Production

C. glutamicum is widely used in amino acid fermentation, which operates at a million tons per annum scale [56]; however, it has not been engineered so far for NMA production. As expected, inspection of the genome revealed that there was no gene(s) encoding for a native enzyme that may N-methylate anthranilate to yield NMA. To study the growth responses of C. glutamicum to anthranilate and NMA, the wild-type strain ATCC13032 (WT) was cultivated with addition of varying anthranilate and NMA concentrations to CGXII minimal medium and 40 g·L⁻¹ glucose. Neither anthranilate nor NMA were utilized or converted by C. glutamicum WT, since their concentrations in supernatants analyzed at the beginning and the end of cultivation were comparable. Maximal biomass concentrations (expressed as ΔOD₆₀₀) were hardly affected by addition of anthranilate or NMA. By extrapolation, the concentrations of anthranilate (about 36 mM) and NMA (about 34 mM), which reduced the specific growth rate in glucose minimal medium to half-maximal, were determined (Figure 2). Based on the observed tolerance, C. glutamicum is a suitable candidate for production of anthranilate and NMA.

![Figure 2](image-url)  
**Figure 2.** Effect of externally added NMA (bright blue) and anthranilate (dark blue) on biomass formation (columns) and specific growth rate (lines) of C. glutamicum strain ATCC13032. Each condition with NMA contained the same amount of methanol (1.65 M) in minimal media. Averages and standard deviation of triplicate cultivations are shown.
3.2. Construction of a C. glutamicum Platform Strain for Production of Anthranilate

Since anthranilate, an intermediate of the tryptophan branch in the shikimate pathway, is a direct precursor of NMA, C. glutamicum C1* was engineered for increased supply of shikimate pathway intermediates by eliminating bottlenecks and minimizing formation of by-products (Figure 1). Hence, in sequential steps, aerCD146 encoding feedback resistant 3-deoxy-α-arabino-heptulosonate-7-phosphate (DAHP) synthase from E. coli [57] was inserted into the locus of tdh coding for vanillin dehydrogenase, which oxidizes vanillin and other aromatic aldehydes such as protocatechic aldehyde [58]. Next, an in-frame deletion of ldhA to reduce l-lactate formation (AR002) and an sugR deletion to increase glycolytic gene expression and sugar uptake [59] were introduced to yield strain ARO03.

Upon transformation with pEKEx3 as an empty vector control and pEKEx3-trpE_FBR for expression of feedback-resistant anthranilate synthase from E. coli [60], strains were evaluated regarding their growth behavior, anthranilate production, and formation of by-products. After 48 h of shake flask cultivation, ARO03(pEKEx3) exhibited decreased biomass formation and increased trehalose and α-ketoglutarate accumulation as compared to ARO01(pEKEx3). Expression of trpE_FBR further decreased biomass formation (i.e., 16.4% less than in empty vector). Comparing strains C1* to ARO03 carrying pEKEx3-trpE_FBR revealed a stepwise increase both in anthranilate and in shikimate production (Figure 3). For example, ARO03 strain harboring pEKEx3-trpE_FBR produced 17.6 ± 1.0 mM anthranilate and 6.8 ± 0.8 mM shikimate as compared to C1*(pEKEx3-trpE_FBR) that accumulated only 9.0 ± 0.2 mM anthranilate and 1.7 ± 0.1 mM shikimate.

![Figure 3](image-url)

**Figure 3.** Production of shikimate (maroon bars) and anthranilate (light blue bars), maximal specific growth rate (gray diamonds) and biomass formation (yellow squares) by C. glutamicum strains C1* and ARO01 to ARO09 carrying either pEKEx3 (left panel) or pEKEx3-trpE_FBR (right panel) were grown in shake flasks in CGXII minimal medium with 40 g·L⁻¹ glucose for 48 h. Means and arithmetic errors of duplicate cultures are shown.

To further increase the carbon flux towards shikimate, several further metabolic engineering steps were undertaken. In ARO04, the gene aroR, which codes for a translational regulatory leader peptide and is located upstream of DHAP synthase gene aroF [61], was replaced by an ilvC promoter followed by an optimized RBS in order to relieve negative translational control of aroF by phenylalanine and tyrosine. As described previously [36], the qsuABCD operon was replaced by qsuC transcribed from the constitutive strong tuf promoter in strain ARO05. This blocked conversion of...
3-dehydroshikimate (3-DHS) to the unwanted by-product protocatechuate (PCA) on the one hand and increased the flux from 3-dehydroquinate (3-DHQ) to 3-DHS on the other hand. The replacement of *ppc* encoding phosphoenolpyruvate (PEP) carboxylase by a second copy of endogenous *aroB* encoding 3-DHQ synthase in ARO06 probably increased supply of PEP as precursor for the shikimate pathway, and overexpression of *aroB* increased conversion of DHAP to 3-DHQ. To increase supply of erythrose-4-phosphate (E4P) as second precursor of the shikimate pathway [62], the native promoter upstream of transketolase gene *tkt* was exchanged by the constitutive strong promoter P*_{tuf}* with an artificial RBS. Since *tkt* is co-transcribed with other genes of the pentose phosphate pathway as operon *tkt-tal-zwf-opcA-pgl*, this promoter exchange is expected to increase flux into the pentose phosphate pathway towards E4P in strain ARO07.

Upon transformation with pEKEx3-trpE*^{FBR}* and pEKEx3-trpE*^{FBR}*, ARO07 produced only slightly more anthranilate (18.2 ± 0.1 mM) than ARO03(pEKEx3-trpE*^{FBR})*, but less shikimate, trehalose, and α-ketoglutarate (Figure 4). Growth was comparably fast (µ of 0.14 ± 0.01 h^{-1} compared to 0.13 ± 0.01 h^{-1}), but a higher biomass was reached (OD_{600} of 24.4 ± 1.0 compared with 16.1 ± 0.1) (Figure 3).

In ARO08, shikimate dehydrogenase gene *aroE* was overexpressed from the strong constitutive promoter P*_{tuf}* and used to replace *iolR*. In the absence of IolR, the inositol catabolism operon (cg0197-cg0207), cg1268, and PEP carboxykinase gene *pck* are deregulated [63,64], and *iolT1*, which codes for a non-phosphoenolpyruvate dependent phosphotransferase transporter (non-PTS) inositol uptake system, is derepressed. Non-PTS uptake of glucose is known to improve availability of PEP. The final strain, ARO09, is a sugR-positive derivative of ARO08. ARO09(pEKEx3-trpE*^{FBR}*) grew faster than ARO07(pEKEx3-trpE*^{FBR}*) (Figure 3) and accumulated less trehalose as unwanted by-product. The maximum anthranilate titer of 22.0 ± 1.4 mM (equivalent to about 3.1 g·L^{-1} anthranilate) was achieved with ARO09(pEKEx3-trpE*^{FBR}*) after 48 h of shake flask cultivation. This titer was 2.5 times more than that obtained with C1*(pEKEx3-trpE*^{FBR})*. Taken together, an anthranilate producing *C. glutamicum* strain converting 12.7% of carbon from glucose (Figure 4) to about 3.1 g·L^{-1} of anthranilic acid, the direct precursor for NMA, was constructed.

3.3. Establishing Fermentative Production of NMA by *C. glutamicum*

NMA is synthesized from anthranilate in a single SAM-dependent methylation reaction at its amino group (Figure 1). Therefore, the anthranilate producing *C. glutamicum* strain ARO09(pEKEx3-trpE*^{FBR}*) was used for heterologous expression of the anthranilate N-methyltransferase gene *anmt* from *R. graveolens*. Transformation of ARO09(pEKEx3-trpE*^{FBR}*) with pGold-*anmt* yielded strain NMA104. To improve SAM regeneration, the endogenous S-adenosylhomocysteinease gene *sahH* was expressed as synthetic operon with *anmt* from plasmid pGold-*anmt-sahH* and used to transform ARO09(pEKEx3-trpE*^{FBR}*) yielding strain NMA105. As negative control, pGold was introduced into ARO09(pEKEx3-trpE*^{FBR}*) yielding strain NMA103 (Table 1). For comparison, the shikimate producing strain ARO09(pEKEx3) was transformed with pGold, pGold-*anmt*, and pGold- *anmt-sahH* yielding strains NMA100, NMA101, and NMA102, respectively (Table 1).

In order to test for NMA production, strains NMA100 to NMA105 were cultivated in CGXII minimal medium supplemented with 40 g·L^{-1} glucose as carbon source. HPLC analysis of supernatants after cultivation for 48 h revealed that NMA100 and NMA103 did not produce NMA, which was expected since they lacked *anmt* from *R. graveolens* (Figure 5). Expression of *anmt* alone or in combination with endogenous *sahH* resulted in production of about 0.5 mM NMA by strains NMA101 and NMA102, respectively. This indicated functional expression of *anmt* from *R. graveolens* in *C. glutamicum*.
Coexpression of trpE<sub>FBR</sub> to boost anthranilate production with Anmt alone (strain NMA104) resulted in production of 1.7 ± 0.1 mM (0.25 ± 0.02 g·L<sup>-1</sup>) NMA. The finding that the anthranilate concentration was reduced from 20.8 ± 0.0 mM as obtained with NMA103 to 17.3 ± 0.9 mM (NMA104) indicated that conversion of anthranilate to NMA was incomplete (at about 10 mol%). Upon coexpression of trpE<sub>FBR</sub> with both Anmt and sahH in strain NMA105, 15.8 ± 1.9 mM anthranilate remained as unconverted precursor (Figure 5), and a significantly increased NMA titer of 2.2 ± 0.2 mM was obtained. This maximal titer in shake flasks corresponds to 0.34 ± 0.02 g·L<sup>-1</sup>. Thus, metabolic engineering of C. glutamicum for NMA production was achieved.
3.4. Fed-Batch Production of NMA in Bioreactors

For industrial applications, a production in larger volumes is preferable, which runs under controlled conditions to obtain a constant production titer. The stability of the NMA production of the metabolically engineered strain NMA105 was investigated in a fed-batch cultivation. Starting with a working volume of 2 L CGXII minimal medium containing 40 g L⁻¹ glucose as carbon source, 160 mL feed (400 g L⁻¹ and 150 g L⁻¹ (NH₄)₂SO₄) was added in a controlled manner depending on the rDOS (see Section 2.2). In total, 104 g glucose was consumed during 48 h fed-batch cultivation with no residual substrate concentrations detectable in the cultivation broth. The strain showed slow growth to OD₆₀₀ 5 in the first 24 h. In the following phase, growth was faster (growth rate of 0.12 h⁻¹, which was comparable to the growth rate observed in shaking flasks), and a maximal optical density of 53 was reached (Figure 6). High concentrations of by-products accumulated, i.e., 1.4 g L⁻¹ of the intermediate shikimate and 2.6 g L⁻¹ of the direct precursor anthranilate (Figure 6). Compared to production in shaking flasks (Figure 5), a reduced product yield on glucose (4.8 mg g⁻¹ as compared to 8.4 mg g⁻¹ in shaking flask) and a comparable volumetric productivity were observed, but NMA accumulated to an about 1.5-fold higher titer (0.5 g L⁻¹ as compared to 0.34 g L⁻¹). Taken together, the fed-batch fermentation with the newly constructed C. glutamicum strain NMA105 showed stable production of NMA in bioreactors at the 2 L scale (Figure 6). A final titer of 0.5 g L⁻¹ with a volumetric productivity of 0.01 g L⁻¹ h⁻¹ and a yield of 4.8 mg g⁻¹ glucose was achieved.

**Figure 5.** Production of anthranilate (light blue) and NMA (dark blue) by C. glutamicum strains NMA100 to NMA105. Cultivation was performed in minimal medium supplemented with 40 g L⁻¹ glucose as carbon source. 1 mM IPTG was added for induction of gene expression. Means and standard deviations of triplicate cultures determined after 48 h cultivation are depicted. Significance has been determined for NMA concentrations based on a two-sided unpaired Student’s t-test (*: p < 0.05; ns: not significant).
or the anthraniloyl-CoA anthraniloyltransferase from \textit{C. glutamicum} anthranilate overproducer.

\textit{Anmt} from \textit{N} acridone alkaloids.

As synthetic consortium [67,68], or developed here may in the future be used in combination with this engineered 4-hydroxy-1-methyl-2(1H)-quinolone [66] were established in \textit{E. coli} expressing ANMT led to production of the flavoring compound O-methyl-N-methylanthranilate [15]. Hypothetically, ANMT could also be an interesting candidate to produce the pharmacologically interesting compounds O-propyl- or O-isopropyl-N-methylanthranilate [22,23]. In the biosynthesis of acridone alkaloids, e.g., in \textit{R. graveolens}, N-methylation of anthranilate catalyzed by ANMT is a key step preceding CoA activation and, thus, separating primary metabolism (here tryptophan synthesis) from secondary metabolism [16,19]. Recently, production of about 26 mg·L\(^{-1}\) 1,3-dihydroxy-10-methylacridone [66] and about 18 mg·L\(^{-1}\) 4-hydroxy-1-methyl-2(1H)-quinolone [66] were established in \textit{E. coli} coexpressing ANMT from \textit{R. graveolens}, anthranilate coenzyme A ligase from \textit{P. aeruginosa}, and acridone synthase of \textit{R. graveolens} or the anthraniloyl-CoA anthraniloyltransferase from \textit{P. aeruginosa}. In these biosynthesis pathways, one molecule of NMA is required per one molecule 1,3-dihydroxy-10-methylacridone or 4-hydroxy-1-methyl-2(1H)-quinolone [65,66]. The NMA-producing \textit{C. glutamicum} strain NMA105 developed here may in the future be used in combination with this engineered \textit{E. coli} strain, possibly as synthetic consortium [67,68], or \textit{C. glutamicum} NMA105 itself may be engineered for production of acridone alkaloids.

Biosynthesis of \textit{N}-alkylated amino acids can be catalyzed by other enzymes besides \textit{N}-methyltransferases. However, while reductive amination using free ammonia is known for many

---

**Figure 6.** NMA production by \textit{C. glutamicum} strains NMA105 in bioreactors operated in fed-batch mode. The cultivation (initial volume of 2 L) was performed in minimal medium supplemented with 40 g·L\(^{-1}\) glucose (dark grey line with squares). The feed (dark grey line) contained 400 g·L\(^{-1}\) glucose and 150 g·L\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\). 1 mM IPTG was added for induction of gene expression during inoculation. OD\(_{600}\) (yellow) and concentrations of anthranilate (light blue), shikimate (maroon), and NMA (dark blue) in the culture broth are depicted. One of two representative fermentations is shown.

4. Discussion

\textit{N}-methylanthranilate production was achieved by applying the plant enzyme \textit{N}-methylanthranilate transferase ANMT of \textit{R. graveolens} in a newly metabolically engineered \textit{C. glutamicum} anthranilate overproducer. \textit{N}-methylanthranilate is known as precursor for several industrially and medically relevant compounds. ANMT of \textit{R. graveolens} showed a narrow substrate specificity when various amino benzoic or benzoic acids or phenolic derivatives were tested as substrates [16]. However, feeding \textit{O}-methylanthranilate (OMA) to \textit{E. coli} expressing ANMT led to production of the flavoring compound \textit{O}-methyl-N-methylanthranilate [15]. Hypothetically, ANMT could also be an interesting candidate to produce the pharmacologically interesting compounds \textit{O}-propyl- or \textit{O}-isopropyl-N-methylanthranilate [22,23]. In the biosynthesis of acridone alkaloids, e.g., in \textit{R. graveolens}, \textit{N}-methylation of anthranilate catalyzed by ANMT is a key step preceding CoA activation and, thus, separating primary metabolism (here tryptophan synthesis) from secondary metabolism [16,19]. Recently, production of about 26 mg·L\(^{-1}\) 1,3-dihydroxy-10-methylacridone [65] and about 18 mg·L\(^{-1}\) 4-hydroxy-1-methyl-2(1H)-quinolone [66] were established in \textit{E. coli} coexpressing ANMT from \textit{R. graveolens}, anthranilate coenzyme A ligase from \textit{P. aeruginosa}, and acridone synthase of \textit{R. graveolens} or the anthraniloyl-CoA anthraniloyltransferase from \textit{P. aeruginosa}. In these biosynthesis pathways, one molecule of NMA is required per one molecule 1,3-dihydroxy-10-methylacridone or 4-hydroxy-1-methyl-2(1H)-quinolone [65,66]. The NMA-producing \textit{C. glutamicum} strain NMA105 developed here may in the future be used in combination with this engineered \textit{E. coli} strain, possibly as synthetic consortium [67,68], or \textit{C. glutamicum} NMA105 itself may be engineered for production of acridone alkaloids.
enzymes, only few enzyme classes accept alkyl amines for N-alkylation, e.g., opine dehydrogenases, N-methyl amino acid dehydrogenases, ketimine reductases, pyrroline-5-carboxylate reductases, or imine reductases [12]. These processes differ regarding the substrate spectra of the involved enzymes. For example, anthranilate N-methylation described here as well as N-methylglutamate production established in *Pseudomonas putida* using N-methylglutamate synthase and γ-glutamylmethy lamide synthetase of the methylamine assimilation pathway of *Methylobacterium extorquens* [13] have narrow substrate spectra (e.g., GMAS from *Methylovorus mays* also forms γ-glutamyllethylamine, also known as theanine [69]) compared with N-alkylation using the imine reductase DpkA of *Pseudomonas putida* [12]. Several methylated or ethylated amino acids could be produced by *C. glutamicum* using the wild-type or a mutant version of DpkA and either MMA or ethylamine as substrates [14,34,35]. With respect to aromatic amino acids, N-methyl-L-phenylalanine could be obtained from phenylpyruvate by enzyme catalysis using DpkA and MMA [12]; however, production of NMA via DpkA by N-alkylation of a carbonyl precursor of NMA has not been described.

The NMA process described here showed lower titers (0.5 g·L⁻¹) than the processes depending on reductive alkylation using MMA (about 32 g·L⁻¹ N-methylalanine [34] and about 9 g·L⁻¹ sarcosine [14]). This may be due to (a) higher activity of DpkA compared with ANMT, (b) better provision of the precursors pyruvate and glyoxalate than of anthranilate, and/or (c) the requirement of SAM for ANMT as compared to MMA for DpkA. Indeed, purified DpkA has a much higher activity (about 40 U·mg⁻¹) [70] than purified ANMT (about 0.04 U·mg⁻¹) [16]. Moreover, while ARO09(pEKE3-trpE<sub>FBB</sub>) produced 3 g·L⁻¹ anthranilate (Figure 3), the precursor strains used for production of N-methylalanine and sarcosine produced up to 45 g·L⁻¹ pyruvate [71] and about 5 g·L⁻¹ glycolate [72], respectively. Third, reductive methylation using DpkA requires addition of MMA as methyl donor to the medium. This is beneficial since MMA has a low price, is readily available, is tolerated well by *C. glutamicum* [34], and because stoichiometric excess of MMA can be used to drive reductive N-methylation by mass action law.

Compared to NMA production by an engineered *E. coli* strain expressing the N-methyltransferase of *R. graveolens* [15], the NMA production by engineered *C. glutamicum* using the same enzyme described here resulted in about 12 times higher titers in shaking flask cultivation (370 mg·L⁻¹ as compared to 29 mg·L⁻¹ sarcosine [14]). This may be due to the fact that, in this study, *C. glutamicum* was metabolically engineered for improved supply of the direct NMA precursor anthranilate as, e.g., strain ARO09(pEKE3-trpE<sub>FBB</sub>) produced about 3 g·L⁻¹ anthranilate. Moreover, while the *E. coli* relied on native SAM regeneration [15], in *C. glutamicum* the endogenous gene for SAM regeneration *sahH* was overexpressed to increase SAM regeneration, and NMA production was improved 1.36-fold (compare 0.34 ± 0.02 g·L⁻¹ for NMA105 with 0.25 ± 0.02 g·L⁻¹ for NMA104 in Figure 5). Two bottlenecks observed with the *C. glutamicum* strain engineered here may be overcome by future metabolic engineering: incomplete conversion of shikimate to anthranilate and incomplete N-methylation of anthranilate by SAM-dependent ANMT. To improve conversion of shikimate to anthranilate from about half to full conversion (compare about 1.4 g·L⁻¹ of shikimate and 2.6 g·L⁻¹ anthranilate produced by NMA105 in bioreactor cultivation; Figure 6), expression of the operon *aroCKB* encoding chorismate synthase, shikimate kinase, and 3-dehydroquinate synthase may be boosted, e.g., by changing the endogenous promoter for the strong promoter *P<sub>tuf</sub>* and using shikimate kinase from *Methanocaldococcus jannaschii* as shown previously [36]. In addition, various studies have shown that deletion of the chorismate mutase will increase the carbon flux towards tryptophan biosynthesis [36,40,73].

SAM-dependent N-methylation of anthranilate by ANMT from *R. graveolens* represents the second bottleneck. ANMT from *R. graveolens* shows high affinity for its substrates (K<sub>M</sub> of 7.1 µM for anthranilate and K<sub>S</sub> of 3.3 µM for SAM), and inhibition by its product NMA has not been described [16]. On the other hand, the inherently low activity of ANMT as compared, e.g., to DpkA (see above) may limit conversion of anthranilate to NMA. Importantly, regeneration of the methyl donor SAM (Figure 1A) is critical in all SAM-dependent methylation reactions. This is even more important for ANMT from *R. graveolens* because it is inhibited by SAH with a K<sub>I</sub> value of 37.2 µM [74]. As shown
overexpression of one gene of the SAM regeneration system (Figure 1A), S-adenosylhomocysteine (SAH) hydrolase gene sahH, partly overcame SAM limitation since conversion of anthranilate to NMA was improved 1.36-fold (Figure 5). This may be due to reduced inhibition of ANMT from R. graveolens by SAH (see above) and/or better SAM regeneration. Irrespective of sahH overexpression, not more than about 14 mol% of anthranilate was N-methylated to NMA (Figure 5). As shown for OMA production [41], overexpression of SAM synthetase gene metK in addition to sahH improved SAM regeneration, whereas deletion of cystathionin-γ-synthese gene metB and of mcbR and cg3031 that code for transcriptional regulators involved in regulation of methionine biosynthesis were not beneficial. Addition of methionine even reduced the production [41]. These changes and abolishing pathways competing for SAM and its precursor by deletion of homoserine kinase gene thrB along with overexpression of metK and vgb, coding for methionine adenosyltransferase and Vitreoscilla hemoglobin, led to a C. glutamicum strain secreting about 0.2 g L⁻¹ SAM within 48 h [75]. In addition to improving SAM regeneration (as shown here by sahH overexpression), it may be beneficial for NMA production to increase SAM biosynthesis and, therefore, the intracellular concentration of SAM. Thus, possibly, NMA production may be improved by overexpression of SAM biosynthesis genes such as metK, or by de-repression of SAM biosynthesis, e.g., via deletion of mcbR, or by deletion of genes for enzymes competing with use of SAM or of SAM biosynthetic precursors such as thrB.

NMA may inhibit anthranilate biosynthesis since NMA was not produced in addition to anthranilate, while the combined titer of NMA and anthranilate remained similar when comparing strains NMA103, NMA104, and NMA105 (Figure 5). Enzymes that are inhibited by NMA have not been described to date. However, product inhibition of anthranilate synthase by anthranilate is known, e.g., in Streptomyces [76], which belongs to the actinobacteria as C. glutamicum, and in Salmonella typhimurium with a Kᵢ of 0.06 mM anthranilate [77]. Here, we used the E. coli enzyme TrpE, which is known to be inhibited by tryptophan, which binds at a site distant from the active center (allosteric regulation) [78]. In the mutant TrpE[S40F], Trp binding is lost as well as allosteric inhibition by Trp [78]. Product inhibition by anthranilate is expected to involve binding to the active center. Since NMA differs from anthranilate just by the N-methyl group, it is conceivable that NMA inhibits in a similar way as anthranilate. This may explain that upon NMA production the anthranilate titer decreased (Figure 5).

NMA also affected growth of C. glutamicum (34 mM or 5 g L⁻¹ reduced the growth rate to half-maximal; Figure 2), but to a lesser extent than OMA, for which a complete growth inhibition was observed at 2 g L⁻¹ OMA [41]. Inhibition of growth by OMA was overcome by application of a tributyrin-based extraction method [41]. This approach likely cannot be transferred directly to the NMA process since OMA contains a methylated carboxy group, whereas the amino group is methylated in NMA. Adaptive laboratory evolution (ALE) is an efficient method to select more tolerant strains and has been applied to C. glutamicum to select strains with improved tolerance to methanol [79–81] or lignocellulose-derived inhibitors [82]. Taken together, this study characterized NMA production by metabolically engineered C. glutamicum, and a first bioreactor process leading to a final titer of 0.5 g L⁻¹ NMA with a volumetric productivity of 0.01 g L⁻¹ h⁻¹ and a yield of 4.8 mg g⁻¹ glucose was achieved. This strain provides the basis to develop an industrially competitive NMA process and shows potential to enable access to a fermentative route to pharmaceutically relevant secondary metabolites such as the acridone alkaloids.

**Supplementary Materials:** The following figures are available online at http://www.mdpi.com/2076-2607/8/6/866/s1, Figure S1: Carbon flux analysis of anthranilate producing C. glutamicum ARO strains, Figure S2: Carbon flux analysis of C. glutamicum ARO strains harboring the plasmid pEKEx3, Table S1: Codon harmonized nucleotide sequence (5’-3’) of the plant gene ANMT.

**Author Contributions:** T.W., N.A.M., A.B., K.C., L.F., A.K., and M.M. constructed strains. T.W., N.A.M., A.B., K.C., L.F., A.K., and M.M. performed the experiments. T.W., A.B., K.C., L.F., A.K., M.M., J.M.R., and V.F.W. analyzed the data. J.-H.L., K.C., and V.F.W. provided resources. T.W., A.B., K.C., L.F., A.K., M.M., and J.M.R. drafted the manuscript. T.W. and V.F.W. reviewed and edited the manuscript. V.F.W. finalized the manuscript. All authors agreed to the final version of the manuscript.
Funding: Funding by ERACoBiotech via grant INDI E (BMEL 22023517) is gratefully acknowledged. Support for the Article Processing Charge by the Deutsche Forschungsgemeinschaft and the Open Access Publication Fund of Bielefeld University is acknowledged.

Acknowledgments: We want to thank Anne-Laure Ricord for cloning expression plasmids and Thomas Schäffer for his support during fed-batch bioreactor cultivation.

Conflicts of Interest: The authors declare no conflicts 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. Liu, L.-K.; Abdelwahab, H.; del Campo, J.S.M.; Mehra-Chaudhary, R.; Sobrado, P.; Tanner, J.J. The Structure of the Antibiotic Deactivating, N-hydroxylating Rifampicin Monoxygenase. *J. Biol. Chem.* 2016, 291, 21553-21562. [CrossRef] [PubMed]

2. Zhao, D.; Yu, Y.; Shen, Y.; Liu, Q.; Zhao, Z.; Sharma, R.; Reiter, R.J. Melatonin Synthesis and Function: Evolutionary History in Animals and Plants. *Front. Endocrinol.* 2019, 10, 249. [CrossRef] [PubMed]

3. Chen, Y.; Scanlan, J.; Song, L.; Crombie, A.T.; Rahman, T.; Schäfer, H.; Murrell, J.C. γ-Glutamylmethylamide Is an Essential Intermediate in the Metabolism of Methylamine by *Methylocella silvestris*. *Appl. Environ. Microbiol.* 2010, 76, 4530-4537. [CrossRef] [PubMed]

4. Chatterjee, J.; Rechenmacher, F.; Kessler, H. N-Methylation of Peptides and Proteins: An Important Element for Modulating Biological Functions. *Angew. Chem. Int. Ed.* 2012, 52, 254-269. [CrossRef] [PubMed]

5. Gazdik, M.; O’Neill, M.; Lopaticki, S.; Lowes, K.N.; Smith, B.J.; Cowman, A.F.; Boddey, J.A.; Gasser, R. The effect of N-methylation on transition state mimic inhibitors of the *Plasmodium* protease, plasmspepsin V. *MedChemComm* 2015, 6, 437-443. [CrossRef] [PubMed]

6. Aurelio, L.; Brownlee, R.T.C.; Hughes, A.B. Synthetic Preparation of N-Methyl-α-amino Acids. *Chem. Rev.* 2004, 104, 5823-5846. [CrossRef]

7. de Marco, R.; Leggio, A.; Liguori, A.; Marino, T.; Perri, F.; Russo, N. Site-Selective Methylation of N-Nosyl Hydrazone of N-Nosyl Protected α-Amino Acids. *J. Org. Chem.* 2010, 75, 3381-3386. [CrossRef]

8. Belcastro, E.; di Gioia, M.L.; Greco, A.; Leggio, A.; Liguori, A.; Perri, F.; Siciliano, C.; Viscomi, M.C. N-Methyl-N-nosyl-β-amino Acids. *J. Org. Chem.* 2007, 72, 4798-4802. [CrossRef]

9. Freidinger, R.M.; Hinkle, J.S.; Perlow, D.S. Synthesis of 9-fluorenylmethyloxycarbonyl-protected N-alkyl amino acids by reduction of oxazolidinones. *J. Org. Chem.* 1983, 48, 77-81. [CrossRef]

10. di Gioia, M.L.; Leggio, A.; Malagrinò, F.; Romio, E.; Siciliano, C.; Liguori, A. N-Methylated α-Amino Acids and Peptides: Synthesis and Biological Activity. *Mini-Rev. Med. Chem.* 2016, 16, 1. [CrossRef]

11. Sharma, A.; Kumar, A.; Monaim, S.A.H.A.; Jad, Y.E.; El-Faham, A.; de la Torre, B.G.; Albericio, F. N-methylation in amino acids and peptides: Scope and limitations. *Biopolymers* 2018, 109, e23110. [CrossRef] [PubMed]

12. Hyslop, J.F.; Lovelock, S.L.; Watson, A.J.B.; Sutton, P.W.; Roiban, G.-D. N-Alkyl-α-amino acids in Nature and their biocatalytic preparation. *J. Biotechnol.* 2019, 293, 56-65. [CrossRef] [PubMed]

13. Mindt, M.; Walter, T.; Risse, J.M.; Wendisch, V. Fermentative Production of N-Methylglutamate From Glycerol by Recombinant *Pseudomonas putida*. *Front. Bioeng. Biotechnol.* 2018, 6, 159. [CrossRef] [PubMed]

14. Mindt, M.; Hannibal, S.; Heuser, M.; Risse, J.M.; Sasikumar, K.; Nampoothiri, K.M.; Wendisch, V. Fermentative Production of N-Alkylated Glycine Derivatives by Recombinant *Corynebacterium glutamicum* Using a Mutant of Imine Reductase DpkA From *Pseudomonas putida*. *Front. Bioeng. Biotechnol.* 2019, 7, 232. [CrossRef]

15. Lee, H.L.; Kim, S.-Y.; Kim, E.J.; Han, D.Y.; Kim, B.-G.; Ahn, J.-H. Synthesis of Methylated Anthranilate Derivatives Using Engineered Strains of *Escherichia coli*. *J. Microbiol. Biotechnol.* 2019, 29, 839-844. [CrossRef]

16. Rohde, B.; Hans, J.; Martens, S.; Baumert, A.; Hunziker, P.; Matern, U. Anthranilic N-methyltransferase, a branch-point enzyme of acridone biosynthesis. *Plant J.* 2007, 53, 541-553. [CrossRef]

17. Mugford, S.T.; Louveau, T.; Melton, R.; Qi, X.; Bakh, S.; Hill, L.; Tsurushima, T.; Honkanen, S.; Rossier, S.J.; Lomonossoff, G.P.; et al. Modularity of plant metabolic gene clusters: A trio of linked genes that are collectively required for acylation of triterpenes in oat. *Plant Cell* 2013, 25, 1078–1092. [CrossRef]

18. Baumert, A.; Schmidt, J.; Gröger, D. Synthesis and mass spectral analysis of coenzyme a thioester of anthranilic acid and its N-methyl derivative involved in acridone alkaloid biosynthesis. *Phytochem. Anal.* 1993, 4, 165-170. [CrossRef]
19. Michael, J.P. Acridone Alkaloids. In The Alkaloids: Chemistry and Biology; Elsevier BV: Amsterdam, The Netherlands, 2017; pp. 1–108.

20. Piboonprai, K.; Khumkhrong, P.; Khongkow, M.; Yata, T.; Ruangrungsri, N.; Chansriniyom, C.; Impridee, T. Anticancer activity of arborinine from Glycosmis parva leaf extract in human cervical cancer cells. Anticancer Research 2012, 32, 4057–4061. [CrossRef] [PubMed]

21. Arndt, A.; Auchter, M.; Ishige, T.; Wendisch, V.F.; Eikmanns, B.J. Ethanol catabolism in Corynebacterium glutamicum. Microorganisms 2018, 7, 282. [CrossRef]

22. Blombach, B.; Seibold, G. Carbohydrate metabolism in Corynebacterium glutamicum and applications for the metabolic engineering of l-lysine production strains. Appl. Microbiol. Biotechnol. 2010, 86, 1313–1322. [CrossRef] [PubMed]

23. Shin, J.; Park, S.H.; Oh, Y.H.; Choi, J.W.; Lee, M.H.; Cho, J.S.; Jeong, K.J.; Joo, J.C.; Yu, J.; Park, S.J.; et al. Metabolic engineering of Corynebacterium glutamicum for enhanced production of 5-aminovaleric acid. Microb. Cell Fact. 2016, 15, 174. [CrossRef]

24. Mindt, M.; Risse, J.M.; Gruß, H.; Sewald, N.; Eikmanns, B.J.; Wendisch, V. One-step process for production of 5-aminovalerate from glucose and alternative carbon sources. Biotechnol. J. 2018, 10, 8075–8090. [CrossRef] [PubMed]

25. Mindt, M.; Friehs, K.; Wendisch, V. A new metabolic route for the fermentative production of 5-aminovaleric acid from sugars and methylamine using recombinant Corynebacterium glutamicum as biocatalyst. Sci. Rep. 2018, 8, 12895. [CrossRef] [PubMed]

26. Kallscheuer, N.; Marienhagen, J. Corynebacterium glutamicum as platform to produce hydroxybenzoic acids. Microb. Cell Factories 2018, 17, 70. [CrossRef]

27. Michael, J.P. Acridone Alkaloids. In The Alkaloids: Chemistry and Biology; Elsevier BV: Amsterdam, The Netherlands, 2017; pp. 1–108.

28. Michael, J.P. Acridone Alkaloids. In The Alkaloids: Chemistry and Biology; Elsevier BV: Amsterdam, The Netherlands, 2017; pp. 1–108.

29. Jorge, J.M.; Pérez-García, F.; Risse, J.M.; Friehs, K.; Wendisch, V. Fermentative production of L-pipecolic acid from glucose and alternative carbon sources. Biotechnol. J. 2017, 12, 1600646. [CrossRef]

30. Pérez-García, F.; Peters-Wendisch, P.; Wendisch, V. Engineering Corynebacterium glutamicum for fast production of l-lysine and l-pipecolic acid. Appl. Microbiol. Biotechnol. 2016, 100, 8075–8090. [CrossRef] [PubMed]

31. Shin, J.; Park, S.H.; Oh, Y.H.; Choi, J.W.; Lee, M.H.; Cho, J.S.; Jeong, K.J.; Joo, J.C.; Yu, J.; Park, S.J.; et al. Metabolic engineering of Corynebacterium glutamicum for enhanced production of 5-aminovaleric acid. Microb. Cell Fact. 2016, 15, 174. [CrossRef]

32. Pérez-García, F.; Peters-Wendisch, P.; Wendisch, V. Engineering Corynebacterium glutamicum for fast production of l-lysine and l-pipecolic acid. Appl. Microbiol. Biotechnol. 2016, 100, 8075–8090. [CrossRef] [PubMed]

33. Purwanto, H.S.; Kang, M.-S.; Ferrer, L.; Han, S.-S.; Lee, J.-Y.; Kim, H.-S.; Lee, J.-H. Rational engineering of the shikimate and related pathways in Corynebacterium glutamicum for 4-hydroxybenzoate production. J. Biotechnol. 2018, 282, 92–100. [CrossRef]

34. Mindt, M.; Risse, J.M.; Gruß, H.; Sewald, N.; Eikmanns, B.J.; Wendisch, V. 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. [CrossRef] [PubMed]

35. Mindt, M.; Heuser, M.; Wendisch, V. xylose as preferred substrate for sarcosine production by recombinant Corynebacterium glutamicum. Bioresour. Technol. 2019, 281, 135–142. [CrossRef] [PubMed]

36. Purwanto, H.S.; Kang, M.-S.; Ferrer, L.; Han, S.-S.; Lee, J.-Y.; Kim, H.-S.; Lee, J.-H. Rational engineering of the shikimate and related pathways in Corynebacterium glutamicum for 4-hydroxybenzoate production. J. Biotechnol. 2018, 282, 92–100. [CrossRef]

37. Kallscheuer, N.; Marienhagen, J. Corynebacterium glutamicum as platform to produce hydroxybenzoic acids. Microb. Cell Factories 2018, 17, 70. [CrossRef]
40. Veldmann, K.H.; Minges, H.; Sewald, N.; Lee, J.-H.; Wendisch, V. Metabolic engineering of Corynebacterium glutamicum for the fermentative production of halogenated tryptophan. J. Biotechnol. 2019, 291, 7–16. [CrossRef]
41. Luo, Z.W.; Cho, J.S.; Lee, S.Y. Microbial production of methyl anthranilate, a grape flavor compound. Proc. Natl. Acad. Sci. USA 2019, 116, 10749–10756. [CrossRef]
42. Baumgart, M.; Unthan, S.; Kloß, R.; Radek, A.; Polen, T.; Tenhaf, N.; Müller, M.F.; Kübler, A.; Siebert, D.; Brühl, N.; et al. Corynebacterium glutamicum Chassis C1*: Building and Testing a Novel Platform Host for Synthetic Biology and Industrial Biotechnology. ACS Synth. Biol. 2017, 7, 132–144. [CrossRef]
43. Hanahan, D. Studies on transformation of Escherichia coli with plasmids. J. Mol. Biol. 1983, 166, 557–580. [CrossRef]
44. Eggeling, L.; Bott, M. Handbook of Corynebacterium glutamicum; CRC Press: Boca Raton, FL, USA, 2005; ISBN 978-1-4200-3969-6.
45. Simon, R.; Priefe, U.; Pühler, A. A Broad Host Range Mobilization System for In Vivo Genetic Engineering: Transposon Mutagenesis in Gram Negative Bacteria. Biotechnology 1983, 1, 784–791. [CrossRef]
46. Green, M.R.; Sambrook, J.; Sambrook, J. Molecular Cloning: A Laboratory Manual, 4th ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, USA, 2012; ISBN 978-1-936113-41-5.
47. Codon Usage Database. Available online: https://www.kazusa.or.jp/codon/ (accessed on 20 May 2020).
48. 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. [CrossRef] [PubMed]
49. De Novo DNA: The Future of Genetic Systems Design and Engineering. Available online: https://www.denovodna.com/software/design_rbs_calculator (accessed on 22 May 2020).
50. Schäfer, A.; Tauch, A.; Jäger, W.; Kalinowski, J.; Thierbach, G.; Pühler, 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. [CrossRef]
51. Heider, S.A.E.; Peters-Wendisch, P.; Netzer, R.; Stafnes, M.; Brautaset, T.; Wendisch, V. Production and glucosylation of C50 and C40 carotenoids by metabolically engineered Corynebacterium glutamicum. Appl. Microbiol. Biotechnol. 2013, 98, 1223–1235. [CrossRef] [PubMed]
52. Blomback, B.; Riester, T.; Wieschalka, S.; Ziert, C.; Youn, J.-W.; Wendisch, V.; Eikmanns, B.J. Corynebacterium glutamicum Tailored for Efficient Isobutanol Production. Appl. Environ. Microbiol. 2011, 77, 3300–3310. [CrossRef]
53. Engels, V.; Wendisch, V. The DeoR-Type Regulator SugR Represses Expression of ptsG in Corynebacterium glutamicum. J. Bacteriol. 2007, 189, 2955–2966. [CrossRef]
54. Stansen, C.; Uy, D.; Delaunay, S.; Eggeling, L.; Goergen, J.-L.; Wendisch, V. Characterization of a Corynebacterium glutamicum Lactate Utilization Operon Induced during Temperature-Triggered Glutamate Production. Appl. Environ. Microbiol. 2005, 71, 5920–5928. [CrossRef]
55. Kirchner, O.; Tauch, A. Tools for genetic engineering in the amino acid-producing bacterium Corynebacterium glutamicum. J. Biotechnol. 2003, 104, 287–299. [CrossRef]
56. Wendisch, V.; Lee, J.-H. Metabolic Engineering in Corynebacterium Glutamicum; Springer Science and Business Media LLC: Berlin/Heidelberg, Germany, 2020; pp. 287–322.
57. Kikuchi, Y.; Tsujimoto, K.; Kurahashi, O. Mutational analysis of the feedback sites of phenylalanine-sensitive 3-deoxy-n-arabino-heptulosonate-7-phosphate synthase of Escherichia coli. Appl. Environ. Microbiol. 1997, 63, 761–762. [CrossRef]
58. Lee, J.-H.; Wendisch, V. Production of amino acids—Genetic and metabolic engineering approaches. Bioreour. Technol. 2017, 245, 1575–1587. [CrossRef] [PubMed]
59. Hasegawa, S.; Tanaka, Y.; Suda, M.; Jojima, T.; Inui, M. Enhanced Glucose Consumption and Organic Acid Production by Engineered Corynebacterium glutamicum Based on Analysis of a pfkB1 Deletion Mutant. Appl. Environ. Microbiol. 2016, 83. [CrossRef] [PubMed]
60. Sander, T.; Farke, N.; Diehl, C.; Kuntz, M.; Glatter, T.; Link, H. Allosteric Feedback Inhibition Enables Robust Amino Acid Biosynthesis in E. coli by Enforcing Enzyme Overabundance. Cell Syst. 2019, 8, 66–75. [CrossRef]
61. Neshat, A.; Mentz, A.; Rückert, C.; Kalinowski, J. Transcriptome sequencing revealed the transcriptional organization at ribosome-mediated attenuation sites in Corynebacterium glutamicum and identified a novel attenuator involved in aromatic amino acid biosynthesis. J. Biotechnol. 2014, 190, 55–63. [CrossRef]
62. Draths, K.M.; Pompliano, D.L.; Conley, D.L.; Frost, J.W.; Berry, A.; Disbrow, G.L.; Staversky, R.J.; Lievense, J.C. Biocatalytic synthesis of aromatics from D-glucose: The role of transketolase. *J. Am. Chem. Soc.* **1992**, *114*, 3956–3962. [CrossRef]

63. Klaffl, S.; Brocker, M.; Kalinowski, J.; Eikmanns, B.J.; Bott, M. Complex Regulation of the Phosphoenolpyruvate Carboxykinase Gene pck via Characterization of Its GntR-Type Regulator IolR as a Repressor of myo-Inositol Utilization Genes in *Corynebacterium glutamicum*. *J. Bacteriol.* **2013**, *195*, 4283–4296. [CrossRef] [PubMed]

64. Hyeon, J.E.; Kang, D.H.; Kim, Y.I.; You, S.K.; Han, S.O. GntR-Type Transcriptional Regulator PckR Negatively Regulates the Expression of Phosphoenolpyruvate Carboxykinase in *Corynebacterium glutamicum*. *J. Bacteriol.* **2012**, *194*, 2181–2188. [CrossRef]

65. Choi, G.-S.; Choo, H.J.; Kim, B.-G.; Ahn, J.-H. Synthesis of acridone derivatives via heterologous expression of a plant type III polyketide synthase in *Escherichia coli*. *Microb. Cell Fact.* **2020**, *19*, 1–11. [CrossRef]

66. Choo, H.J.; Ahn, J.-H. Synthesis of Three Bioactive Aromatic Compounds by Introducing Polyketide Synthase Genes into Engineered *Escherichia coli*. *J. Agric. Food Chem.* **2019**, *67*, 8581–8589. [CrossRef]

67. Sgobba, E.; Stumpf, A.K.; Vortmann, M.; Jagmann, N.; Krehenbrink, M.; Dirks-Hofmeister, M.E.; Moerschbacher, B.M.; Philipp, B.; Wendisch, V. Synthetic *Escherichia coli*-Corynebacterium glutamicum consortia for L-lysine production from starch and sucrose. *Bioresour. Technol.* **2018**, *260*, 302–310. [CrossRef]

68. Sgobba, E.; Wendisch, V. Synthetic microbial consortia for small molecule production. *Curr. Opin. Biotechnol.* **2020**, *62*, 72–79. [CrossRef] [PubMed]

69. Yamamoto, S.; Morihara, Y.; Wakayama, M.; Tachiki, T. Theanine Production by Coupled Fermentation with Energy Transfer Using $\gamma$-Glutamylmethylamide Synthetase of *Methylovorax mays* No. 9. *Biosci. Biotechnol. Biochem.* **2008**, *72*, 1206–1211. [CrossRef] [PubMed]

70. Watanabe, S.; Tanimoto, Y.; Yamauchi, S.; Tozawa, Y.; Sawayaama, S.; Watanabe, Y. Identification and characterization of trans-3-hydroxy-l-proline dehydratase and $\Delta(1)$-pyrroline-2-carboxylate reductase involved in trans-3-hydroxy-l-proline metabolism of bacteria. *FEBS Open Biol.* **2014**, *4*, 240–250. [CrossRef] [PubMed]

71. Wieschalka, S.; Blombach, B.; Eikmanns, B.J. Engineering *Corynebacterium glutamicum* to produce pyruvate. *Appl. Microbiol. Biotechnol.* **2012**, *94*, 449–459. [CrossRef]

72. Zahoor, A.; Otten, A.; Wendisch, V. Metabolic engineering of *Corynebacterium glutamicum* for glycolate production. *J. Biotechnol.* **2014**, *192*, 566–375. [CrossRef]

73. Li, P.-P.; Liu, Y.-J.; Liu, S.-J. Genetic and biochemical identification of the chorismate mutase from *Corynebacterium glutamicum*. *Microbiology* **2009**, *155*, 3382–3391. [CrossRef]

74. Maier, W.; Baumert, A.; Gröger, D. Partial Purification and Characterization of S-Adenosyl-L-Methionine: Anthranilic Acid N-Methyltransferase from Ruta Cell Suspension Cultures. *J. Plant Physiol.* **1995**, *145*, 1–6. [CrossRef]

75. Han, G.; Hu, X.; Qin, T.; Li, Y.; Wang, X. Metabolic engineering of *Corynebacterium glutamicum* ATCC13032 to produce S-adenosyl-1-methionine. *Enzym. Microb. Technol.* **2016**, *83*, 14–21. [CrossRef]

76. Francis, M.M.; Vining, L.C.; Westlake, D.W. Characterization, and regulation of anthranilate synthetase from a chloramphenicol-producing streptomycete. *J. Bacteriol.* **1978**, *134*, 10–16. [CrossRef]

77. Henderson, E.J.; Nagano, H.; Zalkin, H.; Hwang, L.H. The anthranilate synthetase-anthranilate 5-phosphoribosylpyrophosphate phosphoribosyltransferase aggregate. Purification of the aggregate and regulatory properties of anthranilate synthetase. *J. Bacteriol.* **1970**, *134*, 1416–1423.

78. Caligiuri, M.G.; Bauerle, R. Identification of amino acid residues involved in feedback regulation of the anthranilate synthetase complex from *Salmonella typhimurium*. Evidence for an amino-terminal regulatory site. *J. Biol. Chem.* **1991**, *266*, 8328–8335.

79. Leßmeier, L.; Wendisch, V. Identification of two mutations increasing the methanol tolerance of *Corynebacterium glutamicum*. *BMC Microbiol.* **2015**, *15*, 216. [CrossRef]

80. Wang, Y.; Fan, L.; Tuyishime, P.; Liu, J.; Zhang, K.; Gao, N.; Zhang, Z.; Ni, X.; Feng, J.; Yuan, Q.; et al. Adaptive laboratory evolution enhances methanol tolerance and conversion in engineered *Corynebacterium glutamicum*. *Commun. Boil.* **2020**, *3*, 217. [CrossRef] [PubMed]
81. 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. [CrossRef] [PubMed]

82. Wang, X.; Khushk, I.; Xiao, Y.; Gao, Q.; Bao, J. Tolerance improvement of Corynebacterium glutamicum on lignocellulose derived inhibitors by adaptive evolution. *Appl. Microbiol. Biotechnol.* 2017, 102, 377–388. [CrossRef] [PubMed]

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).