Article

Metabolic Engineering of *Corynebacterium glutamicum* for Sustainable Production of the Aromatic Dicarboxylic Acid Dipicolinic Acid

Lynn S. Schwardmann 1, Aron K. Dransfeld 1,*, Thomas Schäffer 2 and Volker F. Wendisch 1,*

Abstract: Dipicolinic acid (DPA) is an aromatic dicarboxylic acid that mediates heat-stability and is easily biodegradable and non-toxic. Currently, the production of DPA is fossil-based, but bioproduction of DPA may help to replace fossil-based plastics as it can be used for the production of polyesters or polyamides. Moreover, it serves as a stabilizer for peroxides or organic materials. The antioxidative, antimicrobial and antifungal effects of DPA make it interesting for pharmaceutical applications. In nature, DPA is essential for sporulation of *Bacillus* and *Clostridium* species, and its biosynthesis shares the first three reactions with the L-lysine pathway. *Corynebacterium glutamicum* is a major host for the fermentative production of amino acids, including the million-ton per year production of L-lysine. This study revealed that DPA reduced the growth rate of *C. glutamicum* to half-maximal at about 1.6 g L\(^{-1}\). The first de novo production of DPA by *C. glutamicum* was established by overexpression of dipicolinate synthase genes from *Paenibacillus sonchi* genomovar *riograndensis* SBR5 in a *C. glutamicum* L-lysine producer strain. Upon systems metabolic engineering, DPA production to 2.5 g L\(^{-1}\) in shake-flask and 1.5 g L\(^{-1}\) in fed-batch bioreactor cultivations was shown. Moreover, DPA production from the alternative carbon substrates arabinose, xylose, glycerol, and starch was established. Finally, expression of the codon-harmonized phosphite dehydrogenase gene from *P. stutzeri* enabled phosphite-dependent non-sterile DPA production.

Keywords: dipicolinic acid; *Corynebacterium glutamicum*; metabolic engineering; sustainable production; nonsterile fermentation; phosphite

1. Introduction

DPA or pyridine-2,6-dicarboxylic acid is a naturally occurring aromatic dicarboxylic acid. Due to its metal-chelating properties, it serves as ligand for lanthanides [1], e.g., to transfer enhanced stability to micelles [2], and is used in complexes with transition metals, such as copper [3], or the actinide uranium. The latter provides the basis for potential applications in nuclear energy systems [4]. DPA also prevents calcium salt precipitation in silver halide photographic solutions [5]. In chemical industry, it serves as precursor for the synthesis of pyridines and piperidines [6] and as a stabilizer of peroxides in aqueous solutions, e.g., in peroxycarboxylic acids and gels of organic peroxides by reducing the decomposition rate [7–9]. Furthermore, its antioxidative effect makes DPA interesting for use in pharmacy [10,11]. It confers antimicrobial activity, enabling its application as an antimicrobial activity reagent [12,13], and the recently observed antifungal activity against canker pathogens reduced the symptoms of *Valsa pyri* infections of pear trees, showing its potential for the management of valsa canker [14]. DPA is a non-toxic dicarboxylic acid that shares some properties, including easy biodegradability and heat-stability, with diamines, with which it can be copolymerized to polyamides [15–17]. DPA has the potential
to contribute to a more sustainable production of bio-based polymers by replacing fossil-based monomers as starting material as shown for pyridine based polyesters [10,16,18], furandicarboxylic acid based polyesters [19] and polyamides [20–22].

Naturally, DPA occurs as a secondary metabolite in endospores of gram-positive bacteria, mainly aerobic Bacillus and anaerobic Clostridium species. It is essential for initiation of sporulation under environmentally stressful conditions, such as nutrient starvation, heat, radiation or the presence of toxic compounds and stability of the endospore [23]. In the endospore, it occurs in a chelate with calcium ions and accounts for approximately 10% of the endospore dry weight in Bacillus subtilis [24]. Thus, DPA mediates heat resistance [25] and prevents DNA denaturation of the endospore [26].

Biosynthesis of DPA in sporulating bacteria only requires a single committed step to convert an intermediate of the L-lysine biosynthesis pathway to DPA. For this reaction, both the oxidoreductase SpoVFA and the flavoprotein SpovFB (corresponding to the gene products of dpaA and dpaB) of dipicolinate synthase SpoVF are required for DPA synthesis by B. subtilis [27]. The first heterologous DPA biosynthesis resulted from overexpression of the B. subtilis spoVFAB operon in Escherichia coli [27]. Orthologous enzymes to SpoVF were later identified in Clostridia, but designated EftA [28]. The exact branchpoint intermediate of DPA synthesis remained controversial for a long time and was revealed as (2S,4S)-4-hydroxy-2,3,4,5-tetrahydrodipicolinic acid (HTPA) [29] and not (S)-2,3-dihydrodipicolinate, as suggested earlier [30,31]. HTPA is synthesized by 4-hydroxy-tetrahydrodipicolinate synthase DapA (reclassified from EC 1.3.1.26 to EC 1.17.1.8) in L-lysine biosynthesis (Figure 1A).

Figure 1. Scheme of the metabolic pathway for DPA biosynthesis in engineered C. glutamicum Dpa1 (A) and of the reactions at the branchpoint intermediate HTPA (B). Single catalytic steps are depicted by solid lines and multiple catalytic steps by dashed lines. It includes endogenous genes (blue) and heterologous dipicolinate synthase genes dpaAB from P. sonchi SBR5 (green). Variants of endogenous genes (purple), namely feedback-resistant aspartokinase lycC T311I, pyruvate carboxylase pyc P458S and homoserine dehydrogenase hom V59A, increase L-aspartate conversion and anaplerosis via pyruvate carboxylase, while decreasing drain of aspartate-4-semialdehyde towards threonine and methionine biosynthesis. DPA shares the first three steps of L-lysine biosynthesis via aspartokinase (lysC), aspartate-semialdehyde dehydrogenase (asd) and HTPA synthase (dapA). A red cross indicates the deletion of carboxykinase gene ppc (red) to prevent decarboxylation of oxaloacetate. Glucose uptake is PTS (phosphotransferase system) mediated and L-lysine is exported via LysE. Brackets depict putative spontaneous dehydration of HTPA.
As a branch point metabolite between L-lysine biosynthesis and DPA biosynthesis, HTPA is converted by HTPA reductase DapB towards L-lysine or to DPA by dipicolinate synthase (Figure 1A) [32]. Dipicolinate synthase DpaAB (Figure 1B) has two subunits encoded either by spoVFAB in Bacilli or by dpaAB in Clostridia, where the electron transfer protein EtfA participates in DPA biosynthesis [28]. Dipicolinate synthase catalyzes the dehydration and reduction of HTPA to DPA and a Km value of 0.776 mM HTPA for SpovFAB was determined [32]. It is possible that dehydration occurs spontaneously followed by enzyme-catalyzed reduction by dipicolinate synthase (Figure 1) [28] or that dipicolinate synthase functions as dual reductase-dehydratase similar to, e.g., TerBC in p-terphenyl biosynthesis [33].

Chemical production of DPA involves the oxidation of the methyl-group of 2,6-lutidine, a heterocyclic aromatic organic compound, which is isolated from coal tar and bone oil [34,35]. Albeit chemical synthesis typically allows for high titers easing product purification, there is an increasing demand for sustainable bioprocesses such as production of DPA. One challenge to establish bio-production is the natural restriction in DPA biosynthesis to the sporulation phase and its repression in vegetative cells [27]. This limitation was overcome by a promoter exchange in the natural producer B. subtilis combined with media optimization [36]. Introduction of B. subtilis sporulation genes spoVFAB into an E. coli strain that was engineered for L-lysine production allowed for DPA production by recombinant bacterium not naturally synthesizing DPA [32]. The Gram-positive soil bacterium Corynebacterium glutamicum naturally produces L-glutamate [37] and has been used for decades in the biotechnological industry for fermentative production of the amino acids L-glutamate and L-lysine, which reached 3.2 and 2.6 million tons, respectively [38]. Therefore, L-lysine overproducing C. glutamicum are ideal hosts for production of compounds that can be derived from L-lysine or intermediates of L-lysine biosynthesis. A strong metabolic engineering tool box is available for C. glutamicum including traditional mutagenesis and selection [39], rational strain design [40], genome reduction [41,42] and the CRISPR tools [43], including CRISPR interference [44]. Based on the strong performance of L-lysine producing C. glutamicum strains [38], production of L-lysine derived products has been enabled by extension of L-lysine biosynthesis. This included production of L-pipeolic acid (L-PA) [45], 4-hydroxylysine [46], cadaverine [47], 3-hydroxy-L-cadaverine [46], 5-aminovalerate (5AVA) [48,49], glutarate [22,50,51] or L-2-hydroxyglutarate [52] as well as inclusion of L-lysine into polymers such as cyanophycin [53]. L-lysine biosynthesis was intercepted in L-lysine overproducing strains to enable production of ectoine and hydroxyectoine from the intermediate aspartate-semialdehyde [54,55] or 3-aminopropionate from L-aspartate [56].

Traditional C. glutamicum fermentation uses phosphate as a phosphorus source and glucose as the dominant carbon source despite its competing uses in human and animal nutrition. C. glutamicum naturally grows with the monosaccharides glucose, fructose and ribose [57], the disaccharides sucrose and maltose, the alcohols inositol and ethanol [58–61], the organic acids pyruvate, propionate and lactate [62,63] as well as some amino acids [64]. A flexible feedstock concept has been achieved by metabolic engineering [65,66] and it enabled C. glutamicum access to non-native carbon sources such as starch, glycerol from fats, pentoses from lignocellulosic hydrolysates and amino sugars from shrimp waste [67]. L-lysine and derived compounds were produced from alternative carbon sources, e.g., cadaverine from starch or xylose [68,69] and ectoine from starch, xylose, arabinose, glycerol and glucosamine [55]. Typically, C. glutamicum co-utilizes the alternative with the native carbon sources, e.g., xylose with glucose [70], while yields remain lower than with glucose with the notable exception of sarcosine production [71]. Traditional C. glutamicum fermentations do not suffer from phage lysis but are operated in sterilized steel tanks to avoid microbial contamination. Sterilization is money-, time- and resource-consuming [72,73]. Non-sterile contamination-free fermentation processes are important [74] and have been realized for B. subtilis, E. coli, and C. glutamicum [72,73,75]. The use of phosphite instead
of phosphate combined with expression of the phosphate dehydrogenase gene ptxD from *Pseudomonas stutzeri* allowed non-sterile production of L-lysine by *C. glutamicum* [72,73].

In this work, we describe the first de novo production of the aromatic dicarboxylic acid DPA by *C. glutamicum*. To lay the foundation for sustainable production, DPA was produced from the alternative carbon sources fructose, xylose, arabinose, glycerol, and starch on the one hand and, on the other hand, ptxD from *Pseudomonas stutzeri* was expressed for non-sterile production using phosphate instead of phosphate as source of phosphorus.

### 2. Materials and Methods

#### 2.1. Bacterial Strains and Growth Conditions

The strains and plasmids used in this study are listed in (Table 1). *E. coli* DH5α [76] was used for vector construction and amplification and grown in lysogeny broth (LB) at 37 °C and 180 rpm. According to the vectors, the medium for *E. coli* was supplemented with tetracycline (10 µg·mL⁻¹) and/or kanamycin (50 µg·mL⁻¹), spectinomycin (100 µg·mL⁻¹) or chloramphenicol (30 µg·mL⁻¹).

Precultures of *C. glutamicum* were inoculated from fresh LB agar plates and cultivated in 10 or 50 mL brain-heart infusion broth (BHI) (ROTH, Karlsruhe, Germany) in 50 or 500 mL baffled flasks at 30 °C and 120 rpm on a rotary shaker. If not stated otherwise, production and growth experiments were routinely performed in 50 mL baffled flasks in 10 mL CgXII minimal medium [77] with 40 g·L⁻¹ glucose at 30 °C and 120 rpm on a rotary shaker. Precultures were harvested by centrifugation (3200 × g, 7 min) and washed once in TN buffer (50 mM Tris-HCL, 50 mM NaCl, pH 6.3) prior to the inoculum of the main cultures to an optical density (OD₆₀₀) of 1. Growth was monitored by optical density measurements at 600 nm, using a V-1200 Spectrophotometer (VWR, Radnor, PA, USA) and an OD₆₀₀ of 1 was determined to be equivalent to 0.25 g·L⁻¹ cell dry weight (CDW).

#### Table 1. Strains and plasmids used in this work.

| Strains | Relevant Characteristics | Source |
|---------|-------------------------|--------|
| *E. coli* DH5α | F-thi-1 endA1 hisd17(r-, m-) supE44 lacI939 (F80lacZ1M15) recA1 gyrA96 C. glutamicum wild type, ATCC 13032 | [76] |
| WT | WT carrying (pECXT_Psyn, ptxD) | [78] |
| DM1729Apck | WT with genomic modifications pckF₄₅₈₄ homV₅₉₃₈ lgsC₇₃₁₁ Apck | This study |
| DM1729AsugRAldhα | WT with genomic modifications pckF₄₅₈₄ homV₅₉₃₈ lgsC₇₃₁₁ AsugRAldhα | [79] |
| DM1729Apck(pECXT_Psyn) | DM1729Apck carrying pECXT_Psyn | This study |
| DM1729AsugRAldhα(pECXT_Psyn) | DM1729AsugRAldhα carrying pECXT_Psyn | This study |
| Dpa1 | Dpa1 carrying pEKEx3_dapA | This study |
| Dpa1-pDapA | Dpa1 carrying pEKEx3_dapA | This study |
| Dpa1-DapA | Dpa1 carrying pEKEx3 | This study |
| Dpa1(pEKEx3) | Dpa1 carrying pWVE1 | This study |
| Dpa1(pVWEx1) | Dpa1 carrying pWVE1 | This study |
| Dpa1(pVWEx1_lysC₃₃₁₁) | Dpa1 carrying pWVE1_lysC₃₃₁₁ | This study |
| Dpa1(pEKEx3_ppc) | Dpa1 carrying pWVE1_ppc | This study |
| Dpa1(pVWEx1_ppc) | Dpa1 carrying pWVE1_ppc | This study |
| Dpa1(pVWEx1_araBAD) | Dpa1 carrying pWVE1_araBAD | This study |
| Dpa1(pVWEx1_xylAB) | Dpa1 carrying pWVE1_xylAB | This study |
| Dpa1(pVWEx1_glpFKD) | Dpa1 carrying pWVE1_glpFKD | This study |
| Dpa1(pVWEx1_amyA) | Dpa1 carrying pWVE1_amyA | This study |
| Dpa1-PtxD | Dpa1-PtxD carrying pWVE1 | This study |
| Dpa1-PtxD(pVWEx1) | Dpa1-PtxD carrying pWVE1 | This study |
| Dpa1-PtxD(pVWEx1_glpFKD) | Dpa1-PtxD carrying pWVE1_glpFKD | This study |
Table 1. Cont.

| Plasmids                        | Description                                                                                       | Manufacturer                        |
|---------------------------------|---------------------------------------------------------------------------------------------------|-------------------------------------|
| pUC57_amIf-pxtD                 | Amp<sup>+</sup>, cloning plasmid with sequences of codon-optimized versions of amif from *Helicobacter pylori* and pxtD from *Pseudomonas stutzeri* WM88          | BioCat GmbH                        |
| pECXT99A                        | Tet<sup>+</sup>, Pta, lacF<sup>-</sup>, pGA1 minireplccon, *C. glutamicum* / *E. coli* IPTG-inducible expression shuttle vector | [80]                               |
| pAmy                            | pECXT99A overexpressing *amyA* from *Streptomyces griseus* IMRU 3570                             | [81]                               |
| pECXT_Psyn                      | Tet<sup>+</sup>, pECXT99A derivative for constitutive expression from promoter P<sub>syn</sub>     | [82]                               |
| pECXT_Psyn_pxtD                 | pECXT<sub>Psyn</sub> derivative for expression of codon-optimized version of pxtD from *Pseudomonas stutzeri* WM88 | This study                         |
| pVWEx1_pstA                     | pECXT<sub>Psyn</sub> derivative for expression of dpaAB from *Pseudomonas stutzeri* genomovar *Riograndensis SBR5* | This study                         |
| pECXT_Psyn_dpaAB                | pVWEx1_pstA derivative for expression of dpaAB from *Pseudomonas stutzeri* genomovor *Riograndensis SBR5* and dpaA from *C. glutamicum* WT | This study                         |
| pECXT_Psyn_dpaAB-pxtD           | pVWEx1_pstA also expressing codon-harmonized pxtD from *Pseudomonas stutzeri* WM88               | This study                         |
| pVWEx1                          | Tet<sup>+</sup>, P<sub>vap</sub>, lacQ pHM1519 OriV<sub>C</sub> *C. glutamicum* / *E. coli* expression shuttle vector | [83]                               |
| pVWEx1_pstA                     | pVWEx1 for overexpression of *lpc*<sup>T3111</sup> from *C. glutamicum* ATCC 13032                | [84]                               |
| pVWEx1 araBAD                    | pVWEx1 for overexpression of araBAD from *E. coli* MG1655                                      | [85]                               |
| pVWEx1_glyFD                    | pVWEx1 for overexpression of *glyF*, *glyD* and *glyP* from *E. coli* MG1655                    | [86]                               |
| pVWEx1_amyA                     | pVWEx1 for overexpression of *amyA* from *Streptomyces griseus* IMRU3570                        | This work                         |
| pEKEx3                          | Spe<sup>+</sup>, P<sub>lac</sub>, pBL1 OriV<sub>C</sub> *C. glutamicum* / *E. coli* expression shuttle vector | [87]                               |
| pEKEx3_ppc                      | pEKEx3 for overexpression of *ppc*<sup>P458S</sup> from *C. glutamicum* WT                    | [88]                               |
| pEKEx3_ppc_pyc<sup>P458S</sup>  | pEKEx3 for overexpression of *ppc*<sup>pyc</sup> from *C. glutamicum* WT                     | This study                         |
| pEKEx3_dpaA                     | pEKEx3 for overexpression of dpaA from *C. glutamicum* WT                                       | This study                         |
| pS_dCas9                        | pS_dCas9 plasmid carrying the dapB14 sgRNA                                                        | This study                         |
| pS_dCas9_dapB14                 | pS_dCas9 plasmid carrying the dapB561 sgRNA                                                        | This study                         |
| pS_dCas9_dapB561                |                                                                                                  | This study                         |

The cultivation with alternative carbon sources was performed at a concentration of 20 g·L<sup>-1</sup>. Deviating, soluble starch was added at 10 g·L<sup>-1</sup> due to low solubility and 0.5 g·L<sup>-1</sup> glucose was added as co-substrate to enable *C. glutamicum* to utilize starch. Phosphorous-substituted (P-substituted) CgXII contained the indicated concentration of di-sodium phosphate instead of di-potassium hydrogen phosphate and potassium dihydrogen phosphate. Growth experiments for the determination of the sensitivity to DPA were carried out in a BioLector microcultivation system (m2p-labs, Aachen, Germany) in a volume of 1 mL CgXII minimal medium with 40 g·L<sup>-1</sup> glucose in a 48-well flower plate at 30 °C, 85% humidity and a shaking frequency of 1100 rpm. A signal gain of 15 was used to follow growth by backscatter light signal at 620 nm.

According to the vectors, the medium for *C. glutamicum* was supplemented with tetracycline (5 μ·mL<sup>-1</sup>) and/or kanamycin (25 μ·mL<sup>-1</sup>), spectinomycin (100 μ·mL<sup>-1</sup>) or chloramphenicol (7 μ·mL<sup>-1</sup>). Gene expression from pVWEx1 and pEKEx3-derived plasmids was induced by the addition of 1 mM Isopropyl-β-D-1-thiogalactopyranoside (IPTG) and the CRISPRi system by the addition of 1 mM IPTG and 0.25 μ·mL<sup>-1</sup> anhydrotetracycline to CgXII minimal medium at the beginning of the cultivation.

2.2. Molecular Genetic Techniques and Strain Construction

DNA sequences were amplified using the respective oligonucleotides (Tables S1 and S2), and ALLIn<sup>TM</sup> HiFi DNA Polymerase (highQu GmbH, Kraichtal, Germany) according to the manufacturer’s protocol. DNA of *C. glutamicum* and *P. sonchi* SBR5 served as template for the amplification of dapA and dpaAB respectively, while ptxD was amplified from the plasmid pUC57_amIf-pxtD and amyA was amplified from pAmy [81]. A consensus ribosome binding site (RBS) sequence (GAAAGGAGGCCCTTCAG) was inserted in front of the genes dpaAB, dapA and ptxD via primer overhangs.

The plasmids pECXT_Psyn pVWEx1 and pEKEx3 were linearized by restriction with BamHI, while PstI (NEB, Frankfurt, Germany) was used to linearize pS_dCas9 [89]. Deviating,
pECXT_Psyn_dpaAB-pxtD was constructed by linearization of pECXT_Psyn_dpaAB with XbaI (NEB, Frankfurt, Germany) for the integration of pxtD amplified from pECXT_Psyn_pxtD. Linearized vectors were dephosphorylated (Antarctic phosphatase, New England Biolabs, Frankfurt, Germany) prior to plasmid assembly by Gibson Assembly [90]. DNA concentration was determined at 600 nm, using a V-1200 Spectrophotometer (VWR, Radnor, PA, USA).

The sequences of inserts of plasmids, constructed in this study (Table 1), were confirmed by sequencing with the respective oligonucleotides (Table S1). All oligonucleotides used for DNA amplification and sequencing were obtained from Metabion (Planegg/Steinkirchen, Germany). Plasmids were isolated using a plasmid miniprep kit (GeneJET, Thermo Fisher Scientific, Schwerte, Germany). Standard molecular genetic techniques were carried out as described previously [91]. Competent *E. coli* cells were prepared by CaCl2 method [91] and transformed by heat shock at 42 °C [77], whereas competent *C. glutamicum* cells were transformed by electroporation [77]. Transformants were screened by colony PCR with the respective vector-specific fw and rv verification oligonucleotides (Table S1).

### 2.3. Analytical Procedures

Extracellular amino acids, carbohydrates and DPA were quantified using a high-pressure liquid chromatography system (HPLC) (1200 series, Agilent Technologies Deutschland GmbH, Böblingen, Germany). Cell culture supernatant was taken at the indicated time points, centrifuged (20,200 × g, 20 min) and stored at −20 °C until analysis.

Samples for amino acid analysis were derivatized with *ortho*-phthalaldehyde (OPA) [92], and L-asparagine served as internal standard. Amino acids were separated by reversed phase HPLC, using a pre- (LiChrospher 100 RP18 EC-5µ (40 × 4 mm), CS Chromatographie Service GmbH, Langerwehe, Germany) and a main-column (LiChrospher 100 RP18 EC-5µ, 125 × 4.6 mm, CS Chromatographie Service GmbH, Langerwehe, Germany) and detected with a fluorescence detector (FLD G1321A, 1200 series, Agilent Technologies, Deutschland GmbH, Böblingen, Germany) at an excitation wavelength of 230 nm and an emission wavelength of 450 nm.

Carbohydrates and DPA were separated with an amino exchange column (Aminex, 300 × 8 mm, 10 µm particle size, 25 Å pore diameter, CS Chromatographie Service GmbH, Langerwehe, Germany) with 5 mM H2SO4 under isocratic conditions at a flow rate of 0.8 mL·min⁻¹ for 30 min as described previously [85]. The substances were detected by a refractive index detector (RID G1362A, 1200 series, Agilent Technologies, Deutschland GmbH, Böblingen, Germany). The DPA and L-lysine titers obtained from starch represent those obtained from 10 g·L⁻¹ plus 0.5 g·L⁻¹ glucose after subtraction of titers, obtained from 0.5 g·L⁻¹ glucose alone.

### 2.4. Enzymatic Assay

Cells for crude extract were grown in LB in 500 mL baffled flasks and harvested by centrifugation (20200 × g, 7 min) at 4 °C when stationary phase was reached. All following steps were performed on ice or at 4 °C. The pellet was resuspended in reaction buffer (100 mM MOPS, pH 7), washed three times and solved in 5 mL reaction buffer for cell disruption by sonication (UP 200S, Dr. Hielscher GmbH, Teltow, Germany) for 9 min at 60% amplitude and a duty cycle of 0.5 s. Total crude protein concentrations were determined by the method of Bradford, and bovine serum standard was used as reference [93].

Oxidative PtxD activity was measured using 4 mM NAD⁺ and 13 mM di-sodium phosphate as described previously [73,94]. Absorption was followed for 3 min at 340 nm. One unit (U) of phosphite dehydrogenase activity was defined as the quantity of enzyme required to convert 1 µmol of NADH per min at 25 °C.

### 2.5. Gene Repression by CRISPRi

CRISPRi-mediated gene repression was based on the previously developed vector pS-dCas9 [89]. Two single guide RNAs (sgRNAs) were designed with the CRISPy-web-tool [95], based on the genome sequence of *C. glutamicum* WT [78]. The targets comprise
a 20 bp region, homologous to the non-template strand of *dapB*. The double-stranded sgRNA inserts were generated from single-stranded oligonucleotides by an annealing oligo method, as described elsewhere for Gibson plasmid assembly with PstI restricted pS_dCas9 [89].

### 2.6. Cultivation in a Fermenter

Fermentative production was performed in a baffled bioreactor with a total volume of 7 L (NLFNLE, Bioengineering AG, Switzerland) with two six-bladed Rushton turbines. A sparger at the bottom provided a constant airflow of 5 NL·min⁻¹. The stirrer rate was set to 200 rpm and increased to 350 rpm after 18 h to maintain a relative dissolved oxygen concentration above 30%. The temperature was kept at 30 °C at the headspace overpressure of 0.2 bar. The pH was kept at 7, adjusted by the automatic addition of chloric acid (2 M) and ammonia (25% (w/v)). A CgXII preculture was used to inoculate the initial working volume of 2.5 L of CgXII minimal medium, omitting MOPS buffer and containing 40 g·L⁻¹ glucose to an OD₆₀₀ of 1. To prevent foaming, 0.6 mL·L⁻¹ of the antifoam agent Pluronic F-68 were added. The feed, consisting of 100 g·L⁻¹ glucose and 40 g·L⁻¹ (NH₄)₂SO₄, was started at 41 h and 70 h when the glucose was depleted and growth had ceased, respectively. Samples were taken by an autosampler and kept at 4 °C until analysis. Titers and yields of the fermentation were related to the initial fermentation volume of 2.5 L. All values were normalized for the rise in the fermentation volume from feed addition. Final product titers and yields were calculated using the total glucose added, comprising the initial glucose concentration (40 g·L⁻¹) and the feed (39 g·L⁻¹, related to the initial fermentation volume of 2.5 L).

### 2.7. Statistical Analysis

Statistical significance was determined by the unpaired Student’s *t*-tests (two-sided), using a *p*-value of <0.001 (***)) for triplicate cultivations.

### 3. Results

#### 3.1. Investigating of the Suitability of *C. glutamicum* for DPA Production

To evaluate the suitability of *C. glutamicum* to produce DPA, growth experiments were performed to test for a potential product toxicity. *C. glutamicum* WT was grown in the BioLector microcultivation system in glucose minimal medium in the presence of 0–100 mM DPA. Growth was observed for the entire DPA concentration range tested but affected by its presence. The addition of DPA slowed growth, and biomass formation was reduced at concentrations of 10 mM (1.7 g·L⁻¹) DPA or more (Figure 2A). At a DPA concentration of 9.8 ± 0.12 mM (1.63 ± 0.02 g·L⁻¹) the growth rate µ was reduced to half-maximum (Figure 2B). Thus, growth of *C. glutamicum* WT in the presence of DPA is possible but was slowed by increasing DPA concentrations.

#### 3.2. Engineering of L-Lysine Producing Strains for DPA Production from Glucose

Biosynthesis of DPA in *Bacilli* shares the first three steps with the L-lysine biosynthesis pathway up to the branch point intermediate HTPA. In L-lysine biosynthesis, HTPA is formed by HTPA synthase (DapA) before being immediately oxidized by dihydrodipicolinate reductase (DapB) to THDP. Instead, dehydration and reduction of HTPA by dipicolinate synthase, encoded by *dpaAB*, yields DPA [28]. Biosynthesis of DPA has not been described for *C. glutamicum*, a bacterium known for its L-lysine production potential. Inspection of the *C. glutamicum* genome [78] did not provide evidence for DPA biosynthesis genes. Thus, exogenous DPA biosynthesis genes were chosen for expression in L-lysine producing *C. glutamicum* strains.
Here, the two previously described L-lysine producing strains *C. glutamicum* DM1729 ΔsugRΔldhA [55] and *C. glutamicum* DM1729Δpck [79] were chosen as potential basis strains for the production of DPA. *C. glutamicum* strain DM1729 overproduces L-lysine due to three single nucleotide exchanges introduced into the wild type ATCC 13032, namely, lysC<sup>T311I</sup>, pyc<sup>P458S</sup> and hom<sup>V59A</sup>. The L-lysine feedback-resistant aspartokinase, encoded by lysC<sup>T311I</sup> [96,97] and the enhanced activity variant pyruvate carboxylase, encoded by pyc<sup>P458S</sup> [98,99], improve the availability of the precursor oxaloacetate. The attenuated homoserine dehydrogenase (encoded by hom<sup>V59A</sup>) reduces loss of the intermediate L-aspartate-semialdehyde to biosynthesis of homoserine, L-methionine and L-threonine [100,101]. Deletion of the gluconeogenic phosphoenolpyruvate carboxykinase gene pck in DM1729Δpck contributes to a higher oxaloacetate availability, which increased flux into L-lysine biosynthesis (Figure 1A) [102]. Strain DM1729ΔsugRΔldhA lacks repression control of phosphotransferase system and glycolysis genes by global transcriptional repressor SugR (Figure 1A) [103–106]. In addition, this strain does not produce lactate as unwanted by-product due to the deletion of ldhA that encodes fermentative NAD-dependent L-lactate dehydrogenase [107]. The strains DM1729, DM1729ΔsugRΔldhA and DM1729Δpck were chosen deliberately to ensure availability of HTPA for DPA biosynthesis since they, unlike many other L-lysine producing strains, do not possess any modifications downstream of HTPA.

Conversion of HTPA to DPA by dipicolinate synthase is well-known in sporulating bacteria, e.g., *B. subtilis* [27]. In this work, we chose to introduce the dipicolinate synthase from *Paenibacillus sonchi* genovar *riograndensis* SBR5 because its optimal growth temperature of 30–37 °C [108] conforms well with that of *C. glutamicum* (30 °C). The dipicolinate synthase genes dpaAB from *P. sonchi* SBR5 were cloned into the constitutive expression vector pECXT_P<sub>syn</sub>. Transformation of DM1729Δpck and DM1729ΔsugRΔldhA with pECXT_P<sub>syn</sub>_dpaAB yielded strains Dpa1 and Dpa2, respectively. The expression of dipicolinate synthase decreased the maximal growth rate and total biomass formation of both strains (Table 2).
Table 2. Growth, DPA and L-lysine production values of engineered DPA producing strains (and control strains). Cultivation was performed in shake flasks using CgXII containing 40 g·L⁻¹ glucose as regular medium and cultivation for 96 h. Deviating medium composition is indicated. Cultivation of Dpa1 and Dpa1-derived strains on alternative carbon sources was performed in CgXII containing 20 g·L⁻¹ fructose, xylose, arabinose, or glycerol, or 10 g·L⁻¹ starch for 72 h. Values are given as means with standard deviations from triplicate cultivations.

![Figure 3](image-url)

Production of DPA by the negative control strains DM1729Δpck(pECXT_Psyn) and DM1729ΔaugRΔdhaA(pECXT_Psyn) could not be detected, confirming that C. glutamicum naturally lacks the ability of de novo biosynthesis of DPA. The constructed strain Dpa1 produced 2.53 ± 0.04 g·L⁻¹ DPA in CgXII minimal medium containing 40 g·L⁻¹ glucose in 96 h, while Dpa2 only produced 0.36 ± 0.01 g·L⁻¹ (Table 2, Figure 3A). The lower DPA production by strain Dpa2 was accompanied by incomplete glucose consumption. Owing to its higher DPA production and robust growth, the following experiments were performed with Dpa1.
3.3. Product Inhibition May Limit DPA Production

To investigate whether production of DPA by Dpa1 was inhibited by the presence of DPA, growth and production were tested in CgXII with 40 g L\(^{-1}\) glucose, supplemented with 1 or 2 g L\(^{-1}\) DPA. Regardless of the concentration, \(\mu\) was lower when DPA was added to glucose containing CgXII (0.03 ± 0.00 h\(^{-1}\) as compared to 0.09 ± 0.00 h\(^{-1}\)). The addition of DPA perturbed growth, shown by incomplete glucose consumption in 144 h. Its presence at a concentration of 1 or 2 g L\(^{-1}\) reduced the total biomass formation to 75% and 30% and DPA production to 62% and 43%, respectively (Table 2).

The observed sensitivity of \textit{C. glutamicum} WT to DPA suggested that growth and growth-coupled DPA production by Dpa1 may be limited by product inhibition. Hence, lowering substrate concentrations may be a simple tool to raise the product yield \(Y_{DPA/S}\), making DPA production more efficient and sustainable due to lower resource use. To test this, Dpa1 was cultivated in CgXII with glucose concentrations reduced to 50% and 25% (20 and 10 g L\(^{-1}\)). When 25% glucose were used, \(Y_{DPA/S}\) was doubled (from 0.06 to 0.12 g g\(^{-1}\)) as compared to the regular concentration of 40 g L\(^{-1}\) (Figure 3B). Concomitantly, the by-product L-lysine yield was reduced to one third for both 25 and 50% glucose (Figure 3B). The cultivation with reduced glucose content was beneficial for improved product yields per substrate and the reduction of by-product formation.

Regular CgXII minimal medium contains 468 mM nitrogen in the form of urea and ammonium sulphate because it was optimized for nitrogen-demanding L-lysine production. L-lysine contains two nitrogen atoms, whereas DPA contains just one. Therefore, Dpa1 was cultivated in CgXII minimal medium containing 50%, 30% or 10% of the regular nitrogen concentration to aim for a more favorable ratio of DPA to L-lysine formation. As intended, reducing the nitrogen concentration in the medium shifted the DPA to L-lysine ratio towards DPA production and resulted in a six-fold higher DPA to L-lysine ratio with 10% nitrogen (Table 2, Figure 3C). While biomass formation was not significantly affected, the absolute DPA titers were reduced to one third (0.81 ± 0.02 g L\(^{-1}\)) with 10% nitrogen as compared to the regular nitrogen concentration in the medium (Table 2).

3.4. Assessment of the Effect of Increased Availability of Precursors on DPA Production

L-lysine production is known to suffer from bottlenecks regarding its initial enzyme aspartokinase [96], and regarding anaplerosis via PEP carboxylase [109] and pyruvate carboxylase [99] that is required for providing oxaloacetate as precursor. As HTPA is an intermediate of L-lysine biosynthesis the substrate of dipicolinate synthase, pathway engineering strategies applied to increase the flux into L-lysine biosynthesis are relevant for DPA production as long as they target only reactions leading to HTPA, but not improving conversion of HTPA towards L-lysine. To investigate the impact on DPA production, the genes \textit{lysC}\textsuperscript{T311I}, \textit{pyc}\textsuperscript{P458S}, \textit{ppc}, or \textit{ppc} and \textit{pyc}\textsuperscript{P458S} were overexpressed from an IPTG-inducible plasmid in strain Dpa1 that already carries chromosomal copies of \textit{lysC}\textsuperscript{T311I} and \textit{pyc}\textsuperscript{P458S}. However, the resulting strains Dpa1(pVWEx1\_\textit{lysC}\textsuperscript{T311I}), Dpa1(pEKE\textit{Ex3}\_\textit{ppc}), Dpa1(pEKE\textit{Ex3}\_\textit{pyc}\textsuperscript{P458S}) and Dpa1(pEKE\textit{Ex3}\_\textit{ppc}\textit{pyc}\textsuperscript{P458S}) produced less DPA than the control strains Dpa1(pVWE\textit{X1}) and Dpa1(pEKE\textit{Ex3}). The simultaneous overexpression of both \textit{ppc} and \textit{pyc}\textsuperscript{P458S} had the most severe effect (Figure 4A). Slower growth of all strains may be due to the additional metabolic burden of the second plasmid. Thus, DPA production by strain Dpa1 could not be improved by these strategies, which indicated that DPA production was not limited by aspartokinase or anaplerosis in these strains.
Microorganisms 2022, 10, x FOR PEER REVIEW 11 of 24

Figure 4. Production of DPA by Dpa1 derived strains overexpressing ppc (yellow), pycP458S (orange), ppc and pycP458S (red) or lysC1311 (violet) from a second plasmid besides pECXT_Psyn_dpaAB. Control strains Dpa1(pVWEx1) and Dpa1(pEKEx3) are shown in grey (A). Comparison of overexpressing native dapA either from pEKEx3_dapA as a second plasmid besides pECXT_Psyn_dpaAB (strain Dpa1-pDapA; turquoise) or in a synthetic operon with dpaAB on plasmid pECXT_Psyn_dpaAB-dapA (strain Dpa1-DapA) (light blue) (B). Cultivation was performed in CgXII containing 40 g L−1 glucose for 144 h (A) or 96 h (B). Concentrations are given as means with standard deviations from triplicate cultivations.

Another possible limitation is the availability of HTPA, the substrate for dipicolinate synthase and the last intermediate shared with L-lysine biosynthesis. HTPA synthase (DapA) yields HTPA by condensing pyruvate with aspartate. Therefore, dapA was overexpressed either from a second IPTG-inducible plasmid (strain Dpa1-pDapA) or in a synthetic operon with dpaAB (strain Dpa1-DapA). However, biomass formation was perturbed and DPA accumulation by strains Dpa1-pDapA and Dpa1-DapA after 144 h was 11% and 36% to that of the control strains Dpa1(pEKEx3) and Dpa1, respectively (Figure 4B). Thus, HTPA availability did not limit DPA production by these strains.

Besides the target molecule DPA, Dpa1 formed 53.66 ± 12.46 g L−1 L-lysine as by-product (Table 2), which reflects its background of an engineered L-lysine producer. As an alternative to the overexpression of dapA to improve HTPA synthesis, its drain due to conversion by HTPA reductase DapB towards L-lysine biosynthesis may be reduced. To this end, dapB was repressed using the CRISPRi method. Two sgRNAs sequences, targeting dapB proximal or distant to the translational start site, were chosen to repress dapB. The resulting plasmids were used to transform strain Dpa1 yielding strains Dpa1(pS_dCas9_dapB14) and Dpa1(pS_dCas9_dapB561), respectively. Strain Dpa1(pS_dCas9) served as a negative control as it expressed dCas9 but lacked a sgRNA. CRISPRi mediated repression of dapB reduced L-lysine formation from 13.53 ± 0.73 g L−1 to 3.53 ± 0.06 g L−1 and 4.47 ± 0.12 g L−1, respectively, while DPA production was increased (Table 2). Strain Dpa1(pS_dCas9_dapB14) grew to a significantly higher biomass concentration (p < 0.001; 5.46 ± 0.26 g L−1 compared to 4.13 ± 0.12 g L−1 by Dpa1(pS_dCas9)) and produced significantly more DPA (p < 0.001; 1.91 ± 0.02 g L−1 compared to 1.73 ± 0.04 g L−1 by Dpa1(pS_dCas9)) (Table 2). Strain Dpa1(pS_dCas9_dapB561) did not grow to a significantly higher biomass concentration (p < 0.001; 4.50 ± 0.37 g L−1 compared to 4.13 ± 0.12 g L−1 by Dpa1(pS_dCas9)) but produced about twice as much DPA (3.28 ± 0.12 g L−1 compared to 1.73 ± 0.04 g L−1 by Dpa1(pS_dCas9)) (Table 2). While the quantitative extents of CRISPRi-mediated repression
of dapB on DPA, L-lysine and biomass concentrations varied depending on the used sgRNA targeting dapB, this strategy proved beneficial for improving DPA production.

3.5. Fermentative Production of DPA in Reactor Scale

In order to test the robustness of DPA production, a lab-scale fed-batch fermentation of Dpa1 was performed in a 7 L bioreactor using 2.5 L CgXII minimal medium containing 40 g·L⁻¹ glucose as seed medium. When glucose was depleted or cell growth had ceased, the addition of a total of 1.75 L feed, containing 100 g·L⁻¹ glucose and 40 g·L⁻¹ ammonium sulphate, was started manually. During the batch phase, biomass formation was twice as high as in shake flask cultivations, reaching 10.1 g·L⁻¹ at 39 h (Figure 5) (as compared to 4.48 ± 0.51 (Table 2)). At 41 h, 0.75 L of feed medium were added. Biomass formation increased little, but production of both DPA and L-lysine continued (Figure 5). At 70 h, another 1 L of feed medium was added. DPA and L-lysine production resumed and continued until cultivation end at 90 h (Figure 5). In this bioreactor culture, DPA was produced to final titer of 1.47 g·L⁻¹ at a yield on glucose of 0.0135 g·g⁻¹ with a volumetric productivity of 0.016 g·L⁻¹·h⁻¹.

![Figure 5. Fed-batch cultivation of Dpa1 in 2.5 L initial volume of CgXII minimal medium, containing 40 g·L⁻¹ glucose for 90 h. The feed contained 100 g·L⁻¹ glucose and 40 g·L⁻¹ ammonium sulphate and is depicted by the purple line. The relative dissolved oxygen concentration (rDOs) is shown (blue line). Glucose (brown), L-lysine (light green), DPA (green) and biomass (black) concentrations are indicated by symbols and lines. All concentrations were calculated to the initial fermentation volume.](image)

3.6. Establishing DPA Production from Alternative Carbon Sources

Besides glucose, amino acid production with C. glutamicum uses fructose-containing molasses or second generation feedstocks [66]. Fructose is a natural source of carbon and energy for C. glutamicum, while metabolic engineering was required for access to the polymer starch as well as to monomeric compounds of second-generation feedstocks such as glycerol, arabinose and xylose [65]. In this regard, Dpa1 was grown on fructose and established metabolic engineering strategies were applied to allow DPA production from glycerol, xylose, arabinose, and starch. The carbon sources fructose, glycerol, arabinose, and xylose were added in a concentration of 20 g·L⁻¹ and starch was added at 10 g·L⁻¹. In all cases, DPA production was observed. DPA yields attained from fructose, xylose, arabinose, glycerol, and starch did not reach those from glucose, and L-lysine titers surpassed those of the target compound DPA by two to six times (Table 2, Figure 6A).
Figure 6. Production of DPA (A) and ratio of production of DPA to L-lysine (B) by (engineered) Dpa1 from glucose, arabinose, xylose, glycerol and starch with glucose as co-substrate. Cultivation was performed in CgXII containing 20 g L\(^{-1}\) (10 g L\(^{-1}\) for starch) of the respective carbon source for 72 h. Concentrations are given as means with standard deviations from triplicate cultivations.

The cultivation with starch instead of glucose resulted in a more favorable DPA to L-lysine ratio (Figure 6B). Thus, DPA production from a broad spectrum of feedstocks was demonstrated.

3.7. Exploiting Phosphite Dehydrogenase to Establish Non-Sterile DPA Production

With the aim of establishing non-sterile production of DPA, a codon-harmonized version of the \(ptxD\) gene from \(P. stutzeri\) was expressed in \(C. glutamicum\) WT and the specific phosphite dehydrogenase activity was determined in crude extracts. While no activity was detected (<0.005 U·mg\(^{-1}\)) for the empty vector carrying control strains, 0.023 ± 0.002 U·mg\(^{-1}\) were determined for strain WT(pECXT_\(p_{c-k}\)\(p_{t-x}\)D), thus, demonstrating functional expression of \(ptxD\) in \(C. glutamicum\). Indeed, only WT(pECXT_\(p_{c-k}\)\(p_{t-x}\)D) could grow with phosphite as the sole source of phosphorus (Figure S1A). In the regular CgXII minimal medium (phosphate as phosphorous source, no tetracycline), the tetracycline resistance-mediating plasmid was lost after 17 serial dilutions into fresh CgXII liquid medium, since no tetracycline resistant colonies were found upon plating on LB-tetracycline agar plates but rather on LB plates without tetracycline. When phosphate in liquid CgXII minimal medium was replaced by phosphite, the plasmid pECXT_\(p_{c-k}\)\(p_{t-x}\)D was maintained, even in the absence of tetracycline (Figure S1B). This confirmed that phosphite and \(ptxD\) expression can be used as a selectable trait under non-sterile growth conditions.

For co-expression with the dipicolinate synthase genes \(dpaAB\) in a synthetic operon, the plasmid pECXT_\(p_{s-y-n}\)\(dpaAB\)\(\_p_{t-x}\)D was constructed and introduced into DM1729\(dpxk\), resulting in strain Dpa1-PtxD. The phosphorous sources in CgXII minimal medium were replaced with the equivalent amount (13 mM) of di-sodium phosphite. Dpa1, lacking phosphite dehydrogenase activity, did not grow with phosphite as a sole phosphorous source (Figure S2), while Dpa1-PtxD reached 45% of the biomass of Dpa1 from 40 g L\(^{-1}\) glucose (Table 2). The yield of DPA by Dpa1-PtxD from glucose with phosphite (0.04 g g\(^{-1}\)) corresponded to approximately 60% of the yield by Dpa1 and regular CgXII minimal medium (Table 2). Thus, DPA production using phosphite is feasible and the aptitude of the utilization of phosphite as the sole source of phosphorus as selectable trait for DPA production was demonstrated.

Beyond overriding the requirement of antibiotics, it would be preferable to replace glucose with a second-generation feedstock for DPA production. On this account, the
substrate spectrum of Dpa1-PtxD was broadened to glycerol, resulting in strain Dpa1-PtxD(pVWEx1 глpFkD).

In P-substituted CgXII, containing 13 mM phosphate, 1 mM IPTG and 40 g·L\(^{-1}\) 

glycerol with the omission of antibiotics, this strain achieved a similar concentration (1.64 ± 0.07 g·L\(^{-1}\)) and yield (0.04 ± 0.00 g·g\(^{-1}\)) as Dpa1-PtxD in P-substituted CgXII from glucose (Table 2), whereas the control strain Dpa1-PtxD(pVWEx) did not grow with glycerol as sole carbon source (Figure S3).

4. Discussion

In this study, we established the first de novo production of DPA by C. glutamicum from glucose and from alternative carbon sources. Moreover, utilization of phosphate instead of phosphate rendered the strain compatible with non-sterile DPA production, and we obtained the proof-of-concept of the process robustness for fermentative DPA production in fed-batch bioreactor cultivation.

Endospores of Bacilli and Clostridia contain little of the secondary metabolite DPA and sensitive methods (gold nanoparticles and nanoclusters for fluorescence detection) were developed to detect DPA [110–112]. Gram-scale DPA production in B. subtilis required addition of glutamate as a precursor to three-fold higher concentrations than the final product titer [36]. DPA production from glucose without the requirement to add an amino acid as a precursor was first implemented in recombinant E. coli. It reached a concentration of 4.7 g·L\(^{-1}\) DPA with a yield of about 0.11 g·g\(^{-1}\) and an overall volumetric productivity of 65 mg·L\(^{-1}\)·h\(^{-1}\) [32]. The C. glutamicum strain constructed here produced 2.54 g·L\(^{-1}\) DPA with a yield on glucose of about 0.06 g·g\(^{-1}\) and an overall volumetric productivity of 26 mg·L\(^{-1}\)·h\(^{-1}\). These titers are considerably lower than the L-lysine titers of up to 100 g·L\(^{-1}\) obtained with E. coli and C. glutamicum L-lysine producing strains [38]. Production of L-lysine-derived products capitalizes on this fact and titers for cadaverine [47] or L-PA [45] are very high, as well. Product titers are lower the longer the extension of L-lysine biosynthesis is, e.g., two or three reaction steps to 5AVA [48,113], five steps to glutarate [22] or six steps to L-2-hydroxy-glutarate [32], with a product titer of 3.5 g·L\(^{-1}\) obtained in the latter example. Similarly, interception of L-lysine biosynthesis by converting the intermediate aspartate-semialdehyde to ectoine [114] or of the intermediate L-aspartate to 3-aminopropanoate [56] led to high product titers (65 g·L\(^{-1}\) and 32.3 g·L\(^{-1}\), respectively).

Overexpression of genes that have been used in L-lysine production, such as the alleles that improve aspartokinase (lysC\(^{T311I}\)) and pyruvate carboxylase (pyc\(^{P458S}\)) activity [115–117], did not increase DPA production in this work, indicating that flux into the L-lysine pathway andoxaloacetate availability is not limiting DPA production. Moreover, the availability of HTPA, the substrate for dipicolinate synthase, does not limit DPA production in the observed concentration range, as shown by overexpression of the HTPA synthase gene that did not improve DPA titers, in contrast to previous studies [118,119]. The low DPA product titer indicates that the one-step conversion of the L-lysine biosynthesis intermediate HTPA to DPA is a major limitation. The well-studied dipicolinate synthase from B. subtilis [32] may be an alternative to the respective enzyme from P. sonchi SBR5 used in this study. Little is known about dipicolinate synthases from paenibacilli [120], and screening for alternative sources of dipicolinate synthases may help to improve DPA production. In this respect, it has to be noted that the Km value of dipicolinate synthase from B. subtilis of 0.776 mM [32] is comparable to that of DapB (0.529 mM) in E. coli [121], the L-lysine biosynthesis enzyme competing for the shared substrate HTPA. Thus, dipicolinate synthases with higher affinities may prove helpful for increasing DPA production.

Improving HTPA supply for dipicolinate synthase by overexpression of the gene for HTPA synthesis dapA did not enhance DPA production but reduced the growth rate. Previously, overexpression of native dapA in C. glutamicum increased the flux towards the L-lysine pathway while lowering the flux into the competing homoserine pathway. However, this entailed a reduced growth rate and elevated L-valine, and L-alanine formation [118,122]. Therefore, the maintenance of the highly sensitive flux balance at this strategic branchpoint
must be considered but fine-tuning and adjustment of \( dpaA \) expression levels may prove beneficial for DPA production. At the same time, \( dapB \) constitutes a promising target to decrease the drain of HTPA into L-lysine biosynthesis by knockdown of the gene. CRISPRi-mediated repression of \( dapB \) reduced L-lysine formation and increased the DPA titer and yield. Thus, decreased utilization of HTPA towards lysine biosynthesis by DapB upon CRISPRi targeting of its gene was superior to increased HTPA biosynthesis as consequence of \( dapA \) overexpression. This may be due to the fact that Km values for HTPA are very low, at around 0.005 mM for bacterial DapB enzymes [123]. Km values of DPA synthase for HTPA have not been described. It has to be noted that during sporulation of \( B. subtilis \), RNA polymerase sigma factor SigK is promoting transcription of \( dpaAB \) and \( dapA \); \( dapB \) is not part of the SigK sigmulon [124]. Thus, this transcriptional regulatory pattern of SigK-dependent expression of \( dpaAB \) and \( dapA \) in the mother cell ensures that HTPA is synthesized for conversion to DPA but is not used by DapB in L-lysine biosynthesis. Notably, DPA is transported from the mother cell to the forespore, crossing two membranes, i.e., the outer and inner forespore membranes. The transporter SpoVV is located in the outer forespore membrane [125], and SpoVA in the inner forespore membrane [126]. Expression of \( spoVV \) and \( spoVA \) is orchestrated with SigE-dependent transcription of \( spoVV \) during engulfment prior to SigG-dependent expression of \( spoVA \) after completion of engulfment, finally followed by SigK-dependent expression of \( dpaAB \) and \( dapA \) in the mother cell [125].

Transport engineering is a valid metabolic engineering strategy [127]. Production of L-lysine derived products benefitted from deletion of \( lysE \), which codes for the export system for L-lysine, L-arginine and L-citrulline [128,129], since loss of L-lysine is precluded [54,130]. However, the growth rate may be decreased if the L-lysine biosynthesis pathway is clogged upon deletion of \( lysE \) [130]. This was also observed when L-lysine biosynthesis was interrupted for ectoine production and \( lysE \) was deleted [55]. This growth impediment indicates that deletion of \( lysE \) is only beneficial if production of these compounds is already almost as high as L-lysine production by the parent strains. In \( C. glutamicum \), DPA production shown here revealed that DPA is exported, but the export system is not known. There are no homologs of \( spoVV \) and \( spoVA \) (s. above) encoded in the \( C. glutamicum \) genome. Transport engineering by deregulated expression of \( spoVV \) during vegetative growth led to secretion of about 65 mg L\(^{-1}\) DPA [125].

The decreased production with the presence of increased concentrations of DPA in the cultivation medium suggests that inherent characteristics of DPA may perturb growth of the production host, e.g., divalent ion chelating. The enzyme 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase of the shikimate pathway from \( B. subtilis \) is inhibited by DPA as it contains iron and zinc ions essential for activity [131]. The slight inhibitory effect of DPA on growth of \( C. glutamicum \) WT is not yet understood, however, the structure of its 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase also contains a divalent cation, namely \( Mn^{2+} \) [132]. In recent years, adaptive laboratory evolution has emerged as an excellent approach to select for improved growth, tolerance and production [133–136]. Tolerance of \( C. glutamicum \) to methanol [137,138], anthranilate [139] and indole [140] was improved by this strategy that may be applicable to increasing tolerance to DPA. When engineered such that DPA production would be required for growth using a metabolic engineering strategy known as flux enforcement, adaptive laboratory evolution can be employed to enhanced production, as has been shown for glutarate production by \( C. glutamicum \) [51]. In addition, media optimization, e.g., with regard to the C/N substrate ratio [141] or concentrations of supplements [36], may improve DPA production.

DPA production was also shown from alternative carbon sources. Production from xylose, arabinose and fructose was reduced considerably as compared to glucose. In the case of fructose, the titer was reduced to 65% of that from glucose (Figure 6A). Similarly, L-lysine production from fructose is lower than from glucose [116], since the carbon flux via the oxidative pentose phosphate pathway is lower on fructose than on glucose, resulting in lower NADPH availability [142]. The biosyntheses of L-lysine and DPA require four and three NADPH molecules per product molecule, respectively. This is also the reason
why less L-lysine and DPA is formed from the carbon sources arabinose and xylose as these pentose sugars enter the pentose phosphate pathway without concomitant NADPH formation [70,143]. By contrast, the more reduced glycerol supported higher production of DPA (Table 2, Figure 6), putrescine [144] and L-lysine [86]. Thus, DPA production from glycerol and pentoses that can be generated from fats and second-generation feedstocks such as lignocellulose or agricultural waste materials, is possible. Recent examples for the production based on agricultural sidestreams by recombinant *C. glutamicum* are cis, cis-muconic acid from lignin [145], 2-hydroxy-glutarate and 5AVA from rice straw hydrolysate or wheat sidestream [52,146,147].

Biorefinery concepts also operate using microbial consortia that may be designed for that purpose [148]. Synthetic consortia with *C. glutamicum* have been used to access chitin and starch with mutually dependent substrate converter and producer strains [67,149]. In biorefinery concepts, the omission of antibiotics in a non-sterile environment may be particularly beneficial to reduce media complexity and cost. DPA production exploiting *ptxD* and phosphite as selective trait was shown here and is expected to be compatible with second-generation feedstock-based production of DPA in non-sterile processes.

While DPA production was stable at the 2.5 L bioreactor scale, more substrate was converted to biomass and less to DPA when compared to the shake flask cultures. It is tempting to speculate that the cation chelating effect of DPA on growth in the bioreactor is different than in shake flasks. Alternatively, or in addition, changed fluxes in the lysine pathway, which also provides *meso*-diaminopimelic acid for cell wall peptidoglycan biosynthesis, may affect the cell wall strength and, thus, have different consequences under the different mechanical forces of shake flasks and bioreactors. The molecular reason(s) and possible adaptive regulatory mechanisms remain to be identified.

This study established a proof-of-principle of *de novo* production of DPA by *C. glutamicum* from glucose and from alternative carbon sources compatible with non-sterile conditions. To achieve commercially relevant titers, yields and productivities, the discussion above may guide the further improvement of strain and process.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms10040730/s1, Figure S1: Growth curves of *C. glutamicum* (pECXT_Psyn_ptxD) and control strain *C. glutamicum* (pECXT_Psyn); Figure S2. Growth curves of *C. glutamicum* Dpa1-PtrX and control strain Dpa1; Figure S3. Growth curves of *C. glutamicum* Dpa1-PtrX(pVWEx1_glpFDK) control strain Dpa1-PtrX(pVWEx1); Table S1. Oligonucleotides used in this work; Table S2. DNA sequences used in this work.

**Author Contributions:** L.S.S. and V.F.W. designed the experiments. V.F.W. acquired funding. V.F.W. coordinated the study. L.S.S. and A.K.D. constructed strains. L.S.S. performed the experiments. L.S.S. and V.F.W. analyzed the data. T.S. and L.S.S. performed bioreactor experiments. L.S.S. drafted the manuscript. V.F.W. finalized the manuscript. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was supported in part by the BMBF project ForceYield (031B0825C).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** All data are present in the manuscript and its Supplement.

**Acknowledgments:** We thank Angelika Urban, Ahmed Zahoor and Daniel Hagedorn for help with construction of the plasmids pECXT_Psyn_dpaAB, pVWEx1_amyA and pEKEx3_ppc-pycP458S. We thank Florian Meyer and Joe Risse for scientific discussions about bioreactor cultivations.

**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. Li, Q.-F.; Ge, G.-W.; Sun, Y.; Yu, M.; Wang, Z. Influence of counter ions on structure, morphology, thermal stability of lanthanide complexes containing dipicolinic acid ligand. Spectrochim. Acta. A. Mol. Biomol. Spectrosc. 2019, 214, 333–338. [CrossRef]

2. Wang, J.; de Kool, R.H.M.; Velders, A.H. Lanthanide-Dipicolinic Acid Coordination Driven Micelles with Enhanced Stability and Tunable Function. Langmuir 2019, 35, 12251–12259. [CrossRef] [PubMed]

3. Kirillova, M.V.; Kirillov, A.M.; Guedes da Silva, M.F.C.; Pombeiro, A.J.L. Self-Assembled Two-Dimensional Water-Soluble Dipicolinate Cu/Na Coordination Polymer: Structural Features and Catalytic Activity for the Mild Peroxidation Oxidation of Cycloalkanes in Acid-Free Medium. Eur. J. Inorg. Chem. 2008, 2008, 3423–3427. [CrossRef]

4. Xu, C.; Tian, G.; Teat, S.J.; Rao, L. Complexation of U(VI) with dipicolinic acid: Thermodynamics and coordination modes. Inorg. Chem. 2013, 52, 2750–2756. [CrossRef] [PubMed]

5. Mihaara, Y.; Nagaoka, S.; Okazaki, M.; Fuji Photo Film Co. Ltd. Photographische Silberhalogenidemulsion. EU Patent EP0116346B1, 31 May 1989.

6. Buchdahl, M.R.; Soine, T.O. The synthesis of some substituted pyridine and piperidine compounds from dipicolinic acid. J. Am. Pharm. Assoc. Am. Pharm. Assoc. 1952, 41, 225–229. [CrossRef]

7. Blumbergs, J.H.; Latourette, H.K.; FMC Corp. Stabilization of Peroxy Carboxylic Acids. US Patent US3130169A, 21 April 1964.

8. Clark, D.E. Peroxides and peroxide-forming compounds. Chem. Health Saf. 2001, 8, 12–22. [CrossRef]

9. Greenspan, F.P.; Mackellar, D.G.; Buffalo Electro Chem Co. Stabilization of Peroxidic Acid. US Patent US2609391A, 2 September 1952.

10. Murakami, K.; Tanemura, Y.; Yoshino, M. Dipicolinic acid prevents the copper-dependent oxidation of low density lipoprotein. J. Nutr. Biochem. 2003, 14, 99–103. [CrossRef]

11. Murakami, K.; Yoshino, M. Dipicolinic Acid as an Antioxidant: Protection of Glutathione Reductase from the Inactivation by Copper. Biomed. Res. 1999, 20, 321–326. [CrossRef]

12. Siddiqi, Z.A.; Khalid, M.; Kumar, S.; Shahid, M.; Noor, S. Antimicrobial and sod activities of novel transition metal complexes of pyridine-2,6-dicarboxylic acid containing 4-picoline as auxiliary ligand. Eur. J. Med. Chem. 2010, 45, 264–269. [CrossRef]

13. Soleimani, E. Synthesis, characterization and anti-microbial activity of a novel macrocyclic ligand derived from the reaction of Propanediol and Various Aromatic Dicarboxylic Acids. J. Macromol. Sci. Part A 2003, 40, 791–805. [CrossRef]

14. Song, X.-G.; Han, M.-H.; He, F.; Wang, S.-Y.; Huang, Z.-G.; Liu, D.; Liu, F.-Q.; Laborda, P.; et al. Antifungal Mechanism of Dipicolinic Acid and Its Efficacy for the Biocontrol of Pear Valsa Canker. Front. Microbiol. 2020, 11, 958. [CrossRef] [PubMed]

15. Banerji, S.K.; Regmi, T.P. Biodegradation of the chlorate 2,6-pyridine dicarboxylic acid (PDA) used for soil metal extraction. Waste Manag. 1998, 18, 331–338. [CrossRef]

16. Roupakias, C.P.; Papageorgiou, G.Z.; Karayannidis, G.P. Synthesis and Thermal Behavior of Polyesters Derived from 1,3-Propanediol and Various Aromatic Dicarboxylic Acids. J. Macromol. Sci. Part A 2003, 40, 791–805. [CrossRef]

17. Sims, G.K.; O’Loughlin, E.J.; Crawford, R.L. Degradation of pyridines in the environment. Crit. Rev. Environ. Control 1989, 19, 309–340. [CrossRef]

18. Pellis, A.; Comerford, J.W.; Weinberger, S.; Guebitz, G.M.; Clark, J.H.; Farmer, T.J. Enzymatic synthesis of lignin derivable pyridine via a Novel Synthetic Pathway. Front. Microbiol. 2012, 3, 958. [CrossRef]

19. Nguyen, A.Q.D.; Schneider, J.; Reddy, G.K.; Wendisch, V.F. Fermentative Production of the Diamine Putrescine: System Metabolic Engineering of Corynebacterium glutamicum. Metabolites 2015, 5, 211–231. [CrossRef]

20. Pérez-García, F.; Jorge, J.M.P.; Dreyzas, A.; Risse, J.M.; Wendisch, V.F. Efficient Production of the Dicarboxylic Acid Glutarate by Corynebacterium glutamicum via a Novel Synthetic Pathway. Front. Microbiol. 2018, 9, 2589. [CrossRef]

21. Leggett, M.J.; McDonnell, G.; Denyer, S.P.; Setlow, P.; Maillard, J.-Y. Bacterial spore structures and their protective role in biocide resistance. J. Appl. Microbiol. 2012, 113, 485–498. [CrossRef]

22. Sunde, E.P.; Setlow, P.; Hederstedt, L.; Halle, B. The physical state of water in bacterial spores. Proc. Natl. Acad. Sci. USA 2009, 106, 19334–19339. [CrossRef] [PubMed]

23. Slieman, T.A.; Nicholson, W.L. Role of Dipicolinic Acid in Survival of Bacillus subtilis Spores Exposed to Artificial and Solar UV Radiation. Appl. Environ. Microbiol. 2001, 67, 1274–1279. [CrossRef] [PubMed]

24. Lindsay, J.A.; Murrell, W.G. Solution spectroscopy of dipicolinic acid interaction with nucleic acids: Role in spore heat resistance. Curr. Microbiol. 1986, 13, 255–259. [CrossRef]

25. Daniel, R.A.; Errington, J. Cloning, DNA Sequence, Functional Analysis and Transcriptional Regulation of the Genes Encoding Dipicolinic Acid Synthetase Required for Sporulation in Bacillus subtilis. J. Mol. Biol. 1993, 232, 468–483. [CrossRef]
51. Prell, C.; Busche, T.; Rückert, C.; Nolte, L.; Brandenbusch, C.; Wendisch, V. F. Adaptive laboratory evolution accelerated glutarate production by Corynebacterium glutamicum. Metab. Eng. 2018, 48, 208–217. [CrossRef]

52. McClintock, M.K.; Fahnhorst, G.W.; Hoye, T.R.; Zhang, K. Engineering the production of dipicolinic acid in Bacillus subtilis. J. Med. Chem. 2016, 59, 4710–4716. [CrossRef]

53. Wördemann, R.; Wiefel, L.; Wendisch, V. F.; Steinbüchel, A. Incorporation of alternative amino acids into cyanophycin by different Bacillus species. Front. Bioeng. Biotechnol. 2019, 7, 153. [CrossRef]

54. Dévé, S.R.A.; Blunt, J.W.; Gerrard, J.A. NMR studies uncover alternate substrates for dihydrodipicolinate synthase and suggest that dihydrodipicolinate reductase is also a dehydratase. J. Med. Chem. 2010, 53, 4808–4812. [CrossRef]

55. Blickling, S.; Renner, C.; Laber, B.; Pohlenz, H.-D.; Holak, T.A.; Huber, R. Reaction Mechanism of Escherichia coli Dihydrodipicolinate Synthase Investigated by X-ray Crystallography and NMR Spectroscopy. Biochemistry 1997, 36, 24–33. [CrossRef]

56. Ygargi, Y.; Gilvarg, C. The Condensation Step in Diaminopimelate Synthesis. J. Biol. Chem. 1965, 240, 4710–4716. [CrossRef]

57. McClintock, M.K.; Fahnhorst, G.W.; Hoye, T.R.; Zhang, K. Engineering the production of dipicolinic acid in Bacillus subtilis. J. Med. Chem. 2016, 59, 4710–4716. [CrossRef]

58. Wendisch, V. F. Metabolic engineering advances and prospects for amino acid production. Metab. Eng. 2020, 58, 17–34. [CrossRef]

59. Zelder, O.; Hauer, B. Environmentally directed mutations and their impact on industrial biotransformation and fermentation processes. Curr. Opin. Microbiol. 2000, 3, 248–251. [CrossRef]

60. Wendisch, V. F.; Lee, J.-H. Metabolic Engineering in Corynebacterium glutamicum. In Corynebacterium glutamicum—Biology and Biotechnology;INU, M., Toyoda, K., Eds.; Microbiology Monographs; Springer International Publishing: Cham, The Netherlands, 2020; pp. 287–322, ISBN 978-3-030-39267-3. [CrossRef]

61. Unthan, S.; Baumgart, M.; Radek, A.; Siebert, D.; Brühl, N.; Bartsch, A.; Bott, M.; Wiechert, W.; Marin, K.; et al. Chassis organism from Corynebacterium glutamicum—A top-down approach to identify and delete irrelevant gene clusters. Biotechnol. J. 2015, 10, 290–301. [CrossRef]

62. Wendisch, V. F. Genome-reduced Corynebacterium glutamicum fit for biotechnological applications. In Minimal Cells: Design, Construction, Biotechnological Applications; Lara, A.R., Gosset, G., Eds.; Springer International Publishing: Cham, The Netherlands, 2020; pp. 95–116, ISBN 978-3-030-31891-0. [CrossRef]

63. Cho, J.S.; Choi, K.R.; Prabowo, C.P.S.; Shin, J.H.; Yang, D.; Jang, J.; Lee, S.Y. CRISPR/Cas9-coupled recombining for metabolic engineering of Corynebacterium glutamicum. Metab. Eng. 2017, 42, 157–167. [CrossRef]

64. Cleto, S.; Jensen, J.V.; Wendisch, V. F.; Lu, T.K. Corynebacterium glutamicum Metabolic Engineering with CRISPR Interference (CRISPRi). ACS Synth. Biol. 2016, 5, 375–385. [CrossRef]

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

66. Prell, C.; Vonderbank, S.-A.; Meyer, F.; Pérez-Garcia, F.; Wendisch, V. F. Metabolic Engineering of Corynebacterium glutamicum for de novo production of 3-hydroxydacetaraine. Curr. Res. Biotechnol. 2022, 4, 32–46. [CrossRef]

67. Mimituška, T.; Sawal, H.; Hatsu, M.; Yamada, K. Metabolic Engineering of Corynebacterium glutamicum for Cadaverine Fermentation. Biotechnol. Biochem. 2007, 71, 2130–2135. [CrossRef] [PubMed]

68. Jorge, J.M.P.; Pérez-García, F.; Wendisch, V. F. A new metabolic route for the fermentative production of 5-aminovanillate from glucose and alternative carbon sources. Bioreourc. Technol. 2017, 245, 1701–1709. [CrossRef]

69. Shin, J.H.; 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-aminovanillic acid. Microb. Cell Fact. 2016, 15, 174. [CrossRef]

70. Han, T.; Kim, G.B.; Lee, S.Y. Glutaric acid production by systems metabolic engineering of an L-lysine–overproducing Corynebacterium glutamicum organism from Escherichia coli. Microb. Cell Fact. 2013, 12, 110. [CrossRef]

71. Pérez-García, F.; Ziert, C.; Risse, J.M.; Wendisch, V. F. Improved fermentative production of the compatible solute ectoine by Corynebacterium glutamicum from glucose and alternative carbon sources. J. Biotechnol. 2017, 258, 59–68. [CrossRef]

72. Song, C.W.; Lee, J.; Ko, Y.-S.; Lee, S.Y. Metabolic engineering of Escherichia coli for the production of 3-aminopropionic acid. Metab. Eng. 2015, 30, 121–129. [CrossRef] [PubMed]
83. Peters-Wendisch, P.G.; Kreutzer, C.; Kalinowski, J.; Pátek, M.; Sahm, H.; Eikmanns, B.J. Pyruvate carboxylase from Corynebacterium glutamicum: Characterization, expression and inactivation of the pyc gene. Microbiol. Rev. Engl. 1998, 144 (Pt 4), 915–927. [CrossRef]
84. LeBmeier, L.; Pfeifenschneider, J.; Carnicer, M.; Heux, S.; Portais, J.-C.; Wendisch, V.F. Production of carbon-13-labeled cadaverine by engineered Corynebacterium glutamicum using carbon-13-labeled methanol as co-substrate. Appl. Microbiol. Biotechnol. 2015, 99, 10163–10176. [CrossRef]
85. Schneider, J.; Niemann, K.; Wendisch, V.F. Production of the amino acids L-glutamate, L-lysine, L-ornithine and L-arginine from arabinose by recombinant Corynebacterium glutamicum. J. Biotechnol. 2011, 154, 191–198. [CrossRef]
86. 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. [CrossRef]
87. Stanssens, C.; Uy, D.; Delaunay, S.; Eggelings, L.; Goergen, J.-L.; Wendisch, V.F. Characterization of a Corynebacterium glutamicum lactate utilization operon during temperature-triggered glutamate production. Appl. Environ. Microbiol. 2005, 71, 5920–5928. [CrossRef] [PubMed]
88. Ziert, C. Metabolic Engineering of Corynebacterium glutamicum for the Production of L-Aspartate and Its Derivatives β-Alanine and Etcode. Doctoral Dissertation, Bielefeld University, Germany, 2014. Available online: https://pub.uni-bielefeld.de/record/2691217 (accessed on 10 March 2022).
89. Göttl, V.; Schmitt, I.; Braun, K.; Peters-Wendisch, P.; Wendisch, V.F.; Henke, N.A. CRISPRi-library guided target identification for engineering carotenoid production by Corynebacterium glutamicum. Microorganisms 2021, 9, 670. [CrossRef]
90. Gibson, D.G.; Young, L.; Chuang, R.-Y.; Venter, J.C.; Hutchison, C.A.; Smith, H.O. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat. Methods 2009, 6, 343–345. [CrossRef] [PubMed]
91. Schneider, J.; Wendisch, V.F. Putrescine production by engineered Corynebacterium glutamicum. Appl. Microbiol. Biotechnol. 2010, 88, 859–868. [CrossRef] [PubMed]
92. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 1976, 72, 248–254. [CrossRef]
93. Hung, J.E.; Fogle, E.J.; Garg, N.; Chekan, J.R.; Nair, S.K.; Donk, W.A. van der Chemical Rescue and Inhibition Studies to Determine the Role of Arg301 in Phosphite Dehydrogenase. PLoS ONE 2014, 9, e87134. [CrossRef] [PubMed]
94. Sambrook, J.; Fritsch, E.F.; Maniatis, T. Molecular Cloning: A Laboratory Manual; Cold Spring Harbor Laboratory Press: New York, NY, USA, 1989; ISBN 0-87969-309-6.
95. Engels, V.; Wendisch, V.F. The DeoR-Type Regulator SugR Represses Expression of the L-Lactate Utilization Operon Induced during Temperature-Triggered Glutamate Production. J. Mol. Microbiol. Biotechnol. 2007, 15, 2955–2966. [CrossRef] [PubMed]
96. Eikmanns, B.J.; Metzger, M.; Reinscheid, D.; Kircher, M.; Sahm, H. Amplification of three threonine biosynthesis genes in Corynebacterium glutamicum strain for efficient L-lysine production. Mol. Microbiol. 2005, 3, 295–300. [PubMed]
97. Engels, V.; Lindner, S.N.; Wendisch, V.F. The Global Repressor SugR Controls Expression of Genes of Glycolysis and of the L-Lactate Dehydrogenase LdhA by Corynebacterium glutamicum. J. Mol. Microbiol. Biotechnol. 2001, 3, 1197–1204. [CrossRef] [PubMed]
98. Ikeda, M.; Ohnishi, J.; Hayashi, M.; Mitsuhashi, S. A genome-based approach to create a minimally mutated Corynebacterium glutamicum strain for efficient L-lysine production. J. Ind. Microbiol. Biotechnol. 2006, 33, 610–615. [CrossRef]
99. Peters-Wendisch, P.G.; Schiel, B.; Wendisch, V.F.; Katsoulidis, E.; Möckel, 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. [PubMed]
100. Engels, V.; Wendisch, V.F. The DeoR-Type Regulator SugR Represses Expression of ptsG in Corynebacterium glutamicum. J. Bacteriol. 2007, 189, 2955–2966. [CrossRef] [PubMed]
101. Follettie, M.T.; Shin, H.K.; Sinskey, A.J. Organization and regulation of the Corynebacterium glutamicum hom-thrB and thrCloci. Mol. Microbiol. 1998, 2, 53–62. [CrossRef]
102. Riedel, C.; Rittmann, D.; Dangel, P.; Möckel, B.; Petersen, S.; Sahm, H.; Eikmanns, B.J. Characterization of the phosphoenolpyruvate carboxykinase gene from Corynebacterium glutamicum and significance of the enzyme for growth and amino acid production. J. Mol. Microbiol. Biotechnol. 2001, 3, 573–583.
103. Engels, V.; Lindner, S.N.; Wendisch, V.F. The Global Repressor SugR Controls Expression of Genes of Glycolysis and of the L-Lactate Dehydrogenase LdhA in Corynebacterium glutamicum. J. Bacteriol. 2008, 190, 8033–8044. [CrossRef] [PubMed]
104. Engels, V.; Wendisch, V.F. The DeoR-Type Regulator SugR Represses Expression of ptsG in Corynebacterium glutamicum. J. Bacteriol. 2007, 189, 2955–2966. [CrossRef] [PubMed]
105. Gaigalat, L.; Schlüter, J.-P.; Hartmann, M.; Mormann, S.; Tauch, A.; Pühler, A.; Kalinowski, J. The DeoR-type transcriptional regulator SugR acts as a repressor for genes encoding the phosphoenolpyruvate:sugar phosphotransferase system (PTS) in Corynebacterium glutamicum. BMC Mol. Biol. 2007, 8, 104. [CrossRef] [PubMed]
106. Toyoda, K.; Teramoto, H.; Inui, M.; Yokawa, H. The IdhA Gene, Encoding Fermentative L-Lactate Dehydrogenase of Corynebacterium glutamicum, is under the Control of Positive Feedback Regulation Mediated by LldR. J. Bacteriol. 2009, 191, 4251–4258. [CrossRef] [PubMed]
107. Blombach, B.; Riester, T.; Wieschauka, S.; Ziert, C.; Youn, J.-J.; Wendisch, V.F.; Eikmanns, B.J. Corynebacterium glutamicum Tailored for Efficient J-Isobutanol Production. Appl. Environ. Microbiol. 2011, 77, 3300–3310. [CrossRef] [PubMed]
108. Brito, L.F.; Irla, M.; Kalinowski, J.; Wendisch, V.F. Detailed transcriptome analysis of the plant growth promoting Paenibacillus ringogradensis SBR5 by using RNA-seq technology. *BMC Genom.* 2017, 18, 846. [CrossRef]

109. Eikmanns, B.J.; Follettie, M.T.; Griot, M.U.; Sinskey, A.J. The phosphoenolpyruvate carboxylase gene of Corynebacterium glutamicum: Molecular cloning, nucleotide sequence, and expression. *Mol. Gen. Genet.* 1989, 218, 330–339. [CrossRef] [PubMed]

110. Baig, M.M.F.; Chen, Y.-C. Gold nanoparticle-based colorimetric sensing of dipicolinic acid from complex samples. *Anal. Bioanal. Chem.* 2018, 410, 1805–1815. [CrossRef] [PubMed]

111. Koo, T.M.; Ko, M.J.; Park, B.C.; Kim, M.S.; Kim, Y.K. Fluorescent detection of dipicolinic acid as a biomarker in bacterial spores employing terbium ion-coordinated magnetite nanoparticles. *J. Hazard. Mater.* 2021, 408, 124870. [CrossRef] [PubMed]

112. Li, X.; Luo, J.; Jiang, X.; Yang, M.; Rasooly, A. Gold nanocluster-europium(III) ratiometric fluorescence assay for dipicolinic acid. *Mikrochim. Acta.* 2021, 188, 26. [CrossRef]

113. Rohles, C.M.; Gießelmann, G.; Kohlstedt, M.; Wittmann, C.; Becker, J. Systems metabolic engineering of Corynebacterium glutamicum for the production of the carbon-5 platform chemicals 5-aminovalerate and glutarate. *Microb. Cell Fact.* 2016, 15, 154. [CrossRef]

114. Gießelmann, G.; Dietrich, D.; Jungmann, L.; Kohlstedt, M.; Jeon, E.J.; Yim, S.S.; Sommer, F.; Zimmer, D.; Mühlhaus, T.; Schroda, M.; et al. Metabolic Engineering of Corynebacterium glutamicum for High-Level Ectoine Production: Design, Combinatorial Assembly, and Implementation of a Transcriptionally Balanced Heterologous Ectoine Pathway. *Biotechnol. J.* 2019, 14, e1800417. [CrossRef] [PubMed]

115. Becker, J.; Klopprogge, C.; Zelder, O.; Heinzel, E.; Wittmann, C. Amplified Expression of Fructose 1,6-Bisphosphatase in Corynebacterium glutamicum Increases In Vivo Flux through the Pentose Phosphate Pathway and Lysine Production on Different Carbon Sources. *Appl. Environ. Microbiol.* 2005, 71, 8587–8596. [CrossRef]

116. Georgi, T.; Rittmann, D.; Wendisch, V.F. Lysine and glutamate production by Corynebacterium glutamicum on glucose, fructose and sucrose: Roles of malic enzyme and fructose-1,6-bisphosphatase. *Metab. Eng.* 2005, 7, 291–301. [CrossRef]

117. Ohnishi, J.; Mitsuhashi, S.; Hayashi, M.; Ando, S.; Yokoi, H.; Ochiai, K.; Ikeda, M. A novel methodology employing Corynebacterium glutamicum genome information to generate a new L-lysine-producing mutant. *Appl. Microbiol. Biotechnol.* 2002, 58, 217–223. [CrossRef]

118. Eggeling, L.; Oberle, S.; Sahm, H. Improved L-lysine yield with Corynebacterium glutamicum: Use of dapA resulting in increased flux combined with growth limitation. *Appl. Microbiol. Biotechnol.* 1995, 49, 24–30. [CrossRef]

119. Vašicová, P.; Pátek, M.; Nešvera, J.; Sahm, H.; Eikmanns, B. Analysis of the Corynebacterium glutamicum dapA Promoter. *J. Bacteriol.* 1999, 181, 6188–6191. [CrossRef] [PubMed]

120. Anandan, K.; Vittal, R.R. Endophytic Paenibacillus amylolyticus KMCE06 Extracted Dipicolinic Acid as Antibacterial Agent Derived via Dipicolinic Acid Synthetase Gene. *Curr. Microbiol.* 2019, 78, 178–186. [CrossRef] [PubMed]

121. Coulter, C.V.; Gerrard, J.A.; Kraunsoe, J.A.E.; Pratt, A.J. Escherichia coli dihydrodipicolinate synthase and dihydrodipicolinate reductase: Kinetic and inhibition studies of two putative herbicide targets. *Pestic. Sci.* 1999, 55, 887–895. [CrossRef]

122. Eikmanns, B.J.; Eggeling, L.; Sahm, H. Molecular aspects of lysine, threonine, and isoleucine biosynthesis in Corynebacterium glutamicum. *Anteine Van Leeuwenhoek* 1993, 64, 145–163. [CrossRef]

123. Anand, V.; Gautam, A.; Sareen, D.; Singh, T.P.; Tewari, R. Molecular cloning, biochemical and biophysical studies of dihydrodipicolinate reductase of Pseudomonas aeruginosa PA01. *Int. J. Integr. Biol.* 2011, 11, 145–152.

124. Eichenberger, P.; Fujita, M.; Jensen, S.T.; Conlon, E.M.; Rudner, D.Z.; Wang, S.T.; Ferguson, C.; Haga, K.; Sato, T.; Liu, J.S.; et al. The Program of Gene Transcription for a Single Differentiating Cell Type during Sporulation in Bacillus subtilis. *PLoS Biol.* 2004, 2, e328. [CrossRef]

125. Ramirez-Guadiana, F.H.; Meeske, A.J.; Rodrigues, C.D.A.; Barajas-Ornelas, R.D.C.; Kruse, A.C.; Rudner, D.Z. A two-step transport pathway allows the mother cell to nurture the developing spore in Bacillus subtilis. *PLoS Genet.* 2017, 13, e1007015. [CrossRef]

126. Wang, S.T.; Setlow, B.; Conlon, E.M.; Lyon, J.L.; Imamura, D.; Sato, T.; Setlow, P.; Losick, R.; Eichenberger, P. The forespore line of Bacillus subtilis. *J. Mol. Biol.* 2006, 358, 16–38. [CrossRef]

127. Pérez-García, F.; Wendisch, V.F. Transport and metabolic engineering of the cell factory Corynebacterium glutamicum. *FEBS Microbiol. Lett.* 2018, 365, 36y166. [CrossRef]

128. Lubitz, D.; Jorge, J.M.P.; Pérez-García, F.; Taniguchi, H.; Wendisch, V.F. Roles of export genes cgmA and lseE for the production of L-arginine and L-citrulline by Corynebacterium glutamicum. *Appl. Microbiol. Biotechnol.* 2016, 100, 8465–8474. [CrossRef]

129. Vrijic, M.; Sahm, H.; Eggeling, L. A new type of transporter with a new type of cellular function: L-lysine export from Corynebacterium glutamicum. *Mol. Microbiol.* 1996, 22, 815–826. [CrossRef] [PubMed]

130. Pérez-García, F.; Max Risse, J.; Friehs, K.; Wendisch, V.F. Fermentative production of L-pipeolic acid from glucose and alternative carbon sources. *Biotechnol. J.* 2017, 12, 1600646. [CrossRef]

131. Wu, J.; Shiehyan, G.Y.; Woodard, R.W. Bacillus subtilis 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase revisited: Resolution of two long-standing enigmas. *Biochem.* 2005, 390, 583–590. [CrossRef] [PubMed]

132. Burschowsky, D.; Thorbjørnsrud, H.V.; Heim, J.B.; Fahrig-Kamarasaité, J.R.; Wüth-Roderer, K.; Kast, P.; Krenkel, U. Inter-Enzyme Allosteric Regulation of Chorismate Mutase in Corynebacterium glutamicum: Structural Basis of Feedback Activation by Trp. *Biochemistry* 2018, 57, 557–573. [CrossRef] [PubMed]
133. Krahn, I.; Bonder, D.; Torregrosa-Barragán, L.; Stoppel, D.; Krause, J.P.; Rosenfeldt, N.; Meiswinkel, T.M.; Seibold, G.M.; Wendisch, V.F.; Lindner, S.N. Evolving a New Efficient Mode of Fructose Utilization for Improved Bioproduction in Corynebacterium glutamicum. Front. Bioeng. Biotechnol. 2021, 9, 669093. [CrossRef]

134. Pfeifer, E.; Gätgens, C.; Polen, T.; Frunzke, J. Adaptive laboratory evolution of Corynebacterium glutamicum towards higher growth rates on glucose minimal medium. Sci. Rep. 2017, 7, 16780. [CrossRef]

135. Stella, R.G.; Wiechert, J.; Noack, S.; Frunzke, J. Evolutionary engineering of Corynebacterium glutamicum. Biotechnol. J. 2019, 14, e1800444. [CrossRef]

136. Yu, X.; Shi, F.; Liu, H.; Tan, S.; Li, Y. Programming adaptive laboratory evolution of 4-hydroxyisoleucine production driven by a lysine biosensor in Corynebacterium glutamicum. AMB Express 2021, 11, 66. [CrossRef]

137. 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]

138. 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. Biol. 2020, 3, 217. [CrossRef]

139. Kuepper, J.; Otto, M.; Dickler, J.; Behnken, S.; Magnus, J.; Jäger, G.; Blank, L.M.; Wierckx, N. Adaptive laboratory evolution of Pseudomonas putida and Corynebacterium glutamicum to enhance anthranilate tolerance. Microbiol. Read. Engl. 2020, 166, 1025–1037. [CrossRef]

140. Walter, T.; Veldmann, K.H.; Götker, S.; Busche, T.; Rückert, C.; Kashkooli, A.B.; Paulus, J.; Cankar, K.; Wendisch, V.F. Physiological Response of Corynebacterium glutamicum to Indole. Microorganisms 2020, 8, 1945. [CrossRef] [PubMed]

141. Kerbs, A.; Mindt, M.; Schwardmann, L.; Wendisch, V.F. Sustainable Production of N-methylphenylalanine by Reductive Methylamination of Phenylpyruvate Using Engineered Corynebacterium glutamicum. Microorganisms 2021, 9, 824. [CrossRef] [PubMed]

142. Kiefer, P.; Heinzle, E.; Zelder, O.; Wittmann, C. Comparative Metabolic Flux Analysis of Lysine-Producing Corynebacterium glutamicum Cultured on Glucose or Fructose. Appl. Environ. Microbiol. 2004, 70, 229–239. [CrossRef] [PubMed]

143. Gopinath, V.; Meiswinkel, T.M.; Wendisch, V.F.; Namboothiri, K.M. Amino acid production from rice straw and wheat bran hydrolysates by recombinant pantose-utilizing Corynebacterium glutamicum. Appl. Microbiol. Biotechnol. 2011, 92, 985–996. [CrossRef] [PubMed]

144. Meiswinkel, T.M.; Rittmann, D.; Lindner, S.N.; Wendisch, V.F. Crude glycerol-based production of amino acids and putrescine by Corynebacterium glutamicum. Bioresour. Technol. 2013, 145, 254–258. [CrossRef]

145. Becker, J.; Kuhl, M.; Kohlstedt, M.; Starck, S.; Wittmann, C. Metabolic engineering of Corynebacterium glutamicum for the production of cis, cis-muconic acid from lignin. Microb. Cell Fact. 2018, 17, 115. [CrossRef]

146. Burgardt, A.; Prell, C.; Wendisch, V.F. Utilization of a Wheat Sidestream for 5-Aminovalerate Production in Corynebacterium glutamicum. Front. Bioeng. Biotechnol. 2021, 9, 772. [CrossRef]

147. Sasikumar, K.; Hannibal, S.; Wendisch, V.F.; Namboothiri, K.M. Production of Biopolyamide Precursors 5-Amino Valeric Acid and Putrescine From Rice Straw Hydrolysate by Engineered Corynebacterium glutamicum. Front. Bioeng. Biotechnol. 2021, 9, 635509. [CrossRef]

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

149. Vortmann, M.; Stumpf, A.K.; Sgobba, E.; Dirks-Hofmeister, M.E.; Krehenbrink, M.; Wendisch, V.F.; Philipp, B.; Moerschbacher, B.M. A bottom-up approach towards a bacterial consortium for the biotechnological conversion of chitin to L-lysine. Appl. Microbiol. Biotechnol. 2021, 105, 1547–1561. [CrossRef] [PubMed]