Metabolic engineering of *Corynebacterium glutamicum* for enhanced production of 5-aminovaleric acid

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**Abstract**

**Background:** 5-Aminovaleric acid (5AVA) is an important five-carbon platform chemical that can be used for the synthesis of polymers and other chemicals of industrial interest. Enzymatic conversion of l-lysine to 5AVA has been achieved by employing lysine 2-monoxygenase encoded by the *davB* gene and 5-aminovaleramidase encoded by the *davA* gene. Additionally, a recombinant *Escherichia coli* strain expressing the *davB* and *davA* genes has been developed for bioconversion of l-lysine to 5AVA. To use glucose and xylose derived from lignocellulosic biomass as substrates, rather than l-lysine as a substrate, we previously examined direct fermentative production of 5AVA from glucose by metabolically engineered *E. coli* strains. However, the yield and productivity of 5AVA achieved by recombinant *E. coli* strains remain very low. Thus, *Corynebacterium glutamicum*, a highly efficient l-lysine producing microorganism, should be useful in the development of direct fermentative production of 5AVA using l-lysine as a precursor for 5AVA. Here, we report the development of metabolically engineered *C. glutamicum* strains for enhanced fermentative production of 5AVA from glucose.

**Results:** Various expression vectors containing different promoters and origins of replication were examined for optimal expression of *Pseudomonas putida* *davB* and *davA* genes encoding lysine 2-monoxygenase and delta-aminovaleramidase, respectively. Among them, expression of the *C. glutamicum* codon-optimized *davA* gene fused with His₆-Tag at its N-Terminal and the *davB* gene as an operon under a strong synthetic H₃₆ promoter (plasmid p36davAB3) in *C. glutamicum* enabled the most efficient production of 5AVA. Flask culture and fed-batch culture of this strain produced 6.9 and 19.7 g/L (together with 11.9 g/L glutaric acid as major byproduct) of 5AVA, respectively. Homology modeling suggested that endogenous gamma-aminobutyrate aminotransferase encoded by the *gabT* gene might be responsible for the conversion of 5AVA to glutaric acid in recombinant *C. glutamicum*. Fed-batch culture of a *C. glutamicum* gabT mutant-harboring p36davAB3 produced 33.1 g/L 5AVA with much reduced (2.0 g/L) production of glutaric acid.

**Conclusions:** *Corynebacterium glutamicum* was successfully engineered to produce 5AVA from glucose by optimizing the expression of two key enzymes, lysine 2-monoxygenase and delta-aminovaleramidase. In addition, production of glutaric acid, a major byproduct, was significantly reduced by employing *C. glutamicum* gabT mutant as a host.

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The metabolically engineered C. glutamicum strains developed in this study should be useful for enhanced fermentative production of the novel C5 platform chemical 5AVA from renewable resources.

**Keywords:** 5-Aminovaleric acid, Corynebacterium glutamicum, l-Lysine, Metabolic engineering, Glutaric acid

**Background**

As a result of increasing pressure on the environment, bio-based production of chemicals, fuels, and materials from renewable non-food biomasses has been attracting much attention [1]. To make such bio-based processes competitive, microorganisms have been metabolically engineered for production of fuels [2–4], amino acids [5–9], polymers [10–12], and other chemicals of industrial importance [13–15]. It is expected that more chemicals and materials of petrochemical origin will be produced through bio-based route employing microorganisms developed by systems metabolic engineering [16, 17].

A non-proteinogenic ω-amino acid, 5-aminovaleric acid (5AVA), has attracted attention as a five carbon (C5) platform chemical because of its potential in polymer synthesis [18–21]. 5AVA can be used to produce δ-valerolactam (2-piperidone) via intramolecular dehydrative cyclization and can be further processed for synthesis of bio-based nylon, such as nylon-5 and nylon-6,5 [18, 20]. Enzymatic conversion of l-lysine to 5AVA has been achieved by employing lysine 2-monoxygenase (E.C. 1.13.12.2, encoded by the davB gene) and 5-aminovaleramidase (E.C. 3.5.1.30, encoded by the davA gene) [19]. We recently reported the development of a whole-cell bioconversion process for conversion of l-lysine to 5AVA by employing recombinant Escherichia coli strains expressing lysine 2-monoxygenase and 5-aminovaleramidase as whole cell biocatalysts [20, 21]. However, it is obviously desirable to use glucose derived from non-food lignocellulosic biomass as a substrate rather than l-lysine [1]. There have been reports on the development of metabolically engineered microorganisms for the production of C3 and C4 ω-amino acids, such as β-alanine [22] and γ-aminobutyrate [23], from glucose. We and others also examined the possibility of producing the C5 ω-amino acid 5AVA by metabolic engineering of E. coli, but the yield and productivity of 5AVA remain very low [18, 20]. Thus, it is necessary to develop a new strategy for more efficient production of 5AVA.

Corynebacterium glutamicum is an organism widely used for the production of amino acids, proteins, monomers for plastic materials, and compounds for cosmetics [24, 25]. Additionally, C. glutamicum has been successfully engineered to produce a different C5-platform chemical, cadaverine (1,5-pentanediamine), and was shown to be a promising host for producing this chemical using different carbon sources, such as glucose and xylose [26–30]. Because C. glutamicum strains capable of producing l-lysine at very high levels have already been commercialized, we decided to exploit C. glutamicum as a host strain for the production of 5AVA.

In this study, we designed and introduced a synthetic pathway for the production of 5AVA into an l-lysine-overproducing C. glutamicum strain. The synthetic pathway consists of two key enzymes, lysine 2-monoxygenase encoded by the davB gene and 5-aminovaleramidase encoded by the davA gene, responsible for the conversion of l-lysine to 5AVA. Various expression systems including vectors and promoters were examined for the most efficient production of 5AVA in C. glutamicum. A reaction responsible for unexpected formation of glutaric acid as a major byproduct was identified and subsequently deleted. The final engineered C. glutamicum strain was used for enhanced production of 5AVA by fermentation with much reduced glutaric acid.

**Results and discussion**

**Construction of the 5AVA synthesis pathway in C. glutamicum by expressing the davAB genes under the control of the tac promoter**

Although 5AVA exists as an intermediate in amino acid degradation pathways in Pseudomonads, metabolic engineering for enhanced production of 5AVA requires strong metabolic flux from the chosen intermediate metabolite toward 5AVA as indicated by many successful examples of systems metabolic engineering [16, 17]. Recombinant E. coli strains employing the davAB genes from the l-lysine catabolic pathway of Pseudomonads were previously shown to produce 5AVA, although at low yield and productivity [18, 21]. Because E. coli strains have a relatively low capacity to provide l-lysine as a precursor for 5AVA, high-level production of 5AVA could not be achieved in recombinant E. coli strains even though the l-lysine catabolic pathway via 5-aminovaleramide provides the shortest route from l-lysine to 5AVA [18, 21]. Thus, C. glutamicum, the well-known, highly efficient l-lysine producing bacterium, was selected as a host strain for 5AVA production in this study to provide strong metabolic flux from glucose to l-lysine, the direct precursor of 5AVA [5, 6]. This is strategically advantageous for producing compounds using l-lysine as a direct precursor.

To extend the metabolic flux from glucose toward 5AVA beyond l-lysine (Fig. 1a), P. putida ATCC 12633
**Fig. 1** Metabolic engineering strategies for SAVA production using *C. glutamicum*. Heterologous expression of the *P. putida* davB gene (encoding *l*-lysine 2-monooxygenase) and the davA gene (encoding delta-aminovaleramidase) results in conversion of *l*-lysine into SAVA. SAVAS, *S*-aminovalerate; ASP *l*-aspartate; ASP-P aspartyl phosphate; ASP-SA aspartate semialdehyde; LYS *l*-lysine.

davAB genes [20, 21] were cloned in two different *E. coli*-*C. glutamicum* shuttle vectors (Additional file 5: Table S1; Fig. 1b) and expressed in *C. glutamicum* BE strain (KCTC 12390BP). In plasmids pKCA212davAB and pJS30 (Fig. 1b), the davA and davB genes were organized in an operon and expressed under the control of the tac promoter, with an additional lacUV5 ribosome-binding site (ttt cac ac agg aa) for the davB gene residing between the coding sequences of the two genes. Plasmid pKCA212davAB was constructed based on an in-house shuttle vector, pKCA212-MCS, whereas pJS30 was derived from pEKEx1 [31]. Plasmid pEKEx1 contains a pBL1 origin of replication, having ~30 copy numbers per cell in *C. glutamicum* [32], and pKCA212-MCS contains a pCC1 origin of replication, also having ~30 to ~40 copies per cell [33]. It was found that *C. glutamicum* BE could produce 17.2 g/L *l*-lysine (yield of 325 mmol/mol glucose) in shake-flask cultivation in 44 h (Fig. 2). It was found through HPLC analysis of metabolites excreted into culture medium that expression of the davAB genes in *C. glutamicum* BE resulted in conversion of *l*-lysine to SAVA, whereas the non-engineered strain did not convert any *l*-lysine into SAVA (Fig. 2).

Slightly decreased *l*-lysine production was observed in recombinant *C. glutamicum* BE strains expressing the davAB genes, although *l*-lysine was still the major product in both engineered strains tested. Expression of the davAB genes using the shuttle vector pEKEx1 (pJS30) was more effective at producing SAVA than using pKCA212-MCS (pKCA212davAB) (Fig. 2). The *C. glutamicum* BE strain harboring pKCA212davAB produced 13.4 g/L *l*-lysine (yield of 271.2 mmol/mol) and 58 mg/L SAVA (yield of 1.5 mmol/mol), whereas the *C. glutamicum* BE strain harboring pJS30 produced 12.4 g/L *l*-lysine (yield of 258.2 mmol/mol) and 641 mg/L SAVA (yield of 16.7 mmol/mol) from glucose. These results demonstrate that heterologous expression of the davAB genes from gram-negative *P. putida* correctly functioned to produce SAVA from glucose using *l*-lysine as a SAVA precursor in gram-positive *C. glutamicum*.

Although the *C. glutamicum* BE strain harboring pKCA212davAB or pJS30 successfully produced SAVA
from glucose, most of l-lysine was not converted into 5AVA, which suggests that metabolic flux from l-lysine to 5AVA was still quite weak as a result of inefficient expression of the \( \text{davAB} \) genes. Thus, we investigated whether 5AVA production could be enhanced by employing \( C.\ \text{glutamicum} \) codon-optimized \( \text{davAB} \) genes. The \( C.\ \text{glutamicum} \) BE strain harboring pJS38, which expresses \( C.\ \text{glutamicum} \) codon-optimized \( \text{davAB} \) genes, produced 3.0 g/L 5AVA in flask cultivation, which represented an increase of almost 370 % over that produced by the \( C.\ \text{glutamicum} \) BE strain harboring pJS30 (Fig. 2). However, 7.9 g/L l-lysine still remained in the culture medium of \( C.\ \text{glutamicum} \) BE strain (pJS38). The yield for l-lysine obtained by \( C.\ \text{glutamicum} \) BE (pJS38) was 193.9 mmol/mol glucose, whereas that for 5AVA was 92.3 mmol/mol glucose.

Notably, 0.14 mg/L glutaric acid was detected in the culture medium of \( C.\ \text{glutamicum} \) BE (pJS38), even though 5AVA aminotransferase and glutarate-semialdehyde dehydrogenase (encoded by \( \text{davT} \) and \( \text{davD} \), respectively), which are the key enzymes for further conversion of 5AVA into glutaric acid using \( \alpha \)-ketoglutarate as an amine acceptor [18, 21], were not expressed in this strain. This result strongly suggests that endogenous enzymes homologous to 5AVA aminotransferase and glutarate-semialdehyde dehydrogenase might be involved in further conversion of 5AVA into glutaric acid in \( C.\ \text{glutamicum} \).

**Examination of 5AVA production by engineered \( C.\ \text{glutamicum} \) strain expressing the \( \text{davAB} \) genes under the control of the \( \text{tac} \) promoter in fed-batch fermentation**

We then investigated the capability of \( C.\ \text{glutamicum} \) BE (pJS38) to produce 5AVA by fed-batch fermentation in a 5-L fermentor (Fig. 3a, c) to examine its potential for large-scale production of 5AVA. During fed-batch cultivation of \( C.\ \text{glutamicum} \) BE (pJS38), l-lysine concentration reached 21.8 g/L after 94 h of cultivation, and then decreased beyond this point. \( C.\ \text{glutamicum} \) BE (pJS38) produced 17.8 g/L 5AVA in 191 h, with an overall yield and productivity of 0.07 g/g (107.3 mmol/mol) and 0.09 g/L/h, respectively (Fig. 3c). The total input of glucose was 790 g (4.4 mol), and the total amount of produced 5AVA was 55.3 g (471 mmol), with the final volume of 3.1 L. The maximum specific growth rate was 0.23 h\(^{-1}\), and the observed maximum specific productivity was 9.2 g/g/h. Initial increases in l-lysine levels followed by a decrease in titler along with constant production of 5AVA indicated that l-lysine-production flux might be strong initially before slowing down in the latter half of cultivation. The maximum l-lysine-specific productivity was 56.7 mg/g/h initially, but gradually decreased to
The amount of the major byproduct, glutaric acid, also increased steadily throughout the entire cultivation period until the concentration reached 5.3 g/L at the end of cultivation (Fig. 3c).

The maximum OD$_{600}$ reached was 95.4, corresponding to the measured dry cell weight concentration of 24.5 g/L, after 189 h of fermentation (Fig. 3a). These results demonstrated that _C. glutamicum_ BE (pJS38) was able to successfully produce 5AVA from renewable resources in a laboratory-scale bioreactor. However, conversion of L-lysine toward 5AVA needs to be further enhanced through stronger expression of the _davAB_ genes.

### Construction of engineered _C. glutamicum_ strain expressing the _davAB_ genes under the control of constitutive promoters to improve 5AVA production

In addition to the _tac_ promoter used in pEKEx1, promoters for SOD (NCgl2826; E.C. 1.15.1.1) and the transcription factor Tu (Tuf; NCgl0480; E.C. 3.6.5.3) have also been widely used in metabolic engineering of _C. glutamicum_ because of their capabilities to support strong gene expression at the chromosome level [34]. Although the _sod_ promoter is known to exhibit weaker plasmid-based expression than the _tac_ promoter [35], weak and medium-strength expression driven by constitutive promoters might be more effective than higher expression levels for producing chemicals of interest under different circumstances [15]. Moreover, use of the constitutive promoters circumvents the requirement for costly additives such as IPTG. Therefore, we replaced the _lacI$^q$_ gene and _tac_ promoter in pJS38 with _P$_{sod}$ or _P$_{tuf}$ (Additional file 5: Table S1). These constructs were then introduced into the _C. glutamicum_ BE strain, and their functions were investigated by flask cultivation. However, expression of the _davAB_ genes under control of the _sod_ promoter (pJS59) and _tuf_ promoter (pJS60) did not result in higher 5AVA production relative to that produced by the parent construct, pJS38 containing the _tac_ promoter (Figs. 2, 4). _C. glutamicum_ BE (pJS59) and _C. glutamicum_ BE (pJS60) produced 556 and 587 mg/L 5AVA,
respectively, with no glutaric acid observed in the culture media after 44 h of flask cultivation.

We also investigated the newly designed synthetic promoter active in \( C. \text{ glutamicum} \), the H36 promoter [36], to see if it can possibly improve 5AVA production; it was successfully employed for the expression of glutamate decarboxylase (GAD) and lysine decarboxylase (LDC) in \( C. \text{ glutamicum} \) strains, resulting in high-level production of gamma-aminobutyrate (GABA) and cadaverine, respectively [30, 37].

We replaced the \( lacI^Q \) and the \( tac \) promoter in pJS38 with the \( P_{H36} \) promoter to construct p36davAB2. However, cultivation of \( C. \text{ glutamicum} \) BE harboring p36davAB2 also did not improve 5AVA production, which resulted in production of 661 mg/L 5AVA under the same culture condition (Fig. 4). These results indicate that expression of the \( \text{davAB} \) genes was still not strong enough to enable efficient conversion of \( \text{l-lysine} \) to 5AVA.

To continue exploring expression vector systems for possible improvement of 5AVA titer, we noticed that the strong H36 promoter originally developed with a different backbone vector, pCES208 [36, 38], might not be optimal for pEKEx1. Engineered \( C. \text{ glutamicum} \) strains harboring a pCES208-based plasmid for expression of target genes under strong synthetic promoters, such as H30 and H36, have been reported to efficiently produce GABA and cadaverine from renewable resources [30, 37]. Therefore, we transferred codon-optimized versions of the \( \text{davAB} \) genes into the pCES208 vector system.

The new construct, p36davAB1, was further modified by inserting a His\(_6\)-Tag into the N-terminal of \( \text{davA} \) gene, resulting in p36davAB3. This was done because there have been reports showing that His\(_6\)-tagged constructs can sometimes be expressed more efficiently [39, 40]. These constructs were transformed into the \( C. \text{ glutamicum} \) BE strain and assessed by flask cultivation. Whereas \( C. \text{ glutamicum} \) BE (p36davAB1) produced only 0.4 g/L 5AVA along with 11.7 g/L \( \text{l-lysine} \), \( C. \text{ glutamicum} \) BE (p36davAB3) produced 6.9 g/L 5AVA, with 5.5 g/L \( \text{l-lysine} \) remaining unconverted (Fig. 5). The 5AVA concentration obtained represents a 130 % increase over that (Fig. 2) obtained with \( C. \text{ glutamicum} \) BE (pJS38). Interestingly, the construct containing the His\(_6\)-tagged variant produced substantially more 5AVA compared to that produced using the construct lacking the His-tag, possibly because of the improved stability afforded by the 5’ modification, which resulted in higher expression of the \( \text{davAB} \) genes in the recombinant \( C. \text{ glutamicum} \) BE strain (Additional file 1: Figure S1).

Comparison of mRNA folding energies (\( \Delta G \)) with the RNA secondary structure prediction program Mfold (http://unafold.rna.albany.edu/?q=mfold/download-mfold) suggested that \( \Delta G \) for the first 30 nucleotides starting from the +1 site of the H36 promoter in p36davAB2 is \(-6.00\) kcal/mol, which is much lower than \( \Delta G \) of \(-0.06\) kcal/mol obtained in p36davAB3. The higher \( \Delta G \) in p36davAB3 indicates that less stable mRNA produced by p36davAB3 might allow the translation machinery to bind more easily than much stable mRNA produced by p36davAB2.
Examination of 5AVA production by engineered *C. glutamicum* expressing the *davAB* genes under the control of the strong H36 promoter in fed-batch fermentation

Having achieved improved 5AVA production in flask culture, fed-batch culture of *C. glutamicum* BE (p36davAB3) was performed next in a 5-L fermentor. *C. glutamicum* BE (p36davAB3) produced 19.7 g/L 5AVA in 157 h, with the overall yield and productivity of 0.08 g/g and 0.16 g/L/h, respectively (Fig. 3d). This strain also accumulated 13.4 g/L glutaric acid as a byproduct at the end of the cultivation. On the other hand, l-lysine accumulation decreased significantly compared to that observed with *C. glutamicum* BE (pJS38). L-Lysine accumulated to 3.7 g/L in 25 h, but production remained between 1 and 2 g/L over the entire cultivation period (Fig. 3d). Citric acid was another major byproduct, but its concentration remained at ~1 g/L throughout cultivation. Notably, the production patterns observed during fed-batch fermentation were different from those observed during flask cultivation. Although large portions of l-lysine remained unconverted at the end of the flask cultivation, very little l-lysine remained in fed-batch fermentation. This indicates that control of pH and provision of sufficient air streams were beneficial for 5AVA production and provided better results during fed-batch fermentation. A sufficient air supply is important for cultivation because lack of sufficient air can result in accumulation of substantial concentrations of lactic and acetic acids in the fermentation broth [30, 31]. These byproducts were not observed in our cultivation conditions, contrary to previous reports. These results suggest that the strategy combining improved expression of the *davA* gene fused with His6-Tag at its N-Terminal and the *davB* gene as an operon under control of the strong synthetic H36 promoter was successful in directing most of the l-lysine pool toward 5AVA, resulting in efficient production of 5AVA.

Construction of an engineered *C. glutamicum gabT* mutant for enhanced production of 5AVA with greatly reduced glutaric acid production

While 19.7 g/L of 5AVA could be produced by fed-batch cultivation of *C. glutamicum* BE (p36davAB3), glutaric acid, a major byproduct, was still produced to a relatively high concentration (up to 13.4 g/L). In order to further enhance 5AVA production, conversion of 5AVA into glutaric acid should be minimized. However, no enzyme responsible for converting 5AVA into glutaric acid is known in *C. glutamicum*. Thus, we performed molecular-docking simulations, which suggested possible interactions between endogenous GabT and 5AVA (Additional file 2: Figure S2, Additional file 3: Figure S3). GabT shares homology (60 % by primary peptide structure) with 4-aminobutyrate aminotransferase (SGR_1829) in *Streptomyces griseus*, which exhibits 60 % relative aminotransfer activity for 5AVA [42]. Although the pyridoxal phosphate moiety was in the correct orientation and position, the orientation of bound 5AVA was twisted, possibly because of the larger size of the substrate being accommodated in the active site. Additionally, the enzyme also shared high homology (Additional file 3: Figure S3) with *P. putida* DavT, which binds 5AVA as its natural substrate (Additional file 4: Figure S4). The major difference in active sites between GabT and DavT is that
DavT contains a glutamine residue (Gln80) rather than a methionine residue, enabling accommodation of the ω-amino group in the binding pocket (Additional file 3: Figure S3). However, GabT from S. griseus, even with the methionine residue at this position, is sufficiently promiscuous to accept 5AVA as a substrate [42]. Thus, we could conclude from the docking simulations that the endogenous C. glutamicum GabT might accommodate 5AVA as a substrate for aminotransfer reactions, leading to the formation of glutaric acid.

Based on the above results, the gabT gene (E.C. 2.6.1.19, encoding 4-aminobutyrate aminotransferase, Ncgl0462) was deleted from the chromosome of C. glutamicum BE to construct C. glutamicum AVA2. C. glutamicum AVA2 produced 17.5 g/L of l-lysine by flask cultivation in 44 h, with no residual 5AVA detected (Fig. 6). This result suggests that deletion of the gabT gene did not inhibit cell growth and l-lysine production. Plasmid p36davAB3 was then transformed into C. glutamicum AVA2 to assess 5AVA production. Fed-batch cultivation of engineered C. glutamicum AVA2 (p36davAB3) in a 5-L fermenter resulted in production of 33.1 g/L 5AVA with greatly reduced glutaric acid (2.0 g/L) and l-lysine (648.3 mg/L) at the end of cultivation. The overall yield and productivity obtained were 0.1 g/g glucose (163.1 mmol/mol) and 0.22 g/L/h, respectively (Fig. 7). Cells grew to an OD₆₀₀ of 134 in 153 h, with a measured dry cell weight of 36.1 g/L, with the maximum specific growth rate was 0.4 h⁻¹. The maximum specific 5AVA productivity was 65.8 mg/g/h, which gradually decreased to 22.1 mg/g/h at the end of cultivation. Additionally, the l-lysine concentration peaked at 28 h, but remained as low as 1 g/L for the remainder of the cultivation. As expected, gabT deletion resulted in a significant decrease in glutaric acid production compared to that observed in the parent strain. However, the continued presence of glutaric acid in the culture broth suggests that unknown aminotransferases still remain in C. glutamicum that are capable of converting 5AVA to glutaric acid, although at lower efficiencies than GabT.

**Conclusions**

In this study, we report development of engineered C. glutamicum strains for the production of 5AVA from glucose. Expression of two key enzymes, lysine 2-monooxygenase and delta-aminovaleramidase, was systematically optimized by examining different promoters, origins of replication, codon usage of the davAB genes, and even 5′ modification of the davA gene with a His-tag, all of which
were found to be important for determining the optimal and stable plasmid-based expression of the davaB genes in C. glutamicum. In addition, production of a major byproduct, glutaric acid, could be significantly reduced by identifying previously unknown enzyme GabT responsible for converting 5AVA to glutaric acid and deleting the corresponding gene from the chromosome. Fed-batch cultivation of the final engineered C. glutamicum AVA2 strain harboring p36davAB3 produced 33.1 g/L 5AVA with greatly reduced glutaric acid (2.0 g/L). The metabolically engineered C. glutamicum strains developed in this study should be useful for enhanced fermentative production of the novel C5 platform chemical, 5AVA, from renewable resources such as glucose.

Methods
Strains and plasmids
All bacterial strains and plasmids used in this study are listed in Additional file 5: Table S1. All DNA manipulations were performed following standard procedures [43]. Primers used in this study (Additional file 6: Table S2) were synthesized at Bioneer (Daejeon, Korea). C. glutamicum BE (KCTC 12390BP) was used as the base strain for 5AVA production. Polymerase chain reaction (PCR) was performed with the C1000 Thermal Cycler (BioRad, Hercules, CA, USA). The general PCR condition for amplifications of target genes using primer sets listed in Additional file 6: Table S2 is as follows: 1 cycle of 95 °C for 5 min; 30 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min 30 s; and a final extension of 72 °C for 5 min. The final reaction volume is 20 μL. The in-house-developed C. glutamicum shuttle vector pKCA212-MCS was constructed by cloning the origin of replication of the cryptic plasmid pCC1 [33] into pKCA212-MCS at the AatII and Xhol sites. The origin of replication of pCC1 was synthesized by GenScript (http://www.genscript.com) based on the reported sequence. Plasmid pKA212-MCS was constructed by replacing the chloramphenicol-resistance gene of pKA312-MCS [11] with a kanamycin-resistance gene obtained from pZA21-MCS (http://www.expressys.com) by restriction digest with AatII and SpeI. The davaB genes from pKE112-DavAB [20, 21] were restriction-digested and ligated into pKCA212-MCS to construct pKCA212davAB using the same restriction enzyme sites (EcoRI/KpnI, KpnI/BamHI). A 16-bp untranslated region (tttcacacaggaaaca) containing a ribosome-binding site was present between the two genes for davaB expression. The same genes were also cloned into pEKE1x to construct pJS30. The codon-optimized versions of davaB genes (Additional file 7: Table S3) with preferred codon usage in C. glutamicum were synthesized by Bioneer (Daejeon, Korea) and cloned into the EcoRI/BamHI restriction enzyme sites in pEKE1x to yield pJS38.

To construct promoter variants of pEKE1x, promoterless pEKE1x was created by removing the tac promoter and the initial 778 bp of the coding sequence of the LacI9 gene from pEKE1x by restriction digestion with EcoRV/EcoRI. The desired promoters were similarly designed as previously described [5, 34, 44] and inserted into the promoterless pEKE1x vector. The region 250 bp upstream of the start codon for the superoxide dismutase gene (NCgl2826, E.C. 1.15.1.1) was amplified by polymerase chain reaction (PCR) from C. glutamicum ATCC 13032 chromosome using primers Psod_F_EcoRV and Psod_R_EcoRI, and then digested and cloned into the EcoRV/EcoRI sites of the promoterless pEKE1x vector to construct pJS57. The 248-bp sequence upstream of the start codon for the gene encoding the elongation factor Tu (Ncgl0480, E.C. 3.6.5.3) was amplified by PCR from C. glutamicum ATCC 13032 chromosome using the primers Ptfu_F_EcoRV (v2) and Ptfu_R_EcoRI, and then digested and cloned into the promoterless pEKE1x vector to yield pJS58. The codon-optimized davaB genes from pJS38 were restriction-digested with EcoRI/BamHI and cloned into the pJS57 and pJS58 vectors at the EcoRI/BamHI restriction enzyme sites to make pJS59 and pJS60, respectively.

Plasmid p36davAB2 was constructed from pEKE1x by cloning the codon-optimized davaB genes. Promoterless pEKE1x was constructed by methods similar to those described in the previous paragraph, except that the genes were cloned into the EcoRV/PstI restriction sites of the vector. The PtufH promoter was amplified by PCR using the JW02H-F and JW02H-R primers from pCES208H36GFP, and the fragments were restriction-digested with EcoRV/EcoRI. A second round of PCR using primers JW02AH-F and JW02AH-R from pJS38 generated codon-optimized davaB gene fragments that were restriction-digested with EcoRI/PstI. The resulting products were then ligated into the EcoRV/PstI restriction sites of the promoterless pEKE1x vector to yield p36davAB2.

Plasmid p36davAB1 was constructed from pCES208 by cloning the codon-optimized davaB genes. Products from the first round of PCR using primers JW01A-F and JW01A-R were used for amplification of the davaA gene, which was then restriction-digested with BamHI/SfiI. The second round of PCR used primers JW01B-F and JW01B-R to amplify the davaB gene, which was then restriction-digested with NotI. These fragments were cloned into the pCES208H36GFP vector [36] by replacing the egfp gene to yield p36davAB1.

Plasmid p36davAB3 was constructed from the pCES208H36GFP vector [36]. The codon-optimized davaA gene fused with His6-Tag at its N-Terminal was amplified using primers JW03A-F and JW03A-R and cloning the codon-optimized davaAB genes from the chromosome. Fed-batch cultivation of the final engineered C. glutamicum AVA2 strain harboring p36davAB3 produced 33.1 g/L 5AVA with greatly reduced glutaric acid (2.0 g/L). The metabolically engineered C. glutamicum strains developed in this study should be useful for enhanced fermentative production of the novel C5 platform chemical, 5AVA, from renewable resources such as glucose.
restriction-digested with BamHI and SfiI. The codon-optimized davB gene was amplified using primers JW01B-F and JW03B-R and restriction-digested with NotI. The two products were then cloned into the pCES208H36EGFP vector by replacing the egfp gene to construct p36davAB3.

Plasmid pJS113 beta was constructed from the pK19mobsacB vector [45]. Primers 113 i1F beta and 113 i1R beta were used to PCR-amplify the upstream region and a portion of the gabT gene from C. glutamicum. Primers 113 i2F beta and 113 i2R beta were then used to PCR-amplify the downstream region and a portion of the gabT gene of C. glutamicum. The two PCR products were joined by a third PCR using primers 113 i1F beta and 113 i2R beta. The final PCR product was cloned into the PstI-digested pK19mobsacB to make pJS113 beta. pJS113 beta was subsequently used to disrupt the gabT gene in the C. glutamicum BE chromosome, resulting in the strain C. glutamicum AVA2. This in-frame deletion left a 330-bp deletion in the 280–609 region of the 1347-bp gabT gene.

E. coli DH5α and TOP10 strains (Additional file 5: Table S1) were used for general cloning purposes. All constructed plasmids introduced into C. glutamicum, except for pJS113 beta, were prepared in unmethylated form using the methylation-deficient E. coli JM110 strain (Stratagene; Agilent Technologies, Santa Clara, CA, USA). pJS113 beta was propagated in E. coli DH5α and TOP10 strains by bacterial conjugation using E. coli S17-1 as a donor [45]. Plasmids were introduced via electroporation as previously described [46]. Cells were transferred to a microcuvette and electroporated using a micropulser. Cells were transformed with about 2 μg of DNA by electroporation (1.8 V and 400 Ω). Pre-chilled preculture medium (900 μL) was added and the transformed cells were allowed for growth recovery for 2 h without shaking in a 30 °C incubator. The transformed cells were then spread onto the agar plates containing kanamycin as a selective marker.

**Culture media**

Cells were cultured in media described below, the compositions of which were modified from previous reports [34, 43]. The pre-culture medium for shake-flask cultivation consisted of 10 g/L beef extract (BD Bacto, Franklin Lakes, NJ, USA), 40 g/L brain–heart infusion (BD Bacto), 20 g/L D-sorbitol, and 10 g/L glucose [41]. The flask culture medium (pH 7.2) consisted of 80 g/L glucose, 1 g/L MgSO4, 1 g/L K2HPO4, 1 g/L KH2PO4, 1 g/L urea, 20 g/L (NH4)2SO4, 10 g/L yeast extract, 100 μg/L biotin, 10 mg/L β-alanine, 10 mg/L thiamine HCl, 10 mg/L nicotinic acid, 1.3 mg/L (NH4)6Mo7O24, 40 mg/L CaCl2, 10 mg/L FeSO4, 10 mg/L MnSO4, 5 mg/L CuSO4, 10 mg/L ZnSO4, and 5 mg/L NiCl2. For fermentation experiments, the seed medium (pH 7.0) consisted of 20 g/L glucose, 1 g/L MgSO4, 10 g/L beef extract, 1 g/L K2HPO4, 1 g/L KH2PO4, 0.5 g/L urea, 10 g/L yeast extract, 100 μg/L biotin, 200 μg/L thiamine HCl, 10 mg/L FeSO4, and 10 mg/L MnSO4. The fermentation medium (1.8 L) contained per liter: 160 g of glucose, 2 g of MgSO4, 2 g of K2HPO4, 2 g of KH2PO4, 2 g of urea, 40 g of (NH4)2SO4, 20 g of yeast extract, 50 mg of CaCl2, 50 μg of biotin, 20 mg of β-alanine, 20 mg of thiamine HCl, 20 mg of nicotinic acid, 1.3 mg of (NH4)6Mo7O24, 10 mg of FeSO4, 10 mg of MnSO4, 5 mg of CuSO4, 10 mg of ZnSO4, 5 mg of NiCl2, and 1 mL of antifoam reagent (AntiFoam 204; Sigma-Aldrich, St. Louis, MO, USA). Each feeding solution (200 mL) contained 90 g of glucose.

**Flask cultivation**

Stock cells stored in glycerol were used to inoculate 5-mL pre-cultures, which were grown at 30 °C with shaking at 200 rpm in an incubator (JSII-300C; JS Research Inc., Gongju, Korea) for 17–18 h. Cells suspended in 250-μL aliquots of pre-culture were harvested by centrifugation (Centrifuge 5415 D; Eppendorf, Hamburg, Germany) and transferred to a 25-mL primary culture in autoclaved 300-mL baffled Erlenmeyer flasks, each containing 1.5 g of CaCO3 to maintain the pH at ~7.0 during cultivation. Primary cultures were grown with shaking in an incubator for 44 h. When appropriate, isopropyl-β-d-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM was used to induce gene expression during the early log phase (OD600 = 0.5–0.6), with 25.0 μg/mL kanamycin added for selective pressure (Ravasi et al. [35]).

**Fed-batch fermentation**

Stock cells stored in glycerol were used to inoculate 5.0-mL pre-cultures, which were grown at 30 °C with shaking in an incubator for 17–18 h. Two 1-mL samples of the pre-culture were transferred to two 1-L Erlenmeyer flasks, each containing 100 mL of seed medium, and grown with shaking (200 rpm) in a 30 °C incubator for 19–20 h. The entire seed culture (200 mL) was added as the inoculum to the 1.8-L primary culture in a fermenter (initial OD600 = 1.5–2.0 in 2 L). IPTG at a final concentration of 1 mM and kanamycin (25.0 μg/L) were also added during fermentation inoculation. A NBS BioFlo 3000 fermenter system (New Brunswick Scientific,
Edison, NJ, USA) equipped with a 6.6-L jar was used for all fed-batch cultivation experiments. The pH was maintained at 7.0 by addition of 28 % (v/v) ammonia solution (Junsei Chemical Co., Ltd., Tokyo, Japan). Temperature and agitation were maintained at 30 °C and 600 rpm, respectively, by a proportional-integral-derivative controller throughout the entire cultivation period. The aeration rate was maintained at 1 L/L/min. Foaming was suppressed by addition of 1:10 diluted antifoam 204 (Sigma-Aldrich). Feeding solution (200 mL) was manually added each time the residual glucose level decreased to <20 g/L.

Analytical procedures
Two high-performance liquid chromatography (HPLC) systems, Agilent 1100 (Agilent Technologies) and Waters Breeze 2 (Waters Corporation, Milford, MA, USA), were used to determine the metabolite concentration in the culture broth. For detection of amino compounds, the supernatant of the culture samples was reacted with o-phthaldehyde as previously described [13] prior to injection into the Eclipse Zorbax-AAA column (Agilent Technologies). Linear gradients of mobile phase A [10 mM Na2HPO4, 10 mM Na2B4O7·10H2O, and 8 mg/L NaN3 (pH 7.2)] and mobile phase B (methanol, acetonitrile, and water at a volumetric ratio of 45:45:10) were used to separate the amino acids in the column. Borate buffer (0.4 M; pH 10.2) was used as a buffering agent rather than pH 9.0 buffer as previously described [13]. The derivatized compounds were detected using a diode-array detector at 338 nm. The column temperature was set to 25 °C, and the flow rate of the pump was set to 0.640 mL/min. The following gradient was applied for resolving the compounds: 0–0.5 min, 0 % B; 0.5–18 min, a linear gradient of B from 0 to 57 %; 18–26 min, a linear gradient of B from 57 to 100 %; 26–31.8 min, a linear gradient of B from 100 to 0 %; 31.8–32 min, 0 % by volume. Glutaric acid was detected using the Waters Breeze 2 HPLC system (Waters Corporation) with a MetaCarb 87H column (Varian; Crawford Scientific, Strathaven, UK) and a constant flow of sulfuric acid solution at 0.5 mL/min. The Waters Breeze 2 system included an isocratic pump (Waters 1515; Waters Corporation), a refractive index detector (Waters 2414; Waters Corporation), and an autosampler (Waters 2707; Waters Corporation).

Cell growth was monitored by measuring the OD600 with an Ultrospec 3000 spectrophotometer (Amersham Biosciences, Uppsala, Sweden). The correlation factor (0.28 g of dry weight of cells per L per OD600 of 1) was experimentally determined and used for biomass concentration calculation of flask-cultivated cells. This correlation factor was in agreement with a previously reported value [47]. Glucose concentration was measured using a 2700 biochemistry analyzer (YSI, Yellow Springs, OH, USA). When necessary, diluted HCl solution was used to neutralize CaCO3 in the cultivation media.

Molecular docking simulation
Molecular docking simulations were performed using Autogrid and Autodock 4.2.5.1 software [48]. Gasteiger charges and hydrogen atoms were added using AutoDockTools 1.5.6. A Lamarckian genetic algorithm with default parameters was used, and no peptide residues were kept flexible. The docking grid was set to encompass the catalytic pocket, but not the entire enzyme. For docking of the natural substrate of 4-aminobutyrate aminotransferase, the substrate molecules were separately saved from a known structure (PDB ID: 4ATQ) [49] as a single molecule in the form of an external aldimine and used as a ligand. For docking with 5AVA aminotransferase, pyridoxal phosphate and 5AVA in the form of an external aldimine were used as the ligand. Torsion about the bond between the pyridine moiety of the pyridoxal phosphate and the Schiff base was not allowed during docking. The docking results were visualized using PyMol 1.6 (https://www.pymol.org/pymol) without additional hydrogen atoms.

Molecular modeling
Homology modeling was carried out with SWISS-MODEL [50]. 4-Aminobutyrate aminotransferase (E.C. 2.6.1.19) of C. glutamicum was homology modeled using the same enzyme from A. aurescens (PDB ID: 4ATQ chain B) [49] as a template. A minor correction was applied for this model (Additional file 2: Figure S2) based on a different enzyme structure (PDB ID: 3LV2). The same enzyme from S. griseus was homology modeled using that from Mycobacterium smegmatis (PDB ID: 3Q8 N chain D) as a template. 5-Aminovalerate aminotransferase (E.C. 2.6.1.48) of P. putida KT2440 was homology modeled using 4-aminobutyrate aminotransferase from E. coli (PDB ID: 1SFF chain A) [51] as a template.
Additional files

Additional file 1: Figure S1. Expression levels of the davA and davB genes in recombinant C. glutamicum GBaT. Selected residues in the PLP-binding pocket from the SWISS-MODEL-predicted molecular structure of C. glutamicum GBaT (magenta) are shown. The file261 residue (orange) near the predicted binding pocket for PLP from the original modeling simulation result protrudes into the binding pocket, thereby hindering subsequent docking simulations. This residue was corrected based on a known crystal structure of a different enzyme (yellow; PDB ID: 3LV2) with the correct Ile orientation at the same position.

Additional file 2: Figure S2. Simulation of molecular docking of 5AVA to homology-modeled DavT from P. putida KT2440. DavT was homology-modeled based on a known structure (PDB ID: 1SFF).

Additional file 3: Figure S3. Molecular docking of external aldime with C. glutamicum GabT. (A) Molecular docking of external aldime (grey; PLP and γ-amino butyrate) to homology-modeled GabT from C. glutamicum (magenta). A known crystal structure of GabT from Arthrobacter aurescens (green; PDB ID: 4ATQ) (Bruce et al. [49]) is superimposed for comparison. (B) Molecular docking of external aldime (blue; PLP and 5AVA) to homology-modeled GabT from C. glutamicum (magenta). Homology-modeled DavT from P. putida (salmon) and homology-modeled GabT from S. griseus (cyan) are superimposed for comparison.

Additional file 4: Figure S4. Simulation of molecular docking of 5AVA to homology-modeled DavT from P. putida KT2440. DavT was homology-modeled based on a known structure (PDB ID: 1SFF).

Additional file 5: Table S1. Strains and plasmids used in this study.

Additional file 6: Table S2. Primers used in this study.

Additional file 7: Table S3. Sequence of codon optimized davA and davB genes from P. putida.

Abbreviations

SAVA: 5-aminovalerate; SOD: superoxide dismutase; IPTG: isopropyl-β-D-thiogalactopyranoside.

Authors' contributions

SYL and SJP conceived the project. JHS, JSC, and JWC performed the experiments. SJP, JHS, SHP, YHO, MHL, KJJ, JCI, and JY analyzed the data. JHS, SHP, and SYL wrote the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

Please contact corresponding author for data requests.

Consent for publication

Our manuscript does not contain any individual person’s data in any form.

Ethics approval and consent to participate

Our manuscript does not report data collected from humans or animals.

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