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Fermentative Production of Halogenated Tryptophan Derivatives with *Corynebacterium glutamicum* Overexpressing Tryptophanase or Decarboxylase Genes

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The aromatic amino acid L-tryptophan serves as a precursor for many valuable compounds such as neuromodulators, indoleamines and indole alkaloids. In this work, tryptophan biosynthesis was extended by halogenation followed by decarboxylation to the respective tryptamines or cleavage to the respective indoles. Either the tryptophanase genes *tnaAs* from *E. coli* and *Proteus vulgaris* or the aromatic amino acid decarboxylase genes *AADCs* from *Bacillus atrophaeus*, *Clostridium sporogenes*, and *Ruminococcus gnavus* were expressed in *Corynebacterium glutamicum* strains producing (halogenated) tryptophan. Regarding indoles, final titers of 16 mg L^{-1} 7-Cl-indole and 23 mg L^{-1} 7-Br-indole were attained. Tryptamine production led to a much higher titer of 2.26 g L^{-1} upon expression of *AADC* from *B. atrophaeus*. AADC enzymes were shown to be active with halogenated tryptophan *in vitro* and *in vivo* and supported production of 0.36 g L^{-1} 7-Br-tryptamine with a volumetric productivity of 8.3 mg L^{-1} h^{-1} in a fed-batch fermentation.

Introduction

Halogenated amino acids are important building blocks in the organic chemical synthesis and serve as precursors of complex molecules.[1] Halogenated tryptophan derivatives such as 7-bromo- and 7-chlorotryptophan (7-Br-tryptophan and 7-Cl-tryptophan) serve as important precursors for industrially relevant compounds. Brominated tryptophan for example is a biosynthetic precursor in complex structures which occur in lower marine invertebrates or sponges,[2] whereas chlorinated tryptophan is integrated into the indolocarbazole antitumor agent rebeccamycin.[3] Fermentative bromination and chlorination of tryptophan were achieved by introducing the FAD-dependent tryptophan 7-halogenase RebH and the NADH-dependent flavin reductase RebF in tryptophan overproducing strains.[4] This strategy was successfully applied using a tryptophan overproducing *Corynebacterium glutamicum* strain,[5] which produced 1.2 g L^{-1} 7-Br-tryptophan[4] in a fed-batch fermentation or 0.1 g L^{-1} 7-Cl-tryptophan[6] when sodium bromide or sodium chloride was added to the cultivation medium.

L-Tryptophan is one of the 20 proteinogenic amino acids in all organism from bacteria to humans.[7] In humans, tryptophan serves as precursor for several biologically active and important molecules such as neuromodulators of the central nervous system,[7] including the neuromediator serotonin (5-hydroxytryptamine) for regulation of gastrointestinal functions, mood, hemodynamics and appetite.[8,9] Tryptophan can also be decarboxylated to the indoleamine tryptamine, which is an important starting substrate for biosynthesis of indole alkaloids in plants.[10] This reaction is catalyzed by the pyridoxal-5′-phosphate (PLP) dependent[11] aromatic amino acid decarboxylases (AADC, EC 4.1.1.28).[12] AADCs play a crucial role in animals for monoaminergic neurotransmission as they decarboxylate aromatic L-amino acids to their catecholamine and indoleamine derivatives such as dopamine and serotonin.[13–15] The animal and bacterial AADCs are known to accept a broad range of substrates, whereas the plant AADCs show a narrow substrate spectrum.[12,16] The bacterial AADC from *Bacillus atrophaeus*, for example, accepts diverse aromatic amino acids, including 5-hydroxytryptophan, the direct precursor for serotonin, and 4-chloro-L-phenylalanine as substrates.[12] In bacteria, tryptophan is also a direct precursor for the synthesis of indole, which is the building block of indole alkaloids and serves as an inter- and intracellular signal controlling different processes like

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plasmid stability, drug resistance and biofilm formation.[17] Indole is synthesized by the PLP-dependent tryptophanase (TnaA; EC 4.1.99.1),[17] which reversibly cleaves L-tryptophan to indole, pyruvate, and ammonia.[18] Most of the known tryptophanases have similar molecular weights, subunit structures, and PLP contents, but they differ in their amino acid sequences,[19,20] the substrate spectrum,[21–23] the pH optimum,[22,24] and the specific enzymatic activity.[25,26] The TnaA of *Escherichia coli* is one of the most studied examples. Expression of *E. coli tnaA* is regulated by tryptophan, such that when the concentration of tryptophan is low, the expression of *tnaA* is downregulated, while the *trp* operon is upregulated.[27,28] At higher concentration of tryptophan, *tnaA* is derepressed and indole production results as consequence. Moreover, the indole biosynthesis is regulated by environmental factors like cell density, carbon source, temperature, and pH control.[17] In plant secondary metabolism, another enzyme, the indole-3-glycerol phosphate lyase, converts indole-3-glycerol phosphate, an intermediate of the tryptophan biosynthetic pathway, to free indole.[29] In daily life, indole has different applications for the production of pharmaceuticals,[30] antioxidants,[31] dyes, pigments,[32] and fragrances.[33]

With halogenated tryptophans as substrate and TnaA or AADC extending tryptophan biosynthesis, fermentative production of halogenated indoles[34] or tryptamines appears feasible. 7-Bromoindole (7-Br-indole) can serve as a precursor for the indole derivative 7-bromoisatin, a precursor of the antimitotic agent diazonamide A.[35–37] 7-Br-indole, chlorinated indole and 5-iodoindole have a strong toxic effect to persister cells of *E. coli*. Formation of bacterial persister cells occurs together with multidrug resistance, so halogenated indoles can serve as potential compounds targeting persister cells.[38,39] Production of 7-chloroindole (7-Cl-indole) and 5-Cl-indole has been shown in a biocatalyzed approach using halogenating enzymes derived from *S. cetonii*, *P. putida*, and *A. hydrophila* respectively.[40] 5-Br-indole was synthesized by a purified putative tryptophanase AetE from *A. hydrillicola* by PLP-dependent conversion of 5-Br-tryptophan *in vitro*.[41] However, fermentative production of halogenated indole derivatives has been described scarcely in literature. For instance, *P. chlororaphis* ACN, a producer of the tryptophan derived dichlorinated metabolite pyrrolnitrin, was genetically modified to fermentatively produce the new compound 3-(2'amino-4'-chlorophenyl)pyrrole.[42] In nuclear medicine, halogenated tryptamines are widely used for the synthesis of halogen substituted derivatives of biologically active compounds for monitoring abnormal brain states occurring in diverse diseases.[43] A further example is the dichlorinated tryptamine 4,6-dichloro-2-methyl-3-aminoethylindole (DCAI) that binds as a small-molecule ligand to oncogenic K-ras inhibiting the SOS-mediated nucleotide exchange activity, and thus, Ras activity.[44,45] Similarly, 7-chlorotryptamine (7-Cl-tryptamine) is a direct precursor of halogenated alkaloid analogues such as 12-chloro-19,20-dihydroakuammicine.[46]

As *C. glutamicum* serves as host for the fermentative production of various amino acids, in particular L-glutamate and L-lysine,[47] it is an ideal host for production of amino acid derivatives such as the diamine putrescine,[48] the cyclic amino acid L-pipecolic acid,[49] the alkylated amino acid *N*-methyl-L-alanine,[50] and the alkylated aromatic amino acids *N*-methylanthranilate[51] and *N*-methyl-L-phenylalanine.[52]

In this study, tryptophan biosynthesis was extended by halogenation followed by either decarboxylation to the respective tryptamines or cleavage to the respective indoles. To this end, either *tnaA*s from *E. coli* and *Proteus vulgaris* or *AADC*s from *B. atrophaeus*, *Clostridium sporogenes*, and *Ruminococcus gnavus* were heterologously expressed in *C. glutamicum* strains that overproduce (halogenated) tryptophan. This work successfully demonstrates the fermentative production of halogenated indoles and tryptamines (Scheme 1).

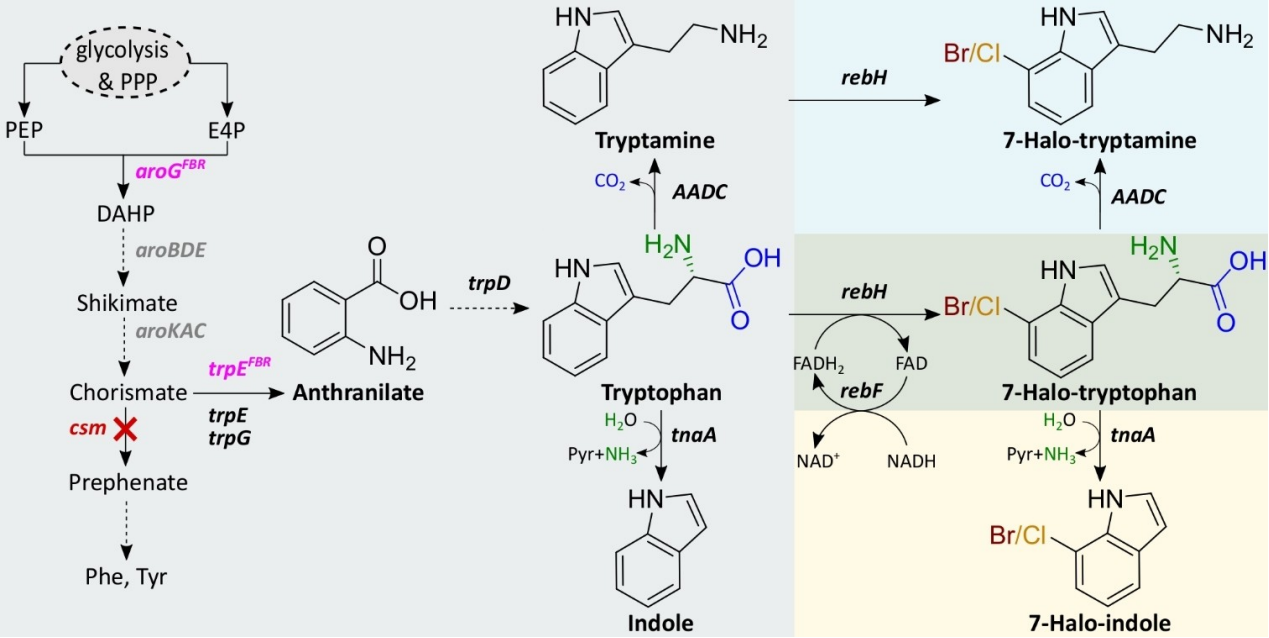
Results and Discussion

Physiological response of *C. glutamicum* to 7-halogenated indole and tryptamine

Indole alkaloids are pharmacologically active molecules[53] and their precursors may be toxic to bacteria as, e.g., shown for inhibition of protein folding in *P. putida*.[54] To test if *C. glutamicum* tolerates these compounds when added to the growth medium, *C. glutamicum* WT was grown in CGXII minimal medium with 40 g L^{-1} glucose as sole carbon source and different concentrations of 7-halogenated indole and tryptamine (Figure 1). The growth of *C. glutamicum* was affected similarly by 5 mM (about 1 g L^{-1}) of 7-Br-indole (Figure 1A, yellow) and 7-Cl-tryptamine (Figure 1B, bright red) resulting in a reduction of the growth rate to half maximum. Lower concentrations of 7-Cl-indole (0.4 g L^{-1}) and 7-bromotryptamine (7-Br-tryptamine) (0.7 g L^{-1}) were needed to reduce the growth rate of *C. glutamicum* to half-maximal (Figure 1). Since methanol solutions of 7-Cl-indole and 7-Br-indole were used, it has to be mentioned that the growth of *C. glutamicum* was reduced significantly by methanol alone (compare 0.33 h^{-1} in the absence of methanol, indicated as red dot in Figure 1A, with growth rates observed with methanol only, i.e., 0 mM 7-Br- or 7-Cl-indole). Despite the influence of low concentrations of halogenated indole and tryptamine on growth, production to the g L^{-1} scale was expected, since it has been shown before that 1.2 g L^{-1} 7-Br-tryptophan could be produced in fed-batch fermentation although the product reduces the growth rate of *C. glutamicum* to half maximal at a concentration of 0.091 g L^{-1}.[4] Taken together, *C. glutamicum* is suited for production of 7-halogenated indole and tryptamine to concentrations in the g L^{-1} range.

Identification of tryptophanases accepting 7-halogenated tryptophans

Firstly, the tryptophanases from *E. coli* and *P. vulgaris* were tested for indole production in *C. glutamicum*. It was successfully demonstrated that both TnaAs were able to synthesize indole from tryptophan *in vivo* (Figure S1), thus, these TnaAs were further investigated regarding their activity



Scheme 1. Scheme of the biosynthesis of halogenated indoles and tryptamines by metabolically engineered *C. glutamicum*. Endogenous genes are depicted in gray, chromosomal integrations in pink, and plasmid gene expression in black. A red cross indicates gene deletions. Thick arrows with gene name indicate individual catalytic steps, dashed arrows represent one or more catalytic steps. The genes encode the following enzymes: *aroG*, DAHP synthase; *aroB*, DHQ synthase; *aroD*, DHQ dehydratase; *aroE*, shikimate dehydrogenase; *aroK*, shikimate kinase; *aroA*, 5-enolpyruvylshikimate-3-phosphate synthase; *aroC*, chorismate synthase; *csm*, chorismate mutase; *trpE*, anthranilate synthase component I; *trpG*, anthranilate synthase component II; *trpD*, anthranilate phosphoribosyl transferase; *rebH*, flavin-dependent tryptophan 7-halogenase; *rebF*, flavin reductase; AADC, aromatic amino acid decarboxylase; *tnaA*, tryptophanase; PPP, pentose-phosphate-pathway; PEP, phosphoenolpyruvate; E4P, erythrose-4-phosphate; DAHP, 3-deoxy-D-arabino-heptulosonate; Phe, L-phenylalanine; Tyr, L-tyrosine; Pyr, pyruvate.

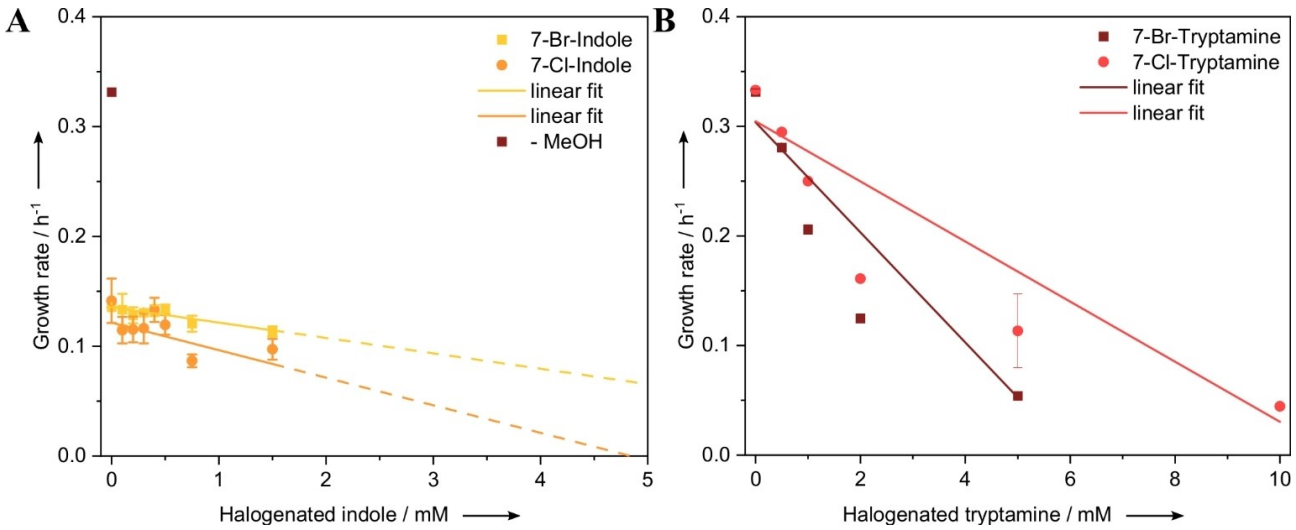


Figure 1. Specific growth rates of *C. glutamicum* WT grown in CGXII minimal medium with 40 g L^{-1} glucose, supplemented with either 7-Br-indole (yellow) or 7-Cl-indole (orange) (A) or 7-Br-tryptamine (dark red) or 7-Cl-tryptamine (bright red) (B). Growth was followed in the BioLector system. Half-maximal growth rates were calculated by extrapolation. Values and error bars represent means and standard deviations of technical triplicates.

with 7-Cl-tryptophan and 7-Br-tryptophan as substrates (Figure S2), since both compounds can be produced by fermentation using *C. glutamicum* strain HalT2.[4,6] Crude extracts were prepared from the control strain WT (pECXT99 A) and its isogenic strains overexpressing *tnaA* from *E. coli* or *P. vulgaris* and TnaA activity was determined with tryptophan, 7-Cl-tryptophan and 7-Br-tryptophan (Figure S2). With the natural substrate tryptophan, TnaA-*Ec* showed higher conversion of tryptophan to indole after 30 min than TnaA-*Pv*, while the empty vector control (WT (pECXT99 A)) showed no activity. With the halogenated tryptophan derivatives, the conversion rate dropped dramatically. Within 48 h, TnaA-*Ec*

and TnaA-*Pv* converted 2.4% and 1.6%, respectively, of the added 7-Cl-tryptophan to 7-Cl-indole. The conversion of 7-Br-tryptophan proceeded faster and within 1 h TnaA-*Ec* converted 2.2% and TnaA-*Pv* 0.95% of the added 7-Br-tryptophan to 7-Br-indole (Figure S2). Thus, both TnaA-*Ec* and TnaA-*Pv* were investigated in further experiments.

Fermentative production of halogenated indoles

C. glutamicum does not possess endogenous tryptophanases, thus, activity of heterologous tryptophanases can be analyzed without interference of endogenous activities. In order to investigate if TnaA-*Ec* and TnaA-*Pv* are suitable for fermentative production of halogenated indole *in vivo*, they were expressed in *C. glutamicum* strain HALT2 yielding strains HaloInd Ec and HaloInd Pv. Expression of the TnaAs was confirmed by SDS-PAGE (Figure S3). Upon cultivation in CGXII medium with 40 g L^{-1} glucose and either 50 mM NaBr or $CaCl_2$ as halide salt donor, HaloInd Ec and HaloInd Pv produced indole and halogenated indoles, while these compounds were not produced by the negative control strain HALT2 psyn (Figure 2). All three strains produced anthranilate, tryptophan and either 7-Br-tryptophan or 7-Cl-tryptophan with less of these compounds accumulating for HaloInd Ec and HaloInd Pv as compared to the control strain lacking a TnaA enzyme. The by-products anthranilate, tryptophan, indole and either 7-Cl-tryptophan or 7-Br-tryptophan accumulated to higher concentrations than the target compounds 7-Br-indole and 7-Cl-indole. For example, HaloInd Ec produced 1.4 ± 0.1 g L^{-1} anthranilate, 0.2 ± 0.01 g L^{-1} tryptophan, 0.06 ± 0.001 g L^{-1} indole, 0.3 ± 0.01 g L^{-1} 7-Br-tryptophan as by-products of 7-Br-indole production which amounted to 0.023 ± 0.001 g L^{-1}. Anthranilate accumulation might have been caused by the inhibitory effect of halogenated tryptophan on the enzyme activity of anthranilate phosphoribosyltransferase TrpD (K_i values of 0.091 g L^{-1} for 7-Br-tryptophan and 0.024 g L^{-1} for 7-Cl-tryptophan).[4] Thus, besides the known bottlenecks of production of halogenated tryptophans by strain HalT2,[4] the preference of the tryptophanases TnaA-*Ec* and TnaA-*Pv* of tryptophan over 7-Br-tryptophan and 7-Cl-tryptophan led to considerable production of indole as by-product. 7-Cl-indole was produced to comparable titers (about 16 mg L^{-1}) by strains HaloInd Ec and HaloInd Pv (Figure 2B). Production of 7-Br-indole based on the tryptophanase from *E. coli* was superior compared to the use of TnaA-*Pv* (14 ± 2 mg L^{-1}) (Figure 2A). HaloInd Ec produced 7-Br-indole (23 ± 0.01 mg L^{-1}) with a volumetric productivity of 0.32 mg L^{-1} h^{-1}.

It is known that the TnaA from *P. vulgaris* has a broad substrate spectrum. The wild-type TnaA from *P. vulgaris* accepts different cysteine derivatives besides its natural substrate tryptophan, like *S*-(*o*-nitrophenyl)-L-cysteine (SOPC), *S*-methyl-L-cysteine, *S*-ethyl-L-cysteine, and *S*-benzyl-L-cysteine, and the chlorinated L-alanine β-chloro-L-alanine.[55] But the substrate affinity (K_M) for most of the substrates was low, except for L-tryptophan, SOPC, and *S*-benzyl-L-cysteine. This indicated potential for further improvement by enzyme engineering. By an amino acid replacement of tyrosine 72 to phenylalanine, the active site of the TnaA was rearranged. Hence, the activity of the mutant TnaA Y72F decreased 50,000-fold for tryptophan. Additionally, the K_M for the previously mentioned substrate decreased by a multiple.[55] This mutation was created to increase the space around the α-carbon atom of the substrate. This may also be helpful for 7-Cl-tryptophan and 7-Br-tryptophan because the K_M for tryptophan was improved considerably and, thus, the substrate affinity for the halogenated tryptophans may increase as well. Additionally, the active site could be further improved by a rearrangement around the indole ring of tryptophan. By an expansion around this area the space for

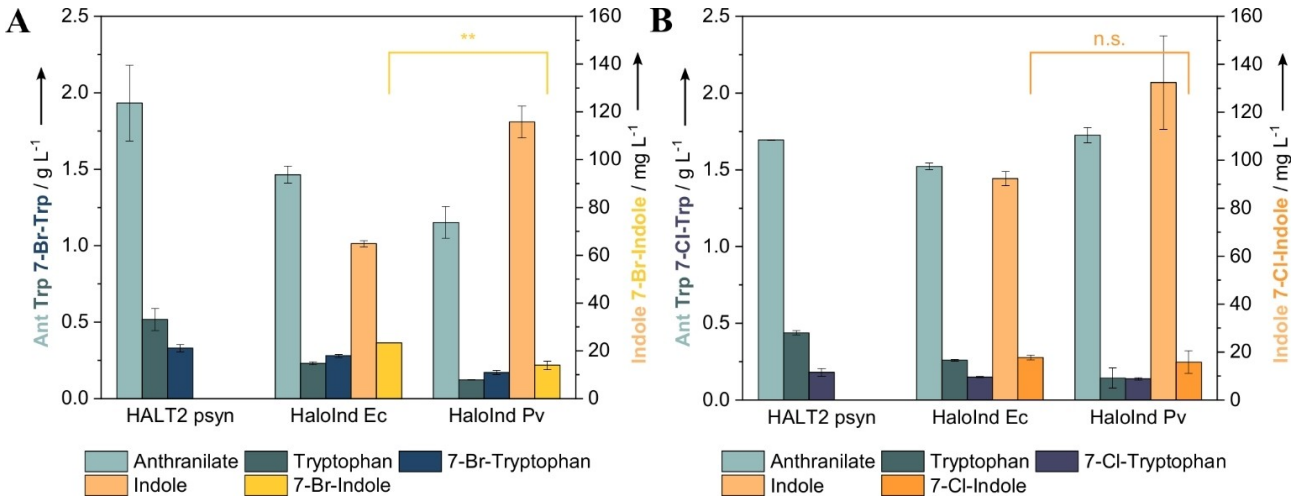


Figure 2. Production of 7-Br-indole (A) and 7-Cl-indole (B) by *C. glutamicum* HALT2 expressing *tnaA* from *E. coli* or *P. vulgaris*. The strains HALT2 psyn (control strain), HaloInd Ec and HaloInd Pv were cultivated in shake flasks in CGXII medium with 40 g L^{-1} glucose and 1 mM IPTG, supplemented with either 50 mM NaBr (A) or $CaCl_2$ (B). Values and error bars represent means and standard deviations of biological triplicates with supernatants analyzed after 72 h. Statistical significance was determined by Student's paired t-test (** $P < 0.01$, n.s., not significant).

other tryptophan derivatives like 5,6-halo-L-tryptophans, methyl-L-tryptophans or hydroxy-L-tryptophans could be created. For the wild-type TnaA from *E. coli* it was shown before that it accepts different tryptophan derivatives, like 4-, 5-, 6-, and 7-methyltryptophan and 4-, 5-, 6-, and 7-chlorotryptophan.[19] In this study, the TnaA from *E. coli* showed the lowest and slowest conversion rate with 7-Cl-tryptophan, which fits the previously tested *E. coli* TnaA. Additionally, a TnaA variant from a tryptophan auxotrophic *E. coli* mutant was tested, but no activity with halogenated tryptophan as substrate was observed.[19] Hence, no improvement of this *E. coli* TnaA mutant was achieved. Another promising *E. coli* TnaA mutant was generated by exchanging histidine 463 by phenylalanine, which is located in the substrate-binding portion of the active site.[56] As consequence, the high activity and substrate specificity towards tryptophan were lost, whereas the substrate spectrum was extended to the tryptophan benzimidazole analog β-(benzimidazol-1-yl)-L-alanine.[57] Shifting substrate specificity from the natural substrate tryptophan towards tryptophan analogues may also be beneficial for conversion of halogenated tryptophans.

Screening of AADCs for tryptamine synthesis

The cleavage of 7-Br-tryptophan and 7-Cl-tryptophan by tryptophanases to pyruvate, ammonia and the respective halogenated indoles did not result in a metabolic pull since anthranilate, tryptophan, 7-Br-tryptophan and 7-Cl-tryptophan remained dominant by-products. Tryptophan can be decarboxylated by AADC and, besides the product tryptamine, this conversion of tryptophan only yields carbon dioxide. Carbon dioxide partitions into the gas phase driving decarboxylation reactions as, e.g., exploited in fermentative production of cadaverine and putrescine, the decarboxylation products of lysine and ornithine.[58,59] The AADC enzyme class was first characterized in mammals to catalyze the formation of dopamine from L-DOPA and serotonin from 5-hydroxytryptophan, but also synthesized amines like tyramine, 2-phenylethylamine and tryptamine in traces.[14] It received attention in the context of the human metabolic disorder amino acid decarboxylase deficiency,[60] but not in a biotechnological context. The plant tryptophan decarboxylase (TDC) from *Catharanthus roseus* has been the only decarboxylase that was used for production of tryptamine and serotonin[61,62] and in contrast to AADCs from mammals, the TDC from plants is specific for amino acids with indole side chains, but does not accept phenol side chains.[63] However, due to the inefficient conversion and low titers reported for the plant enzyme, three AADCs from the bacteria *C. sporogenes*, *B. atropheaus* and *R. gnavus* were chosen instead and screened for tryptamine synthesis. The tryptophan overproducing *C. glutamicum* strain Tp679 was transformed with the vector pECXT-P*syn* carrying either one of the AADC genes or none as a negative control. Expression of the AADCs was confirmed by SDS-PAGE (Figure S3). The resulting strains Tra Cs, Tra Ba, Tra Rg, and Tra psyn were cultivated in CGXII medium with 40 g L^{-1} glucose for 48 h. While the control strain produced 2.44 ± 0.01 g L^{-1} tryptophan, all three AADC expressing strains did not produce tryptophan, but tryptamine and in the case of Tra Rg and Tra Ba some anthranilate (0.37 ± 0.03 g L^{-1} and 0.07 ± 0.01 g L^{-1}, respectively) (Figure 3). Anthranilate accumulation might be caused by inhibition of anthranilate phosphoribosyl transferase TrpD, which should be further investigated experimentally. The highest tryptamine titer of 2.26 ± 0.05 g L^{-1} was reached using strain Tra Ba, followed by Tra Cs (1.55 ± 0.17 g L^{-1}) and Tra Rg (1.08 ± 0.04 g L^{-1}). Tryptamine production by Tra Ba occurred with a volumetric productivity of 47 mg L^{-1} h^{-1}. To the best of our knowledge, this is the first report of efficient fermentative *de novo* tryptamine production.

Fermentative production of tryptamine with TDC from *C. roseus* was attempted, but no tryptamine formation was observed (data not shown), thus, the bacterial AADCs proved superior to the TDC from *C. roseus*. In one study using TDC from *C. roseus*, 53% of 1 mM supplemented tryptophan was decarboxylated to tryptamine[62] while another study aimed at overcoming the conversion bottleneck by applying a co-culture strategy with a tryptophan precursor supplying strain and a tryptamine production strain, yielding 86% conversion of tryptophan, however, producing only 0.19 g L^{-1} tryptamine.[61] The lower conversion by the TDC from *C. roseus* as compared to the bacterial AADCs may be due to the inhibition of the plant TDC by either the substrate L-tryptophan or the product tryptamine. Substrate inhibition has not been described, but D-tryptophan and tryptamine have been identified as non-competitive and competitive inhibitor, respectively.[63] If the bacterial AADCs suffer from substrate or product inhibition, has

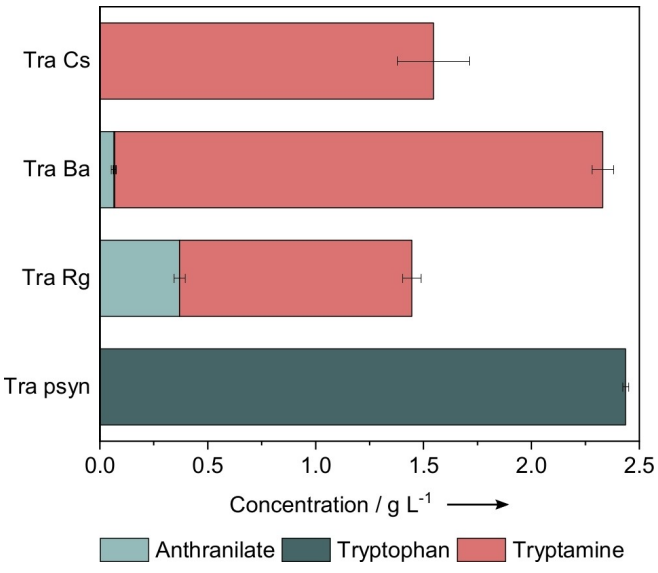


Figure 3. Screening of AADCs from *C. sporogenes*, *B. atrophaeus*, and *R. gnavus* for production of tryptamine in the background strain Tp679 (pCES208-*trpED*). The strains Tra psyn (control strain), Tra Cs, Tra Ba and Tra Rg were cultivated in shake flasks in CGXII medium with 40 g L^{-1} glucose. Values and error bars represent means and standard deviations of biological triplicates with supernatants analyzed after 48 h.

not been investigated. Despite the high V_{max} of 2.71 mM min^{-1} mg^{-1} and low K_M of 0.12 mM of TDC for tryptophan[64] compared to AADCs from *B. atrophaeus* (0.27 mM),[12] *C. sporogenes* (0.12 mM min^{-1}; 2.8 mM), and *R. gnavus* (0.04 mM min^{-1}; 1.1 mM),[65] this type of inhibition of TDC from *C. roseus* makes it less suitable for tryptamine production. The only inhibitor described for AADCs is (*S*)-α-fluoromethyltryptophan, a tryptophan-mimicking compound forming a covalent bond with PLP in the AADCs from *C. sporogenes* and *R. gnavus*.[65] Product inhibition by dopamine, β-phenylethylamine and tyramine as well as slight inhibition by tryptamine was found for the broad substrate spectrum AADC from the bacterium *Micrococcus percitreus*.[66] It remains to be studied if the AADC from *B. atrophaeus* that performed best for *in vivo* tryptamine production and is known to catalyze the decarboxylation of a variety of aromatic L-amino acids with high activity like phenylalanine, 5-hydroxytryptophan and several halogenated phenylalanines[12] is free from substrate and/or product inhibition.

Assaying AADC activity for tryptophan and its halogenated derivatives

AADCs are reported to exhibit a wide substrate spectrum of aromatic L-amino acids, such as phenylalanine, 5-hydroxytryptophan, L-DOPA and halogenated phenylalanine derivatives.[12] However, their activity towards halogenated tryptophans has not been examined yet. Since we aimed to develop a fermentative process, specific activities were measured using crude extracts of strains Tra Cs, Tra Ba, and Tra Rg with the substrates tryptophan, 7-Br-tryptophan, and 7-Cl-tryptophan. As expected, no activity was measured with the crude extract of the negative control Tra psyn (data not shown). The K_M values of AADC-Ba and AADC-Rg determined for tryptophan were three to four times higher than the published values obtained for purified enzymes,[12,65] which may indicate that the crude extracts used here contain (an) interfering compound(s). Importantly, all tested AADCs showed activity with 7-Br-tryptophan and 7-Cl-tryptophan in addition to the natural substrate tryptophan (Table 1, Figure S4). Notably, for the enzymes AADC-Cs and AADC-Ba the K_M and V_{max} values were in a similar range when comparing tryptophan, 7-Cl-tryptophan, and 7-Br-tryptophan as substrates (Table 1). For example, AADC-Ba showed K_M values of 0.9 ± 0.3 mM for tryptophan and 0.7 ± 0.3 mM for 7-Cl-tryptophan (Table 1). Thus, 7-Br-tryptophan and 7-Cl-tryptophan may be accommodated in the substrate binding pocket as well as the native substrate tryptophan. For AADC-Rg activity towards 7-Br-tryptophan and 7-Cl-tryptophan was detected, however, in the case of 7-Br-tryptophan the measured activity was unprecise. While these results were sufficient to guide developing our fermentative approach, future work using the purified enzymes will have to be performed to obtain a detailed characterization of the substrate spectrum of these enzymes.

The substrate binding pocket in the published structure of AADC-Rg (PDB ID: 6EEW)[65] is depicted in Figure 4. Indeed, the cavity that is formed by the residues Phe98, His120 and Leu126 provides sufficient space to accommodate a bromo- or chloro-substituent at C7 of tryptophan.

Extension of tryptophan biosynthesis for fermentative production of halogenated tryptamines

The findings that strains Tra Cs, Tra Ba and Tra Rg supported tryptamine production and that the employed AADCs accepted 7-Cl-tryptophan and 7-Br-tryptophan comparably well as tryptophan raised the question if 7-Cl-tryptamine and 7-Br-tryptamine can be produced by fermentation. Thus, AADC genes were expressed in strain HALT2 yielding strains HaloTra Cs, HaloTra Ba, HaloTra Rg in addition to control strain HALT2 psyn that lacked AADC. These strains were cultivated in CGXII medium with 40 g L^{-1} glucose, supplemented with 1 mM IPTG and either 50 mM NaBr or 50 mM $CaCl_2$ as halide salt. After 72 h, the control strain HALT2 psyn accumulated anthranilate, tryptophan and either 0.81 ± 0.07 g L^{-1} 7-Br-tryptophan or 0.42 ± 0.03 g L^{-1} 7-Cl-tryptophan (Figure 5). Notably, strains HaloTra Cs, HaloTra Ba and HaloTra Rg produced either 7-Cl-tryptamine or 7-Br-tryptamine. For example, strain HaloTra Rg produced 0.15 g L^{-1} 7-Br-tryptamine and strain HaloTra Cs produced 0.22 g L^{-1} 7-Br-tryptamine (Figure 5). Tryptophan, 7-Cl-tryptophan, and 7-Br-tryptophan were not produced as by-products and accumulation of anthranilate was much reduced. However, tryptamine production exceeded production of 7-Br-tryptamine and 7-Cl-tryptamine (Figure 5). This reflects that tryptophan is decarboxylated to tryptamine in a single reaction, while consecutive halogenation and decarboxylation of tryptophan are required for production of 7-Cl-tryptamine and 7-Br-tryptamine. Anthranilate production differed when sodium chloride was replaced

Table 1. Kinetic properties of AADC-Cs, AADC-Ba and AADC-Rg. Enzymatic assays were performed using crude extracts of Tra Cs, Tra Ba and Tra Rg. Reaction mixtures contained 50 mM potassium phosphate buffer pH 7.0, 40 μM PLP and either tryptophan, 7-Br-tryptophan, or 7-Cl-tryptophan in different concentrations. Reactions were carried out in triplicates and stopped using acetonitrile and samples were analyzed via HPLC. The values for V_{max} are given in U mg^{-1} whole protein.

	AADC-Cs		AADC-Ba		AADC-Rg	
	K_M [a]	V_{max} [b]	K_M [a]	V_{max} [b]	K_M [a]	V_{max} [b]
Trp	2.2 ± 0.1	0.5 ± 0.0	0.9 ± 0.3	0.3 ± 0.0	4.7 ± 2.5	2.5 ± 0.8
7-Br-Trp	3.1 ± 2.4	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.0	n.d.	n.d.
7-Cl-Trp	4.5 ± 1.9	0.6 ± 0.1	0.7 ± 0.3	0.3 ± 0.0	11.0 ± 3.9	3.0 ± 0.9

[a] ln [mM]. [b] ln [U mg^{-1}]. n.d., not determined.

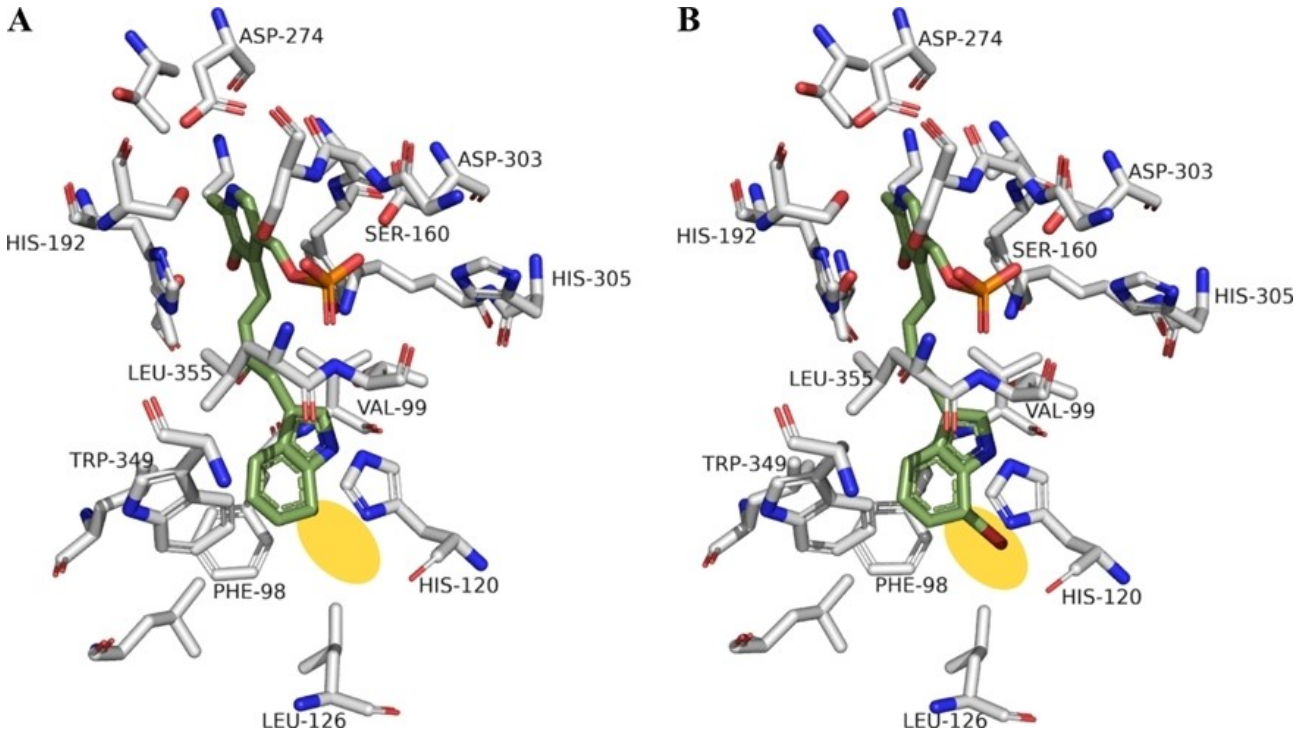


Figure 4. Amino acid residues in the active center of AADC-Rg binding tryptophan (A) or 7-Br-tryptophan (B). Depicted in green are the Schiff base adducts of PLP and the decarboxylated products tryptamine (A) and 7-Br-tryptamine (B). Carbon, white/green; nitrogen, blue; oxygen, red; phosphorus, orange; bromine, dark red. The structure was obtained from (PDB ID: 6EEW).[65]

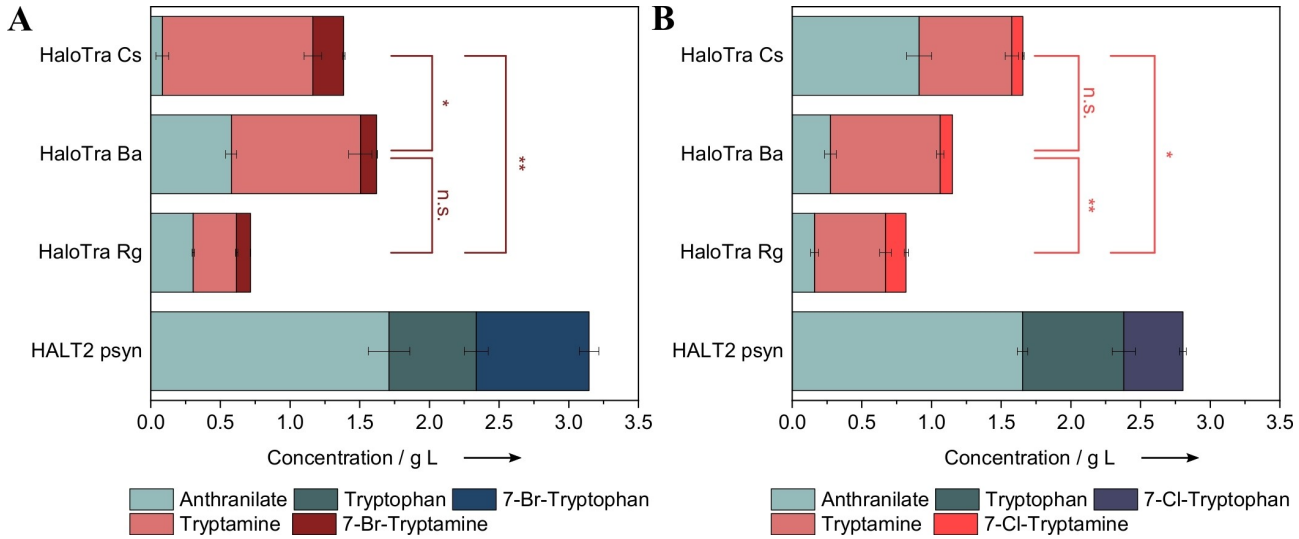


Figure 5. Production of 7-Br-tryptamine (A) and 7-Cl-tryptamine (B) by *C. glutamicum* HALT2 expressing AADCs from *C. sporogenes*, *B. atrophaeus or R. gnavus*. The strains HALT2 psyn (control strain), HaloTra Cs, HaloTra Ba and HaloTra Rg were cultivated in shake flasks in CGXII medium with 40 g L^{-1} glucose and 1 mM IPTG, supplemented with either 50 mM NaBr (A) or $CaCl_2$ (B). Values and error bars represent means and standard deviations of biological triplicates with supernatants analyzed after 72 h. Statistical significance was determined by Student's paired t-test (* $P < 0.5$, ** $P < 0.01$, n.s., not significant).

by sodium bromide in the growth medium. While the effect on anthranilate production currently is not understood, it is known that *C. glutamicum* requires chloride under hyperosmolar conditions,[67] possibly, homeostasis of sodium ions that are cytotoxic and typically expelled from the *C. glutamicum* cells[68] may be affected since export of sodium ions by Na^+/H^+ antiporters is enhanced by simultaneous export of chloride ions via the ClC-type sodium channels.[67,69]

Fed-batch fermentation for the production of brominated tryptamine

Since strain HaloTra Cs produced 7-Br-trypamine to a titer of 0.22 ± 0.01 g L^{-1} with a volumetric productivity of 3.1 mg L^{-1} h^{-1}, it was tested if production is stable when upscaled to bioreactor cultivation. Following the published protocol for fed-batch bioreactor cultivation of HALT2,[4] HaloTra Cs was cultivated in HSG medium with 40 g L^{-1} glucose, 1 mM IPTG and 50 mM NaBr as halide salt. It was shown that using HSG complex medium resulted in much lower intermediate accumulation compared to CGXII minimal medium (Figure S5). The culture reached the stationary growth phase after around 20 h. Feeding initiated at 18 h and was regulated according to the relative dissolved oxygen saturation (rDOS) regulation set to 60% (Figure S6). Relatively high amounts of tryptophan and tryptamine at the beginning of the cultivation were carried over from the pre-culture since the genes for tryptamine biosynthesis were constitutively expressed (pCES208-*trpED* and pECXT-P*syn-AADC-Cs*) (Figure 6). 7-Br-tryptophan started to accumulate at 10 h, i.e., about 4 h before 7-Br-tryptamine started to accumulate. In the time from 12 to 30 h, 7-Br-tryptamine accumulated steadily with a production rate of 6.2 mg L^{-1} h^{-1}. After subsequently increasing the feed rate, 7-Br-tryptamine accumulated faster with a rate of 25.3 mg L^{-1} h^{-1}, however, also anthranilate was produced as byproduct with a rate of 15.1 mg L^{-1} h^{-1} (Figure 6).

Overall, 7-Br-tryptamine was produced to a titer of 0.36 g L^{-1} with a volumetric productivity of 8.3 mg L^{-1} h^{-1}. Since these values exceeded those obtained in shake flask culture, a proof-of-concept for stable fermentative production of 7-Br-tryptamine by the constructed strain was obtained.

As the synthesis of halogenated tryptamine from tryptophan requires a halogenation and a decarboxylation reaction, two different sequences of reactions are possible. We have shown that the bacterial AADCs used in this study equally decarboxylate the native substrate tryptophan as well as its halogenated derivatives. In principle, the halogenation-decarboxylation sequence could be interchangeable *in vivo*. However, in an *in vitro* assay RebH only halogenated tryptamine to 7-Cl-tryptamine with 20% conversion when using 1.5 mM tryptamine as substrate.[70] Therefore, the decarboxylation-halogenation sequence is possible, but less likely to occur due to the comparatively high AADC activities. With the N470S mutant of RebH, the 7-Cl-tryptamine yield increased 2.5-fold while maintaining >99% selectivity for the C7 position.[70] Another mutant was designed in a rational approach in which residues proximal to the carboxyl group of the substrate tryptophan were targeted, yielding the mutant Y455 W that facilitated a much higher preference for tryptamine over tryptophan and increased 7-Cl-tryptamine accumulation in an *in vitro* assay.[71,72] A combination of both mutations in RebH might enable tryptamine halogenation via the decarboxylation-halogenation sequence.

Further directed evolution of RebH generated mutants that switched regioselectivity to either C5 or C6[70] or that accepted other indole compounds like indole 6-carboxylic acid, 5-methylindole, and 5-chloroindole. Recently, seven new bromotryptamine metabolites have been isolated from the sponge *Narrabeena nigra*.[73] These may have bioactivities relevant in the pharmaceutical industry and may be produced using RebH variants. In this respect, directed evolution combined with a substrate walking approach shifted the substrate spectrum of RebH towards more bulky substrates such as tetrahydroharmine, yohimbine, and evodiamine.[74] The different substrate spectra of RebH mutants in combination with the naturally broad substrate spectrum of AADCs hold the potential to greatly expand the product range of halogenated aromatic compounds by metabolically engineered *C. glutamicum*.

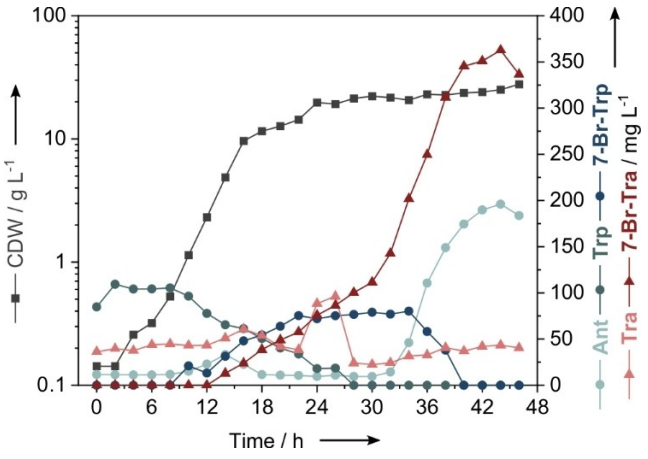


Figure 6. 7-Br-tryptamine production by *C. glutamicum* HaloTra Cs in fed-batch fermentation. The cells were cultivated in HSG medium with 40 g L^{-1} glucose, 1 mM IPTG and 50 mM NaBr. The feed contained 150 g L^{-1} ammonium sulfate, 400 g L^{-1} glucose, 5.14 g L^{-1} NaBr, 0.25 g L^{-1} tyrosine and 0.25 g L^{-1} phenylalanine. Cultivation was performed at 30 °C and pH 7.0, regulated with 10% (w/w) phosphoric acid and 4 M potassium hydroxide. The initial volume was 2 L, 143 mL of feed were added within 46 h. The titers were calculated to the initial volume. Ant, anthranilate; Trp, tryptophan; 7-Br-Trp, 7-bromotryptophan; Tra, tryptamine; 7-Br-Tra, 7-bromotryptamine.

Conclusion

We have expanded the tryptophan biosynthetic pathway of *C. glutamicum* by halogenation combined with either tryptophanase or aromatic amino acid decarboxylase. We identified two tryptophanases and three decarboxylases which were able to accept C7 halogenated tryptophan as substrate *in vivo*. We have demonstrated that tryptamine as well as halogenated indole and halogenated tryptamine can be produced in g L^{-1} and mg L^{-1} scale, respectively. Furthermore, the process was transferred to fed-batch bioreactor cultivation and stable 7-Br-tryptamine production was achieved. These findings add to the notion that *C. glutamicum* is a well-suited host for the production of aromatic compounds for pharmaceutical applications.

Experimental Section

Bacterial strains and growth: Bacterial strains and plasmids used in this study are listed in Table S1. *E. coli* DH5α[75] was used as a host for plasmid construction. *E. coli* and *C. glutamicum* were grown in lysogeny broth (LB) or brain heart infusion (BHI) medium in 500 mL baffled flasks at 120 rpm or 180 rpm at 37 °C or 30 °C, respectively. For growth and production experiments, *C. glutamicum* was inoculated in CGXII minimal medium with 40 g L^{-1} glucose[76] in 100 mL baffled flasks (filling volume 10%) to an optical density (OD_{600}) of 1, supplemented with either 50 mM NaBr or $CaCl_2$ as halide source. All strains derived from Tp679 (pCES208-*trpED*) were cultivated in CGXII minimal medium, supplemented with 1.37 mM tyrosine and 1.50 mM phenylalanine. Growth at 120 rpm was monitored for 72 h by measuring the optical density at 600 nm using a V-1200 spectrophotometer (VWR, Radnor, PA, USA). When necessary, the growth medium was supplemented with kanamycin (25 μg mL^{-1}), spectinomycin (100 μg mL^{-1}), and/or tetracycline (5 μg mL^{-1}). Isopropyl-β-D-1-thiogalactopyranoside (IPTG) (1 mM) was added to induce the gene expression from the vector pEKEx3 and pECXT99 A. To assess possible product toxicity, *C. glutamicum* wild type was grown in the Biolector microfermentation system using minimal medium with 40 g L^{-1} glucose, supplemented with 7-Br/Cl-indole and 7-Br/Cl-tryptamine at different concentrations.

Molecular genetic techniques and strain construction: *E. coli* was used as host organism for gene cloning. Transformation in *E. coli* was done by heat shock at 42 °C for 90 s with the rubidium chloride method.[75] *C. glutamicum* was transformed by electroporation at 2.5 kV, 200 Ω and 25 μF.[76] All used primers are listed in Table S2. The forward primers *Ec-tnaA*-1-fwd and *Pv-tnaA*-1-fwd harbor the artificial ribosome binding site (RBS) sequence (GAAAGGA GGCCCTTCAG). Some genes were amplified in two parts to enable a base pair exchange for better codon harmonization in *C. glutamicum* (Table S3). The primer pairs *Ec-tnaA*-1-fwd/*Ec-tnaA*-1-rv (part I) and *Ec-tnaA*-2-fwd/*Ec-tnaA*-2-rv (part II) were used to amplify *tnaA-Ec* gene from genomic DNA of *E. coli* K12. The primers *Ec-tnaA*-2-fwd/*Ec-tnaA*-1-rv harbor a base pair exchange to optimize the codon usage of arginine 295 for *C. glutamicum.* For amplification of the *tnaA-Pv* gene the genomic DNA of *Proteus vulgaris* (DSM 13387) and the primer pairs *Pv-tnaA*-1-fwd/*Pv-tnaA*-1-rv (part I) and *Pv-tnaA*-2-fwd/*Pv-tnaA*-2-rv (part II) were used. For the codon optimization of the arginine 4, isoleucine 284, arginine 289, arginine 457 and arginine 462 for *C. glutamicum* the primers *Pv-tnaA*-1-fwd, *Pv-tnaA*-1-rv, *Pv-tnaA*-2-fwd, and *Pv-tnaA*-2-rv were used. The *tnaA* genes (consisting of one or two parts) were cloned *via* Gibson assembly[77] in the vector pECXT99 A digested with *Bam*HI. The constructed plasmids pECXT99 A-*tnaA-Ec* and pECXT99 A-*tnaA-Pv* were transformed into *C. glutamicum* WT for enzymatic assay. For production experiments the genes from *E. coli* and *P. vulgaris* were cloned in the *Bam*HI digested pECXT-P*syn* plasmid. For this the codon harmonized *tnaA* gene from *E. coli* was amplified from plasmid pECXT99 A-*tnaA-Ec* with primers P*syn-tnaA-Ec*-fwd and P*syn-tnaA-Ec*-rv. For the amplification of the codon harmonized tryptophanase from *P. vulgaris* the primers P*syn-tnaA-Pv*-fwd and P*syn-tnaA-Pv*-rv were used. As a template the vector pECXT99A-*tnaA-Pv* was used. Additionally, an optimized RBS was calculated using SalisLab (Salis laboratory at Penn State University, https://salislab.net/software) and introduced in front of the tryptophanase genes (underlined in Table S2). The genes encoding AADCs from *B. atrophaeus* (Genbank: JQ400024), *C. sporogenes* (CLOSPO_02083) and *R. gnavus* (RUMGNA_01526) were codon-harmonized[78] and generated by gene synthesis (Thermo Fisher Scientific). The genes were amplified with the primers *AADC-Ba*-fwd and *AADC-Ba*-rv, *AADC-Cs*-fwd and *AADC-Cs*-rv, and *AADC-Rg*-fwd and *AADC-Rg*-rv, respectively. The amplified genes were cloned in the *Bam*HI digested pECXT-P*syn* plasmid. Optimized RBSs were introduced in front of the *AADC* genes (underlined in Table S2).

Enzymatic assay for tryptophanase with tryptophan and its halogenated derivatives: The measurement was performed in triplicates for tryptophan. The formation of 7-Br-indole and 7-Cl-indole in the enzyme assay was measured by HPLC. The reaction mixture contained 10 mM dipotassium phosphate buffer (pH 7.0), 0.1 mM PLP, 1 mM 7-Br-tryptophan/7-Cl-tryptophan, 5% 2-propanol (v/v) and the crude extract. The mixture was incubated at 30 °C and aliquots were taken in regular time intervals. The reaction was stopped with one volume of methanol and the product formation was measured with HPLC.

Enzymatic assay for decarboxylases with tryptophan and its halogenated derivatives: In order to quantify specific activity of the decarboxylases, an *in vitro* activity assay was performed referring to Ref. [12]. For this *C. glutamicum* cells overexpressing the respective decarboxylase were grown in BHI medium. After 24 h of cultivation cells were harvested by centrifugation and cell lysis was carried out with a sonicator (UP 200S, Dr. Hielscher GmbH, Teltow, Germany) at an amplitude of 60% and a duty cycle of 0.5 s for 9 min. To obtain cell free extracts the cell suspension was centrifuged (20,200 × *g*, 60–90 min, 4 °C). Protein concentration was determined using Bradford reagent and bovine serum albumin as a reference. Specific activity assays were carried out at 37 °C in a total volume of 400 μl in a potassium phosphate buffer (50 mM, pH 7.0). As cofactor 40 μM PLP was added to the reaction mixture. The substrates tryptophan, 7-Br-tryptophan or 7-Cl-tryptophan were added to the mixture, based on the published K_M values for tryptophan (0.27 mM for *B. atrophaeus*,[12] 2.8 mM for *C. sporogenes*,[65] and 1.1 mM for *R. gnavus*[65]). Thereby three different concentrations were used of each substrate (1-times, 2-times, or 5-times of the published K_M value for tryptophan for each decarboxylase). Enzyme dependency was shown in the mixture with abundant substrate concentration (5-times K_M), when substrate conversion was halved. After 0, 30 and 60 min, 50 μl aliquots were taken and the reaction was stopped with addition of 450 μl acetonitrile. Protein precipitation was performed by vortexing the aliquots for 2 min at 2680 rpm. After final centrifugation (20,200 × *g*, 10 min), the supernatant was analyzed *via* HPLC as described below. For protein modeling, active site of AADC from *R. gnavus* (PDB ID: 4OBV) was used. Simulations with bonded tryptophan or 7-Br-tryptophan were performed with PyMOL.

Analytic procedure: For the quantification of anthranilate, tryptophan and its derivatives 7-Br/Cl-tryptophan, indole, and 7-Br/Cl-indole, tryptamine, 7-Br/Cl-tryptamine a high-pressure liquid chromatography (HPLC) system was used (1200 series, Agilent Technologies Deutschland GmbH, Böblingen, Germany). Production titers were evaluated by using authentic standard curves. The supernatants of the cell culture or enzyme assay were collected by centrifugation (14,680 rpm, 20 min, RT) and further used for analysis. The separation was carried out by a reversed-phase HPLC using a pre-column (LiChrospher 100 RP18 EC-5 μ (40 mm × 4.6 mm), CS-Chromatographie Service GmbH, Langerwehe, Germany) and a main column (LiChrospher 100 RP18 EC-5 μ (125 mm × 4.6 mm), CS Chromatographie Service GmbH) with a flow rate of 1 ml min^{-1}. As mobile phase the eluents 0.1% TFA buffer (A) and acetonitrile (B) were used with the following gradient: 0 min 10% B, 1 min 10% B, 10 min 70% B, and 14 min 10% B. The detection was carried out with a Diode Array Detector (DAD, 1200 series, Agilent Technologies) at 280 nm and 330 nm.

Fed-batch fermentation: Fed-batch fermentation was performed in a 2 L reactor with a constant overpressure of 0.2 bar as described elsewhere.[4] As a culture medium HSG rich medium supplemented with 50 mM NaBr and 1 mM IPTG (added at timepoint 0 h) was

used. The feeding medium contained 150 g L^{-1} ammonium sulfate, 400 g L^{-1} glucose, 5.14 g L^{-1} NaBr, 0.25 g L^{-1} L-tyrosine, and 0.25 g L^{-1} L-phenylalanine. Automatic control of the stirrer speed kept the rDOS at 30%. The feeding started automatically when rDOS exceeded 60% and stopped when rDOS fell below the set-point. A stable pH of 7.0 was established and controlled by automatic addition of phosphoric acid (10% (w/w)) and potassium hydroxide (4 M). Antifoam 204 was used as antifoam agent. The titer and yield were calculated to the initial volume.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: *Corynebacterium glutamicum* · fermentation · halogenated compounds · metabolic engineering · tryptophan

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