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Metabolic engineering of *Corynebacterium glutamicum* for the fermentative production of halogenated tryptophan

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ABSTRACT

Halogenated compounds, like 7-chloro-L-tryptophan, are important intermediates or components of bioactive substances relevant for the pharmaceutical, chemical and agrochemical industries. About 20% of all pharmaceutical small molecule drugs and around 30% of all active compounds in agrochemistry are halogenated. Chemical halogenation procedures usually are characterized by the use of hazardous or even highly toxic chemicals. Recently, a biocatalytic process for L-tryptophan halogenation at the gram-scale using FAD-dependent halogenase and NADH-dependent flavin reductase enzymes has been described. Many proteinogenic amino acids are produced by fermentation using *Corynebacterium glutamicum*. The fermentative production of L-glutamate and L-lysine, for example, is operated at the million-ton scale. However, fermentative production of halogenated amino acids has not yet been described. In this study, fermentative production of the halogenated amino acid 7-chloro-L-tryptophan from sugars, ammonium and chloride salts was achieved. This required metabolic engineering of an L-tryptophan producing *C. glutamicum* strain for expression of the genes coding for FAD-dependent halogenase RebH and NADH-dependent flavin reductase RebF from *Lechevalieria aerocolonigenes*. Chlorination of L-tryptophan to 7-chloro-L-tryptophan by recombinant *C. glutamicum* was improved by optimizing the RBS of *rebH*. Metabolic engineering enabled production of 7-chloro-L-tryptophan and L-tryptophan from the alternative carbon sources arabinose, glucosamine and xylose.

1. Introduction

Halogenated amino acids are sought after by the pharmaceutical, chemical and agrochemical industries (Diederich and Stang, 2008). They are found in various natural products including the antibiotics chloramphenicol and pyrroindomycin, the plant growth-regulating thienodolin, and the antifungal pyrrolnitrin (Hammer et al., 1997; van Pée and Hölzer, 1999). Rebeccamycin is an indolocarbazole alkaloid antibiotic that inhibits DNA topoisomerase I and is produced naturally by *Lechevalieria aerocolonigenes* (Nishizawa et al., 2005; Onaka et al., 2003). 7-Chloro-L-tryptophan (7-Cl-Trp) is a precursor of rebeccamycin. Halogenation of L-tryptophan (Trp) requires two enzymes encoded in the *reb* cluster of *L. aerocolonigenes*: the FADH-dependent halogenase RebH and NADH-dependent flavin reductase RebF (Nishizawa et al., 2005). Halogenase RebH uses $FADH_2$, molecular oxygen (O_2) and chloride (Cl^-) to halogenate its substrate Trp yielding 7-Cl-Trp. In turn, recycling of the reduced cofactor $FADH_2$ requires nicotinamide adenine dinucleotide (NADH)-dependent flavin reductase RebF. Recently, a biocatalytic process for L-tryptophan halogenation at the gram-scale has been described based on the purified enzymes and cross-linked enzyme aggregates including alcohol dehydrogenase for NADH recycling with isobutanol (Frese and Sewald, 2015; Schnepel and Sewald, 2017). However, production of the halogenated amino acid 7-Cl-Trp by fermentation has neither been described for the native *L. aerocolonigenes* nor for other microorganisms.

Corynebacterium glutamicum is the work horse of fermentative amino acid production (Eggeling and Bott, 2015; Hermann, 2003; Lee et al., 2016; Wendisch et al., 2016). Besides its use in the million-ton scale L-glutamate and L-lysine processes, this bacterium has been engineered for production of various compounds including alcohols (Inui et al., 2004; Jojima et al., 2015; Siebert and Wendisch, 2015; Wendisch et al., 2016), diamines (Wendisch et al., 2018) like putrescine (Schneider and Wendisch, 2010), organic acids (Wieschalka et al., 2013), terpenoids (Heider et al., 2014) such as patchoulol (Henke et al., 2018) or non-

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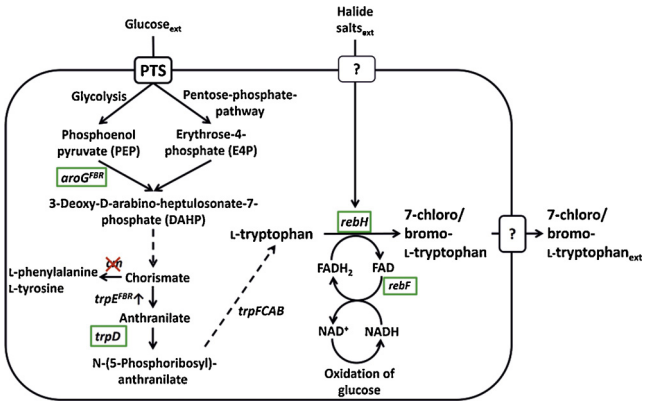

Fig. 1. Schematic representation of reactions in recombinant Trp and 7-Cl-Trp producing *C. glutamicum*. Genes names are shown next to reactions that are represented by arrows. Dashed arrows show several reactions. Overexpression of endogenous genes is indicated (↑), expression of heterologous genes is marked by green boxes, deletions are marked with red crosses. FBR: feedback resistant (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

proteinogenic amino acids (Wendisch, 2016) such as GABA (Jorge et al., 2016, 2017; Kim et al., 2013; Pérez-García et al., 2016). *C. glutamicum* has also been engineered to produce aromatic amino acids including Trp and aromatic compounds amenable from intermediates of the shikimate and chorismate pathways (Lee and Wendisch, 2017). Recently, a metabolic engineering strategy to overproduce Trp and related compounds such as 4-hydroxybenzoate has been described (Purwanto et al., 2018). Strain engineering for Trp production included plasmid-based expression of *trpD* from *E. coli* and genome-based expression of *trpE*^{FBR} coding for feedback-resistant anthranillate synthase, together with deletion of *csm* encoding chorismate mutase and expression of *aroG*^{FBR} encoding feedback-resistant 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase from *E. coli* in the *vdh*-deleted genome locus (Purwanto et al., 2018).

Here, we describe metabolic engineering of 7-Cl-Trp producing strains based on the above described Trp producing strain (Fig. 1). Overexpression of the genes encoding the FADH-dependent halogenase RebH and the NADH-dependent flavin reductase RebF from *L. aerocolonigenes* enabled halogenation of Trp to 7-Cl-Trp. Moreover, production of 7-Cl-Trp and Trp from the alternative carbon sources like arabinose, xylose and glucosamine that do not have competing uses for human and animal nutrition was established.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5α (Hanahan, 1983) was used for cloning of the plasmid constructs. *E. coli* and *C. glutamicum* were usually grown in lysogeny broth medium (LB) in 500 ml baffled flasks at 37 °C and 30 °C, respectively. For growth and production experiments *C. glutamicum* was inoculated in CGXII (Eggeling and Bott, 2005) supplemented with 40 g L^{-1} glucose in 500 ml baffled flasks to an optical density (OD_{600}) of 1 and incubated at 120 rpm (shaking diameter: 16.5 cm) at 30 °C. Growth was followed by measuring the optical density using V-1200 spectrophotometer at 600 nm (VWR, Radnor, PA, USA). For production of halogenated tryptophan, the minimal medium was supplemented with chloride salts. For all strains derived from Tp679 1.5 mM L-phenylalanine and 1.37 mM L-tyrosine were added to the minimal medium. If necessary, the growth medium was supplemented with kanamycin (25 µg ml^{-1}), spectinomycin (100 µg ml^{-1}) and/or tetracycline (5 µg ml^{-1}). To induce the gene expression from the vectors pEKEx3 (Stansen et al., 2005) and pECXT99 A (Kirchner and Tauch, 2003) isopropyl-β-D-1-thiogalactopyranoside (IPTG) (1 mM) was added.

2.2. Molecular genetic techniques and strains construction

Standard molecular genetic techniques were carried out as described elsewhere (Sambrook et al., 1989). *E. coli* was transformed by heat shock (Sambrook et al., 1989) and *C. glutamicum* by electroporation (Eggeling and Bott, 2005). The genes *rebH* and *rebF* were amplified from plasmid pET28-*rebH* and pET21-*rebF*, respectively. For amplification of *rebH* different forward primers (fwd) and one reverse (rv) primer were used: pEKEx3-*rebH*-fwd (CATGCCTGCAGGTCGACTCTAGAGGAAAGGAGGCCCTTCAGATGTCCGGCAAGATTGACAAG)(HalT1), pEKEx3-optimRBS-*rebH*-fwd (GCCTGCAGGTCGACTCTAGAGCACGCTGTAAGGAGTAACCAAAAAAAGGAGGTTTTTTATGTCCGGCAAGATTGACAAG) (HalT2), pECXT99A-*rebH*-fwd (CATGGAATTCGAGCTCGGTACCCGGGGAAAGGAGGCCCTTCAGATGTCCGGCAAGATTGACAAG) (HalT3), pECXT99A-optimRBS-*rebH*-fwd (CATGGAATTCGAGCTCGGTACCCGGGAATATTCCAATTTAAAGGAGGTTATTTATGTCCGGCAAGATTGACAAG)(HalT4) and *rebH*-rv (GAACTCGATCGTCATCTGAAGGGCCTCCTTTCTCAGCGGCCGTGCTGTTGCC). RebF was amplified with *rebF*-fwd (CCTGAGGCAACAGCACGGCCGCTGAGAAAGGAGGCCCTTCAGATGACGATCGAGTTCGACAG) and *rebF*-rv (GAATTCGAGCTCGGTACCCGGGGATCTCATCCCTCCGGTGTCCACA). All *rebH*-fwd primers carry the RBS sequence for *rebH* and primer *rebF*-fwd carries the RBS sequence for *rebF*. The vectors pEKEx3 and pECXT99 A were restricted with *Bam*HI and incubated in Gibson assembly (Gibson et al., 2009) with the two PCR products for construction of pEKEx3-*rebH-rebF*, pEKEx3-optimRBS-*rebH-rebF*, pECXT99A-*rebH-rebF* and pEKEx3-optimRBS-*rebH-rebF*. These plasmids were used for transformation of *C. glutamicum* strains. For construction of the expression plasmid the genes for xylose degradation *xylA* from *Xanthomonas campestris* SCC1758 and *xylB* from *C. glutamicum* ATCC 13,032 were amplified using the plasmid pEKEx3-$xylA_{Xc}$-$xylB_{Cg}$ (Meiswinkel et al., 2013) with the primers pECXT99A-*xylAB*-fwd (CATGGAATTCGAGCTCGGTACCCGGGGAAAGGAGGCCCTTCAGATGAGCAACACCGTTTTCATC) and pECXT99A-*xylAB*-rv (GCCTGCAGGTCGACTCTAGAGGATCCTAGTACCAACCCTGCGTTGC); pECXT99A-*xylAB*-fwd carries the RBS sequence (underlined). The vector pECXT99 A was incubated with *Bam*HI for restriction and incubated with the PCR product in a Gibson assembly for plasmid construction. The constructed plasmid was used to transform *C. glutamicum* strains. For glucosamine degradation *nagB* from *C. glutamicum* ATCC 13032, was amplified using genomic DNA of *C. glutamicum* ATCC,13032 with the primers pECXT99A-*nagB*-fwd (CATGGAATTCGAGCTCGGTACCCGGGGAAAGGAGGCCCTTCAGATGGACATCATCATCTGCAAAG) and pECXT99A-*nagB*-rv (GCCTGCAGGTCGACTCTAGAGGATCCTAGCGCAGCTTTAATTGCTC); pECXT99A-*nagB*-fwd carries the RBS sequence (underlined). The vector pECXT99 A was incubated with *Bam*HI for restriction and incubated with the PCR product in a Gibson assembly for plasmid construction. The constructed plasmid was used to transform *C. glutamicum* strains.

2.3. Determination of halogenase activity

2.3.1. Cultivation and disruption of halogenase overproducing *C. glutamicum* cells

C. glutamicum HalT1 was inoculated to 50 ml LB from an overnight culture. Expression of *rebH* and *rebF* was induced with 1 mM IPTG and the cells were grown for 24 h at 30 °C before the cells were spun down and stored at −20 °C. To disrupt the cells the pellet was resuspended in 2 ml 100 mM Na_2HPO_4, 300 mM NaCl, pH 7.4 (in case of RebF in 10 mM Na_2HPO_4 pH 7.4 buffer) and sonicated for 9 min 55% amplitude and 0.5 cycles on ice in the UP200S Ultrasonic Processor from Hielscher Ultrasound Technology. The supernatant after centrifugation was used as crude extract for the enzymatic assay.

Table 1
Strains and vectors used in this work.

Strains	Description	Source
WT	*C. glutamicum* wild type, ATCC, 13032	ATCC
Tp679	*Δcsm ΔtrpL::P_{ilvCM1} trpE^{fbr}Δvdh::P_{ilvC}aroG^{fbr}*	(Purwanto et al., 2018)
Tp679 (pCES208-*trpD*)	Tp679 carrying pCES208-*trpD*	(Purwanto et al., 2018)
P. putida KT2440	*P. putida* mt-2 hsdRI hsdM*	(Bagdasarian et al., 1981; Nelson et al., 2002)
P. putida (pCIBhis-*prnF*)	*P. putida* KT2440 carrying pCIBhis-*prnF*	This work
E.coli BL21(DE3)	F^- ompT hsdSB(r_B- m_B-) gal dcm (DE3)	Novagen
BL21(DE3) (pET21-ADH)	BL21(DE3) carrying pET21-ADH	This work
Tp679 (pCES208-*trpD*) (pEKEx3)	Tp679 (pCES208-*trpD*) carrying pEKEx3	This work
WT (pEKEx3-*rebH-rebF*)	Wild type carrying pEKEx3-*rebH-rebF*	This work
HalT1	Tp679 (pCES208-*trpD*) carrying pEKEx3-*rebH-rebF*	This work
HalT2	Tp679 (pCES208-*trpD*) carrying pEKEx3-optimRBS-*rebH-rebF*	This work
HalT3	Tp679 (pCES208-*trpD*) carrying pECXT99A-*rebH-rebF*	This work
HalT4	Tp679 (pCES208-*trpD*) carrying pECXT99A-optimRBS-*rebH-rebF*	This work
HalT1 (pECXT99A-*araBAD*)	HalT1 carrying pECXT99A-*araBAD*	This work
HalT1 (pECXT99A-*xylAB*)	HalT1 carrying pECXT99A-*xylAB*	This work
HalT1 (pECXT99A-*nagB*)	HalT1 carrying pECXT99A-*nagB*	This work

Plasmids	Description	Source
pCLBhis-*prnF*	Tet^R, *E. coli/Pseudomonas* shuttle vector (Ptac, *tra*, oriT, oriV, (his)6-tag) overexpressin *prnF* from *P. fluorescens*	(Frese et al., 2014)
pET21-ADH	Amp^R, *E. coli* vector (T7 promotor, *lacI*, pBR322 $Ori_{E.c.}$ (his)6-tag.) overexpressing alcohol dehydrogenase from *Rhodococcus* sp.	(Frese et al., 2014)
pCES208	Kan^R, *C. glutamicum/ E. coli* shuttle vector; pCG1 $OriV_{C.g.}$	(Park et al., 2008)
pCES208-*trpD*	Kan^R, pCES208 overexpressing *trpD* from *E. coli* with PilvC-M1	(Purwanto et al. 2018)
pEKEx3	Spec^R, *C. glutamicum/E. coli* shuttle vector (Ptac, *lacI*, pBL1 $OriV_{C.g.}$)	(Stansen et al., 2005)
pECXT99A	Tet^R, *C. glutamicum/E. coli* shuttle vector (Ptrc, *lacI*, pGA1 $OriV_{C.g.}$)	(Kirchner and Tauch, 2003)
pEKEx3-*rebH-rebF*	Spec^R, pEKEx3 overexpressing *rebH*, *rebF* from *L. aerocolonigenes*	This work
pEKEx3-optimRBS-*rebH-rebF*	Spec^R, pEKEx3 overexpressing *rebH*, *rebF* from *L. aerocolonigenes* with optimized RBS for *rebH*	This work
pECXT99A-*rebH-rebF*	Tet^R, pECXT99 A overexpressing *rebH*, *rebF* from *L. aerocolonigenes*	This work
pECXT99A-optimRBS-*rebH-rebF*	Tet^R, pECXT99 A overexpressing *rebH*, *rebF* from *L. aerocolonigenes* with optimized RBS for *rebH*	This work
pEKEx3-$xylA_{Xc}$-$xylB_{Cg}$	Spec^R, pEKEx3 overexpressing *xylA* from *Xanthomonas campestris* SCC1758 and *xylB* from *C. glutamicum* ATCC, 13032	(Meiswinkel et al., 2013)
pECXT99A-*nagB*	Tet^R, pECXT99 A overexpressing *nagB* from *C. glutamicum* ATCC, 13032	This work
pECXT99A-*araBAD*	Tet^R, pECXT99 A overexpressing *araBAD* from *E. coli* MG1655	(Mindt et al., 2018)
pECXT99A-*xylAB*	Tet^R, pECXT99 A overexpressing *xylA* from *Xanthomonas campestris* SCC1758 and *xylB* from *C. glutamicum* ATCC, 13032	This work

2.3.2. Determination of the specific activity of RebH

The measurements were done in triplicates. The reaction mixture had a final volume of 0.5 ml and contained 5 mM Trp, 1 U/ml ADH from *Rhodococcus* sp. (s. below), 2.5 U/ml PrnF from *P. fluorescens* (s. below), 0.01 mM FAD, 1 mM NAD, 5% isopropanol, 0.005 mM PMSF, 5 mM Na_2HPO_4, 15 mM NaCl and 250 µl of crude extract. It was incubated at 30 °C for 10 min. The reaction was stopped with 187.5 µl methanol. The supernatant was analyzed by HPLC. Product formation was calculated based on the peak area in comparison to a calibration curve of 7-Cl-Trp. Since we and Frese et *al*., 2014, observed difficulties in purification of RebF, we used the easily obtainable PrnF for this in vitro assay.

2.3.3. Cultivation of P. putida KT2440(pCIBhis-prnF) and E. coli BL21(DE3) (pET21-ADH)

For the overproduction of PrnF from *P. fluorescens*, *P. putida* KT2440 was transformed with pCIBhis-*prnF*. For the expression, 500 ml LB were inoculated with 5 ml of the *P. putida* KT2440(pCIBhis-*prnF*) overnight culture. The main culture was cultivated for 1 day at 30 °C with 50 µg ml^{-1} tetracycline. Then the cells were spun down and stored at −20 °C. For overexpression of the alcohol dehydrogenase (ADH), pET21-ADH was transformed in BL21(DE3). After inoculated from an overnight culture to an OD 0.1, the cells were cultivated at 37 °C to an OD of 0.6 before the temperature was lowered to 21 °C and the expression was induced with 0.5 mM IPTG. After 4 h the cells were spun down and stored at −20 °C.

2.3.4. Enzyme purification

All steps during the purification were done at 4 °C. To purify the His_6-tagged PrnF, *P. putida* cells from a 500 ml LB culture were centrifuged and resuspended in TGNI5 buffer (20 mM Tris, 300 mM NaCl, 5% glycerin, 5 mM imidazole). To inhibit the protease activity 1 mM PMSF was added. The cells were disrupted by sonication (UP 200 S, Dr. Hielscher GmbH, Teltow, Germany) on ice at an amplitude of 55% and a duty cycle of 0.5 for 4 min. After centrifugation (20,200 × g, 60 min, 4 °C) the supernatant was filtered and poured in Polypropylene columns 5 ml which a had bed volume of 2 ml Ni-NTA Superflow from Qiagen. The column was washed twice with TNGI5 and once with TNGI20 (20 mM Tris, 300 mM NaCl, 5% glycerin, 20 mM imidazole). The protein was eluted with TNGI buffer which contained 200 mM imidazole. The protein was analyzed using a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For the purification of the ADH, a heat precipitation method was used. The cells were resuspended in 6.6 ml 10 mM K_2HPO_4, pH 7.4, and disrupted 4 min with 55% amplitude and 0.5 cycles on ice in the UP200S Ultrasonic Processor from Hielscher Ultrasound Technology. The crude extract was taken after centrifugation and heated up to 60 °C for 20 min. The enzyme was stored at −20 °C.

2.3.5. Determination of the alcohol dehydrogenase activity

The specific activity of the overexpressed alcohol dehydrogenase (crude extract) was determined in triplicates by monitoring an increase of absorption at λ = 340 nm due to the oxidation of NADH + H^+ to NAD^+ (ε = 6.3 ml $mmol^{-1}$ cm^{-1}) in a final volume of 1 ml. The reaction mixture contained 910 µl of 10 mM K_2HPO_4 pH 7.4, 5% isopropanol (2-Propanol), 250 µM NAD and 20 µl of the crude extract. The cuvette was prewarmed to 30 °C with the Shimadzu UV-1800. The conversion rate of the substrate was determined by regression of the linear range.

2.4. Determination of flavin reductase activity

The cultivation and crude extract preparation are described in capture 2.3.1. The specific activities of the overexpressed flavin reductase RebF in *C. glutamicum* (crude extract) and the purified flavin reductase PrnF (see 2.3) were determined in triplicates by monitoring the decrease of absorption at $\lambda = 340$ nm due to the oxidation of NADH + H^+ to NAD^+ ($\varepsilon = 6.3\,mmol^{-1}\,cm^{-1}$). The reaction mixture with a final volume of 1 ml contained 910 µl of 10 mM Na_2HPO_4 (pH 7.4 buffer), 50 µM FAD, 160 µM NADH and 20 µl of the crude extract (or the purified enzyme). The reaction mixture was prewarmed to 30 °C with the Shimadzu UV-1800. The conversion rate of the substrate was determined by regression in the linear range.

2.5. Analytical procedures

For the quantification of the extracellular and intracellular tryptophan and 7-chloro-tryptophan a high-pressure liquid chromatography (HPLC) system was used (1200 series, Agilent Technologies Deutschland GmbH, Böblingen, Germany). The supernatants of the cell culture were collected by centrifugation (20,238 x g, 20 min, RT) and further used for analysis. For detection of L-tryptophan and the derivatives, samples were derivatised with *ortho*-phthaldialdehyde (OPA) (Schneider and Wendisch, 2010). The amino acid separation was performed by a precolumn (LiChrospher 100 RP18 EC-5 µ (40 mm × 4 mm), CS-Chromatographie Service GmbH, Langerwehe, Germany) and a column (Li- Chrospher 100 RP18 EC-5 µ (125 mm × 4 mm), CS Chromatographie Service GmbH). The detection was carried out with a fluorescence detector (FLD G1321 A, 1200 series, Agilent Technologies) with the excitation and emission wavelengths of 230 nm and 450 nm, respectively.

2.6. Internal metabolites extraction

For the quantification of intracellular amino acids 2 ml of broth for each strain grown in minimal medium were collected at 24 h. 1 ml was centrifuged for 10 min at 13,000 rpm and 4 °C. Pellets were treated with 5% $HClO_4$ in an ice bath for 20 min. After centrifuging at 13,000 rpm for 10 min and 4 °C, the supernatant was neutralized with K_2CO_3 solution and centrifuged again at 13,000 rpm for 10 min and 4 °C. The supernatant was then ready for amino acid quantification or to be stored at −20 °C (Sun et al., 2016). The biomass concentration was determined according to the correlation CDW = 0.353 OD (Bolten et al., 2007).

3. Results and discussion

3.1. C. glutamicum is unable to use Trp as nitrogen and carbon sources

For the production of halogenated tryptophans it is beneficial if the production host cannot degrade the final product. As a first approach, it was tested if *C. glutamicum* can use Trp as sole nitrogen source, *C. glutamicum* WT was grown in CGXII minimal medium lacking urea and ammonium sulfate as nitrogen sources but containing 10 g L^{-1} glucose as carbon source and either 10 mM Trp or 10 mM nitrogen (urea + ammonium sulfate) as nitrogen source. While *C. glutamicum* grew with urea + ammonium sulfate as nitrogen source at a specific growth rate of 0.35 ± 0.01 h^{-1}, Trp did not serve as nitrogen source for growth (Fig. 2). Second, it was tested if Trp can be used as sole carbon source, *C. glutamicum* was grown in CGXII minimal medium with either 10 mM Trp or with 10 mM glucose as sole carbon source. No growth with 10 mM Trp as carbon source was observed, whereas *C. glutamicum* grew with a specific growth rate of 0.31 ± 0.01 h^{-1} with 10 mM glucose (Fig. 2).

From the experiments with Trp it was expected that 7-Cl-Trp would serve neither as a carbon nor as a nitrogen source. To test if *C. glutamicum* WT can metabolize 7-Cl-Trp, the WT was cultivated in the presence of 0.1 mM 7-Cl-Trp in CGXII containing 10 mM nitrogen (urea + ammonium sulfate) as nitrogen source and 10 mM glucose as carbon source. *C. glutamicum* WT grew to a biomass concentration of 1.18 ± 0.03 g/l with a growth rate of 0.17 ± 0.01 h^{-1}. Quantitation of 7-Cl-Trp in the medium supernatant revealed that comparable 7-Cl-Trp concentrations of 0.1 ± 0.01 mM after 0 h, 12 h and 24 h (data not shown). Thus, during growth in minimal medium *C. glutamicum* is unable to metabolize 7-Cl-Trp.

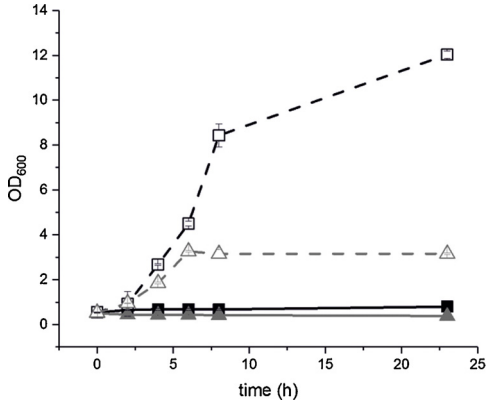

Fig. 2. Growth of *C. glutamicum* WT with Trp as sole nitrogen or carbon source. To test if Trp serves as sole nitrogen source *C. glutamicum* WT was grown in nitrogen source-free CGXII minimal medium and 10 g L^{-1} glucose as carbon source supplemented with either 10 mM Trp (■) or 10 mM nitrogen (urea and ammonium sulfate) (□). To test if Trp serves as sole carbon source *C. glutamicum* WT was cultured in carbon source-free CGXII minimal medium supplemented either with 10 mM Trp (▲) or 10 mM glucose (Δ). Optical densities are reported as means and standard deviations of triplicate cultivations.

The inability of *C. glutamicum* to use Trp as sole nitrogen or carbon source is likely due to the lack of tryptophan degrading enzyme(s). *E. coli*, for example, is able to use tryptophan as sole carbon source by degrading tryptophan to indole, pyruvate and ammonia by tryptophanase (Yanofsky et al., 1991). Alternatively, tryptophan is degraded by a tryptophan oxygenase as e.g. in *Bacillus megaterium*. Tryptophan oxygenase forms *N*-formyl-L-kynurenine from Trp, which subsequently is catabolised to acetate, succinate and ammonium (Bouknight and Sadoff, 1975).

A reduction of the growth rate was observed when 0.1 mM 7-Cl-Trp was added to glucose minimal medium. Therefore, it was tested to what extent 7-Cl-Trp perturbs growth of *C. glutamicum* in glucose minimal medium. The presence of 0.1 mM 7-Cl-Trp reduced the maximal growth rate to 50% and in the presence of 0.5 mM 7-Cl-Trp, no significant growth was observed (data not shown). Therefore, although not metabolized, 7-Cl-Trp inhibits growth of *C. glutamicum*.

3.2. Heterologous expression of halogenase and flavin reductase genes in a Trp overproducing C. glutamicum strain

To enable fermentative production of 7-Cl-Trp by *C. glutamicum,* the genes encoding FADH-dependent halogenase RebH and NADH-dependent flavin reductase RebF from *L. aerocolonigenes* were expressed in Trp overproducing strain Tp679. Since RebH and RebF derive from the same organism and are likely co-evolved for functioning together, we chose to use RebH and RebF in our fementative approach. To confirm that both gene products were functional in the *C. glutamicum* host, crude extracts after growth in LB medium with 1 mM IPTG were assayed for halogenase and flavin reductase activities. While the specific enzyme activity of the purified halogenase RebH has been reported to be 145 mU/mg at 25 °C (turnover number of 0.29 s^{-1}) (Yeh et al., 2007), a specific enzyme activity of 0.90 ± 0.01 mU/mg (turnover

number of 0.002 ± 0.0005 s^{-1}) was measured in crude extracts of *C. glutamicum*. The low activity in crude extracts (about 0.6% activity of the purified enzyme) was in accordance with protein levels observed in SDS-PAGE of these crude extracts (Supplement data Fig. 1). Similarly, the specific enzyme activity of flavin reductase RebF in crude extracts (28 ± 4 mU/mg; turnover number of 0.013 ± 0.002 s^{-1}) was about 1% of that observed for purified flavin reductase RebF (Yeh et al., 2005), which was in accordance with SDS-PAGE analysis of the crude extracts (Supplement data Fig. 1).

For the fermentative production of 7-Cl-Trp by *C. glutamicum* HalT1 which expresses *rebF* and *rebH,* glucose minimal medium CgXII was tested with different chloride salts as halogen substrates for Trp chlorination. It is known that *C. glutamicum* withstands relatively high concentrations of sodium chloride and potassium chloride (half-maximal growth rates at ~490 mM and ~480 mM, respectively) (Zahoor et al., 2014). Hence, low concentrations of chloride salts (potassium, sodium, calcium, magnesium) added to the growth medium of *C. glutamicum* were expected to have little effect on growth. In minimal medium with 50 mM NaCl *C. glutamicum* HalT1 showed a specific growth rate of 0.08 ± 0.01 h^{-1} and produced 20 ± 1 mg L^{-1} 7-Cl-Trp and 2.0 ± 0.1 g L^{-1} Trp (Fig. 3; Table 2). With 50 mM potassium chloride a specific growth rate of 0.08 ± 0.01 h^{-1} and production of 19 ± 2 mg L^{-1} 7-Cl-Trp and 2.0 ± 0.1 g L^{-1} Trp resulted (Fig. 3; Table 2). As the minimal medium CGXII contains calcium chloride and magnesium sulfate (Eggeling and Bott, 2005), the calcium chloride was raised to 25 mM and instead of magnesium sulfate 50 mM magnesium chloride, respectively, were used. With 25 mM $CaCl_2$ a growth rate of 0.17 ± 0.01 h^{-1} resulted and the strain produced 62 ± 7 mg L^{-1} 7-Cl-Trp and 2.5 ± 0.2 g L^{-1} Trp. With 25 mM $MgCl_2$ *C. glutamicum* HalT1 produced 54 ± 1 mg L^{-1} 7-Cl-Trp and 2.3 ± 0.1 g L^{-1} Trp with a specific growth rate of 0.17 ± 0.01 h^{-1}. The negative control strain Tp679 *trpD* (pEKEx3) did not produce 7-Cl-Trp (data not shown) under these conditions. Since $CaCl_2$ resulted in the highest 7-Cl-Trp titers, the following experiments were performed with $CaCl_2$. The improved performance in the presence of calcium ions currently is not understood, but tryptophan 7-halogenase was found to contain calcium in one report (Gribble, 2004).

Taken together, fermentative production of 7-Cl-Trp by recombinant *C. glutamicum* has been enabled. However, 7-Cl-Trp titers are around 50 to 100-fold lower than Trp titers produced by the same strain.

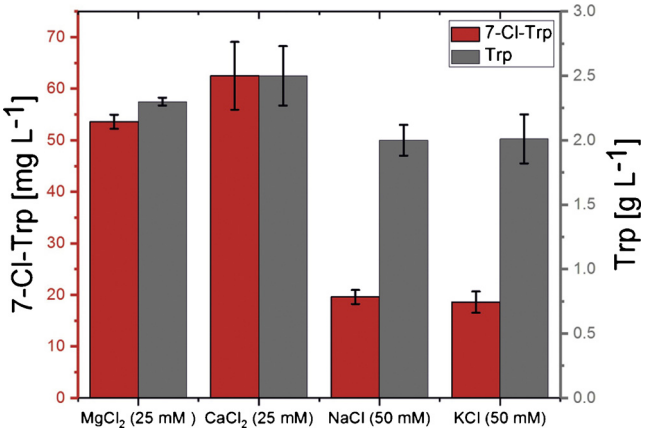

Fig. 3. Influence of various chloride salts on the production of 7-Cl-Trp and Trp by *C. glutamicum* HalT1. Strain HalT1 was grown in CGXII minimal medium with 40 g L^{-1} glucose and the indicated chloride salt concentrations. Means and standard deviations of three replicate cultivations are shown.

3.3. Determination of intra- and extracellular 7-Cl-Trp and Trp concentrations during 7-Cl-Trp production by *C. glutamicum* HalT1

One possible explanation of the finding that 7-Cl-Trp titers are around 50 to 100-fold lower than Trp titers may be inefficient export of 7-Cl-Trp out of the *C. glutamicum* cell. Since the genes of *C. glutamicum* coding for export system(s) for Trp and/or 7-Cl-Trp are unknown (Marin and Krämer, 2007), genetic experiments were not possible. Under the assumption that inefficient 7-Cl-Trp export resulted in low extracellular but high intracellular 7-Cl-Trp concentrations, the intracellular Trp and 7-Cl-Trp content and the supernatant concentrations were quantified during 7-Cl-Trp production by *C. glutamicum* HalT1 (Fig. 4).

C. glutamicum HalT1 was cultivated in CGXII with 4% glucose and 50 mM $CaCl_2$ and grew with a specific growth rate of 0.19 ± 0.01 h^{-1}. In the stationary phase from 24 h to 72 h the biomass concentration decreased from 13.2 ± 0.9 g L^{-1} to 7.8 ± 0.5 g L^{-1} (Fig. 4). During this time the extracellular concentration of 7-Cl-Trp increased from 15 ± 1 mg L^{-1} to 38 ± 1 mg L^{-1} (Figs. 4 and 5). The extracellular Trp concentration was 1.3 ± 0.1 g L^{-1} after 24 h and reached a plateau of about 2 g L^{-1} at 36 h to 72 h. The extracellular 7-Cl-Trp concentration increased gradually from 15 ± 1 mg L^{-1} to 38 ± 1 mg L^{-1} in the period from 24 h to 72 h (Fig. 4). At all time points assayed the extracellular Trp concentration exceeded the extracellular 7-Cl-Trp concentration. For example, at 72 h, the extracellular Trp concentration (2.1 g L^{-1} or about 10 mM) was 65-fold that of 7-Cl-Trp (38 mg L^{-1} or about 0.16 mM; Fig. 4).

After 24 h no intracellular 7-Cl-Trp was detected, although extracellular 7-Cl-Trp was present in the supernatant (s. above; 15 ± 1 mg L^{-1}). At later time points, intracellular 7-Cl-Trp concentrations of 0.34 ± 0.05 mg g[CDW] $^{-1}$ to 0.46 ± 0.05 mg g[CDW] $^{-1}$ were detected (Fig. 4). During this period, intracellular Trp concentrations ranged between 14 and 19 mg g[CDW] $^{-1}$) (Fig. 4). Since the *C. glutamicum* cell volume has been determined at 2 µl mg[CDW] $^{-1}$ (Gutmann et al., 1992), intracellular concentrations of about 34 mM Trp (at 72 h) and about 1 mM 7-Cl-Trp (at 72 h) were detected. Since the intracellular Trp and 7-Cl-Trp concentrations were about three and about six-fold higher than the respective extracellular concentrations, export of these compounds out of the *C. glutamicum* cell may limit production to some extent. However, as observed for the extracellular concentrations, the intracellular concentration of Trp was much larger (34 fold) than that of 7-Cl-Trp. Thus, the major bottleneck of 7-Cl-Trp production by *C. glutamicum* is inefficient halogenation of Trp.

3.4. Provision of intracellular Trp by feeding Trp or the dipeptide Ala-Trp

It may be possible that biosynthetic precursors of Trp inhibit chlorination of Trp to 7-Cl-Trp. The biosynthetic precursors are likely present at increased intracellular concentrations in a Trp overproducing strain. Therefore, instead of using a Trp overproducing strain, the plasmid pEKEx3-*rebH-rebF* for expression of the FADH-dependent halogenase and the flavin reductase genes was used to transform *C. glutamicum* WT. To produce 7-Cl-Trp this strain was grown in CGXII 4% glucose with 50 mM $CaCl_2$, and 2 mM Trp was added to the medium. Since import of Trp occurs via the importer AroP (Wehrmann et al., 1995), Trp was expected to be available intracellularly for halogenation to 7-Cl-Trp, while the intracellular concentrations of its precursors are not expected to be increased. *C. glutamicum* WT(pEKEx3-*rebH-rebF*) grew with a specific growth rate of 0.15 ± 0.01 h^{-1} and produced 21 ± 1 mg L^{-1} 7-Cl-Trp after 48 h. This 7-Cl-Trp concentration (about 0.1 mM) is much lower than the Trp concentration added to the medium (2 mM).

As an alternative, Trp was provided intracellularly by feeding the dipeptide L-alanyl-L-tryptophan (Ala-Trp). Dipeptides like Ala-Trp are taken up actively into the *C. glutamicum* cell by peptide transport systems before they are hydrolyzed by peptide hydrolases (Trötschel et al.,

Table 2
Growth parameters, production yields and volumetric productivities of *C. glutamicum* HalT1 in the presence of different chloride salts.

Chloride salt	Specific growth rate (h^{-1})	Biomass concentration ($g\ L^{-1}$)	7-Cl-Trp Yield (mg g^{-1})	7-Cl-Trp Volumetric productivity (mg $L^{-1}\ h^{-1}$)	Trp Yield ($g\ g^{-1}$)	Trp Volumetric productivity (g $L^{-1}\ h^{-1}$)
$MgCl_2$ (25 mM)	0.17 ± 0.00	10.9 ± 0.5	1.34 ± 0.03	0.74 ± 0.02	0.06 ± 0.00	0.03 ± 0.00
$CaCl_2$ (25 mM)	0.17 ± 0.00	7.8 ± 1.4	1.56 ± 0.16	0.87 ± 0.09	0.06 ± 0.01	0.03 ± 0.00
NaCl (50 mM)	0.08 ± 0.00	10.9 ± 0.3	0.49 ± 0.03	0.27 ± 0.02	0.05 ± 0.0	0.03 ± 0.00
KCl (50 mM)	0.08 ± 0.00	11.5 ± 1.6	0.47 ± 0.05	0.26 ± 0.03	0.05 ± 0.01	0.03 ± 0.00

Means and standard deviations obtained from triplicate shake flask cultivations in CgXII minimal medium after 72 h are given. Product yields are normalized to the glucose concentration utilized.

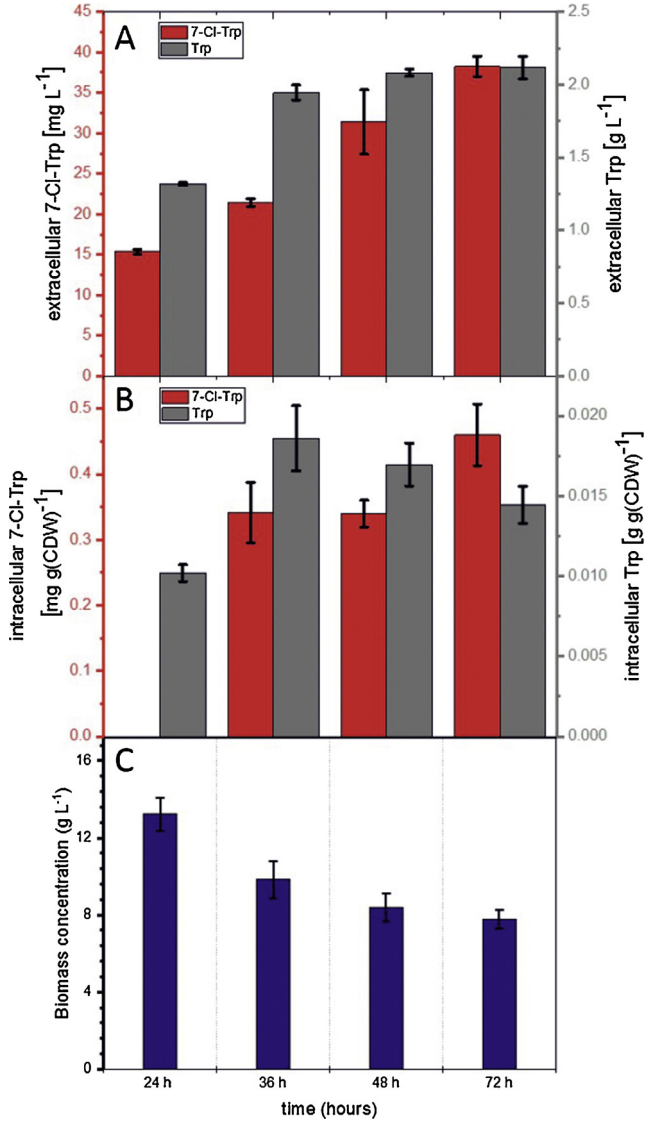

Fig. 4. Extracellular (A) and intracellular (B) product concentrations and biomass concentrations (C) of *C. glutamicum* strain HalT1. Values are given as means and standard deviations of three replicate cultivations in CGXII minimal medium with 40 g L^{-1} glucose.

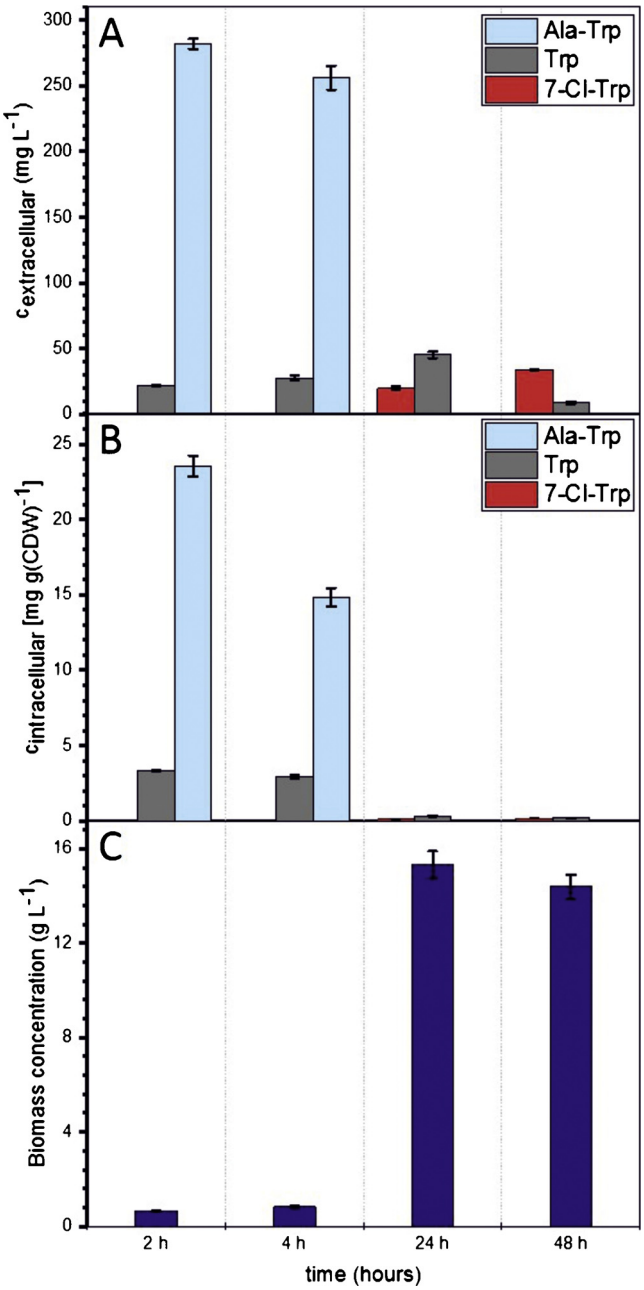

Fig. 5. Extracellular (A) and intracellular (B) product concentrations and biomass concentrations (C) of *C. glutamicum* strain WT (pEKEx3-*rebH-rebF*) after growth in the presence of 1 mM Ala-Trp. Values are given as means and standard deviations of three replicate cultivations in CGXII minimal medium with 40 g L^{-1} glucose.

2005). Whereas alanine is catabolized fast by *C. glutamicum* (Trötschel et al., 2005), Trp is not (s. above, Trp is neither a carbon nor a nitrogen source for growth of *C. glutamicum*). When *C. glutamicum* WT(pEKEx3-*rebH-rebF*) was cultivated in CGXII 4% glucose with 1 mM Ala-Trp and 50 mM $CaCl_2$, it grew with a specific growth rate of 0.15 ± 0.01 h^{-1} to a biomass concentration of 14.4 ± 0.5 g L^{-1} at 48 h (Fig. 5). After 4 h, about 1 mM (256 ± 9 mg L^{-1}) Ala-Trp remained in the supernatant

while its intracellular concentration was about 27 mM (14 ± 1 mg g [CDW]$^{-1}$). At 4 h, the intra- and extracellular Trp concentrations were 7 mM (3 ± 1 mg g[CDW]$^{-1}$) and about 0.14 mM (27 ± 2 mg L^{-1}), respectively, while 7-Cl-Trp was neither detected intracellularly nor in the supernatant (Fig. 5). As expected from the kinetics of dipeptide uptake and hydrolysis (Trötschel et al., 2005), Ala-Trp was completely utilized at 48 h since it could neither be detected intra- nor extracellularly. At 48 h, the intracellular concentrations of Trp and 7-Cl-Trp were very low (below 0.5 mM). 7-Cl-Trp was produced (34 ± 1 mg L^{-1}; 0.14 mM) and its concentration exceeded that of extracellular Trp (9 ± 1 mg L^{-1}; 0.04 mM).

Taken together, the low titers of 7-Cl-Trp that resulted upon addition of Trp or Ala-Trp do not support the notion that biosynthetic precursors of Trp inhibit its chlorination to 7-Cl-Trp.

3.5. Optimization of the 7-Cl-Trp production

To test if expression of the halogenase and flavin reductase genes is limiting production of 7-Cl-Trp, two expression vectors differing by their origins of replication and copy numbers were compared. In addition, the influence of altering the ribosome binding site (RBS) to support a higher translation initiation rate of *rebH* was tested. The synthetic *rebH-rebF* present in pEKEx3- *rebH-rebF* was cloned into pECXT99 A (Kirchner and Tauch, 2003) (Table 1). The sequence of the RBS of *rebH* was optimized using RBS calculator (https://salislab.net/software/). Based on this optimized RBS, the vectors pEKEx3-optimRBS-*rebH-rebF* and pECXT99A-optimRBS-*rebH-rebF* were constructed. Vectors pECXT99A-*rebH-rebF* and pECXT99A-optimRBS-*rebH-rebF* were used to transform Tp679 (pCES208-*trpD*). The resulting strains were HalT3 and HalT4, respectively. (Table 1). The strain HalT3 was cultivated in CGXII 4% glucose and had a specific growth rate of 0.17 ± 0.004 h^{-1}. HalT3 produced 42 ± 2 mg L^{-1} 7-Cl-Trp and 2.3 ± 0.2 g L^{-1} Trp (Fig. 6). This was similar to HalT1 which produced 57 ± 1 mg L^{-1} 7-Cl-Trp and 2.2 ± 0.1 g L^{-1} Trp with a specific growth rate of 0.11 ± 0.01 h^{-1}.

A second option to improve the production of 7-Cl-Trp was to optimize the RBS. The genes *rebH* and *rebF* were cloned with the optimized RBS in the pECXT99 A vector and transformed Tp679 (pCES208-*trpD*). The build strain HalT4 was grown in CGXII with 4% glucose and 50 mM $CaCl_2$. The specific growth rate was 0.16 ± 0.006 h^{-1}, and 66 ± 1 mg L^{-1} 7-Cl-Trp and 2.3 ± 0.02 g L^{-1} Trp were accumulated. An increase of 7-Cl-Trp production by 56% could be observed between HalT3 and HalT4 which arrived from the optimized RBS. Thus, the RBS in combination with the pEKEx3 vector (Stansen et al., 2005), which is the expression vector of HalT1, was optimized as well. The plasmid pEKEx3-optimRBS-*rebH-rebF* was transformed in Tp679 (pCES208-*trpD*). The resulting strain HalT2 was cultivated in CGXII with 4% glucose and 50 mM $CaCl_2$. HalT2 reached a specific growth rate of 0.11 ± 0.004 h^{-1} and a production of 108 ± 2 mg L^{-1} 7-Cl-Trp and 1.1 ± 0.1 g L^{-1} Trp was measured (Fig. 6). Hence, there is an improvement in production of 7-Cl-Trp of 56% from HalT1 to HalT2. The production of Trp decreased by 48% from HalT1 to HalT2. But 1.7 ± 0.1 g L^{-1} of the precursor anthranilate (Ant) were detected in HalT2. This can be caused through an inhibitory effect of halogenated compounds on TrpD (Fig. 1). It is known that halogenated Ant is a competitive inhibitor of PqsA, the initial enzyme of 4-hydroxy-2-alkylquinolines biosynthesis in *Pseudomonas aeruginosa,* and that halogenated Ant competitively inhibits Ant converting TrpD (Lesic et al., 2007). Until now, it is not known if 7-Cl-Trp inhibits TrpD or other enzymes of the Trp pathway in *C. glutamicum*. The intracellular concentration of the cofactor FAD may be limiting although the intracellular concentrations of FAD or FMN are about 4.5 mM OD_{610}^{-1} and 2 mM OD_{610}^{-1}, respectively (Toyoda et al., 2014). The addition of 2 mM FMN to the cultivation medium improved 7-Cl-Trp production by about two fold (62 ± 7 mg L^{-1} 7-Cl-Trp for HalT1 with 50 mM $CaCl_2$, 4% glucose without added FMN as compared to 130 ± 1 mg L^{-1} 7-Cl-Trp when 2 mM FMN were added, Fig. 3 and data not shown). However, the addition of FMN is too costly for fermentative production. Our results clearly show that despite sufficient provision of Trp by the recombinant *C. glutamicum* strains, halogenation proceeds to much lower concentrations as compared to Trp production (Figs. 3 and 4). Likewise, classical biocatalytic halogenation stops at a distinct conversion (Schnepel and Sewald, 2017). For example, Frese et al. (2014) as well as Payne et al. (2013) converted Trp at concentrations of only 0.5 mM, which led to almost complete conversion. Up-scaling required to increase the volume while maintaining the low substrate concentration, e.g. Payne et *al.* 2013 converted 100 mg Trp in a volume of 12 l (Payne et al., 2013). Although operated at the gram scale, bromination of Trp using CLEAs also had to be operated at a low concentration (i.e. 1 g Trp in 5 l; Frese and Sewald, 2015). Possible limitations may be product inhibition of the halogenase, insufficient supply of oxygen and/or redox cofactors. Thus, chlorination of Trp in solution using purified enzymes or preparations such as CLEAs currently is restricted to conversion of few milligrams of substrate (Frese and Sewald, 2015). Fermentation as described here may similarly be limited by product inhibition of the halogenase enzyme, thus, enzyme engineering to overcome such limitation would benefit both biocatalytic and fermentative processes. The expression of *rebH* and *rebF* of the strains HalT1 to HalT4 was shown by SDS-PAGE (Supplement Fig. 1). Strain HalT2, which accumulated the highest concentration of 7-Cl-Trp, showed the highest expression of the *rebH* gene. Thus, future strain development may target further *rebH* overexpression. It may also be important to balance the concentrations of the involved enzymes RebH and RebF. This may be done e.g. by varying translation initiation rates by changing the sequence and/or position of the ribosome binding sites (Henke et al., 2016). Alternatively, fusion proteins of RebH and RebF may show better performance (Payne et al., 2013). Higher FAD concentrations within the cell

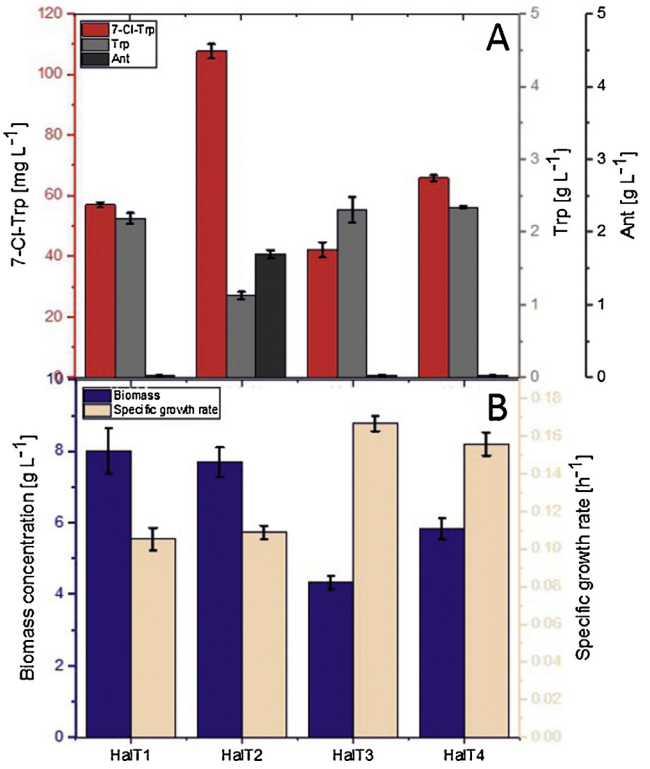

Fig. 6. Product titers and growth parameters of various *C. glutamicum* strains. The *C. glutamicum* strains HalT1, HalT2, HalT3 and HalT4 were grown in CGXII with 4% glucose, 50 mM $CaCl_2$ and were induced with 1 mM IPTG. Means and standard deviations of triplicate cultivations are given for titers of 7-Cl-Trp, Trp, and anthranilate (Ant) (A) and for biomass concentrations and specific growth rates (B).

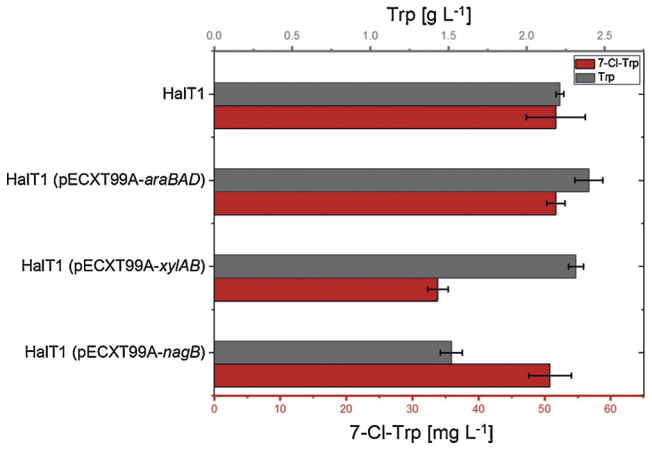

Fig. 7. Production of 7-Cl-Trp and Trp by *C. glutamicum* HalT1 and derived strains with different carbon sources. For HalT1 40 g L^{-1} glucose were used as sole carbon source, 40 g L^{-1} arabinose for HalT1 (pECXT99A-*araBAD*), 40 g L^{-1} Xylose for HalT1 (pECXT99A-*xylAB*) and 40 g L^{-1} glucosamine for HalT1 (pECXT99A-*nagB*). Means and standard deviations of three replicate cultivations are given.

may be reached by deletion of *rshA* (Toyoda et al., 2014) or by overexpression of *ribF* (Taniguchi and Wendisch, 2015). Redox cofactor recycling may be improved by oxygen-deprivation conditions as shown for L-alanine production by recombinant *C. glutamicum* (Jojima et al., 2010). It may be important to identify the transport system for 7-Cl-Trp export. In the case of fermentative L-lysine production, the L-lysine export gene *lysE* was overexpressed in L-lysine producing strains (Unthan et al., 2015). Unfortunately, currently the Trp export system of *C. glutamicum* is unknown (it may also be relevant for 7-Cl-Trp export) (Pérez-García and Wendisch, 2018). Trp transport engineering, however, successfully improved Trp production when the Trp uptake system was deleted (Pérez-García and Wendisch, 2018). Assuming that this system imports 7-Cl-Trp into the *C. glutamicum* cell, deletion of its gene may also be relevant for improved 7-Cl-Trp producing strains.

3.6. 7-Chloro-tryptophan production from alternative carbon sources

Glucose is the preferred carbon and energy source of *C. glutamicum* and a proof-of-concept for glucose-based 7-Cl-Trp production was reached. However, feedstocks that do not have competing uses in human and animal nutrition are to be preferred for large-scale fermentations. Metabolic engineering strategies for access of *C. glutamicum* to the alternative carbon sources arabinose, xylose and glucosamine have been developed and applied to amino acid production (Meiswinkel et al., 2013; Sasaki et al., 2011; Schneider et al., 2011; Uhde et al., 2013).

For access to arabinose *C. glutamicum* HalT1 was transformed with pECXT99A-*araBAD* containing the heterologous operon *araBAD* from *E. coli* for arabinose catabolism (Sasaki et al., 2011; Schneider et al., 2011). The resulting strain grew with a growth rate of 0.08 h^{-1} and produced 52 ± 1 mg L^{-1} 7-Cl-Trp and 2.4 ± 0.1 g L^{-1} Trp (Fig. 7; Table 3). Efficient xylose utilization by *C. glutamicum* required heterologous expression of the xylose isomerase XylA gene from *Xanthomonas campestris* and overexpression of the endogenous gene coding for xylulokinase XylB (Meiswinkel et al., 2013). *C. glutamicum* HalT1 (pECXT99A-*xylAB*) showed a specific growth rate of 0.07 ± 0.01 h^{-1} in minimal medium with 40 g L^{-1} xylose and produced 34 ± 2 mg L^{-1} 7-Cl-Trp and 2.3 ± 0.1 g L^{-1} Trp (Fig. 7; Table 3). Glucosamine, a constituent of chitin and one of the most abundant amino sugars in soil, is a poor growth substrate for *C. glutamicum* (Uhde et al., 2013). For efficient glucosamine utilization the endogenous glucosamine-6-phosphate deaminase gene *nagB* was overexpressed (Uhde et al., 2013). *C. glutamicum* HalT1 (pECXT99A-*nagB*) grew in CGXII with 40 g L^{-1} glucosamine with a specific growth rate of 0.11 h^{-1} and produced 51 ± 3 mg L^{-1} 7-Cl-Trp and 1.5 ± 0.1 g L^{-1} Trp. For comparison, *C. glutamicum* HalT1 grew in CGXII medium with 40 g L^{-1} glucose with a specific growth rate of 0.13 h^{-1} and produced 56 ± 5 mg L^{-1} 7-Cl-Trp and 2.2 ± 0.1 g L^{-1} Trp (Fig. 7, Table 3). Taken together, 7-Cl-Trp production has been shown using glucose as well as the alternative feedstocks glucosamine, arabinose and xylose as growth substrates for the *C. glutamicum* host.

4. Conclusion

Fermentative production of the halogenated amino acid 7-Cl-Trp from sugars, ammonium and chloride salts was enabled by recombinant *C. glutamicum* expressing the genes coding for FAD-dependent halogenase RebH and NADH-dependent flavin reductase RebF from *L. aerocolonigenes*. Chlorination of Trp to 7-Cl-Trp by *C. glutamicum* was improved by optimizing the RBS of *rebH*. Production of 7-Cl-Trp was limited by inhibition of 7-Cl-Trp and the chlorination efficiency. Strains able to produce 7-Cl-Trp and Trp from the alternative carbon sources arabinose, glucosamine and xylose were constructed.

Compliance with ethical standards

The authors declare that they have no conflicts of interest. The research performed did not involve human participants and/or animals.

Acknowledgments

The authors gratefully acknowledge Irene Krahn for construction of the plasmids pECXT99A-*xylAB* and pECXT99A-*nagB*.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2018.12.008.

Table 3
Growth parameters, production yields and volumetric productivities of *C. glutamicum* HalT1 and derived strains with different carbon sources.

Strain	Carbon source	Specific growth rate (h^{-1})	Biomass concentration (g L^{-1})	7-Cl-Trp yield (mg g^{-1})	7-Cl-Trp volumetric productivity (mg L^{-1} h^{-1})	Trp yield (g g^{-1})	Trp volumetric productivity (g L^{-1} h^{-1})
HalT1	Glucose	0.13 ± 0.00	7.5 ± 0.5	1.41 ± 0.11	0.47 ± 0.04	0.05 ± 0.00	0.02 ± 0.00
HalT1 (pECXT99A-*araBAD*)	Arabinose	0.07 ± 0.01	0.9 ± 0.6	1.30 ± 0.03	0.43 ± 0.01	0.06 ± 0.00	0.02 ± 0.00
HalT1 (pECXT99A-*xylAB*)	Xylose	0.07 ± 0.01	8.8 ± 0.6	0.85 ± 0.04	0.28 ± 0.01	0.06 ± 0.00	0.02 ± 0.00
HalT1 (pECXT99A-nagB)	Glucosamine	0.11 ± 0.00	5.4 ± 0.6	1.27 ± 0.08	0.42 ± 0.03	0.04 ± 0.00	0.01 ± 0.00

Means and standard deviations obtained from triplicate shake flask cultivations in CgXII minimal medium with 40 g L^{-1} carbon source after 120 h are given. Product yields are normalized to the carbon source concentration utilized.

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