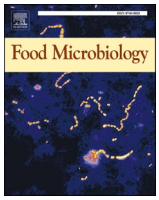
Food Microbiology 116 (2023) 104343

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm



# Ability of *Latilactobacillus curvatus* FAM25164 to produce tryptamine: Identification of a novel tryptophan decarboxylase

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ARTICLE INFO

*Keywords:*
*Latilactobacillus curvatus*
Tryptamine
Tryptophan decarboxylase
Cheese

ABSTRACT

Screenings of cheese isolates revealed that the *Latilactobacillus curvatus* strain FAM25164 formed tryptamine and tyramine. In the present study, it was studied whether a tryptophan decarboxylase, which has rarely been described in bacteria, could be involved in the production of tryptamine. The genome of strain FAM25164 was sequenced and two amino acid decarboxylase genes of interest were identified by sequence comparisons and gene context analyses. One of the two genes, named *tdc1*, showed 99% identity to the *tdcA* gene that has recently been demonstrated by knockout studies to play a role in tyramine formation in *L. curvatus*. The second gene, named *tdc2*, was predicted to have an amino acid decarboxylase function, but could not be assigned to a metabolic function. Its protein sequence has 51% identity with Tdc1 and the *tdc2* gene is part of a gene cluster not often found in publicly available genome sequences of *L. curvatus*. Among others, the gene cluster includes a tryptophan–tRNA ligase, indicating that *tdc2* plays a role in tryptophan metabolism. To study decarboxylase activity, *tdc1* and *tdc2* were cloned and expressed as His6-tagged proteins in *Escherichia coli*. The recombinant *E. coli* expressing *tdc1* produced tyramine, whereas *E. coli* expressing *tdc2* produced tryptamine. The purified recombinant Tdc1 protein decarboxylated tyrosine and 2,3-dihydroxy-L-phenylalanine (L-DOPA), but not tryptophan and phenylalanine. In contrast, the purified Tdc2 was capable of decarboxylating tryptophan but not L-DOPA, tyrosine, or phenylalanine. This study describes a novel bacterial tryptophan decarboxylase (EC 4.1.1.105) that may be responsible for tryptamine formation in cheese.

## 1. Introduction

Biogenic amines are undesirable substances in cheese, as they can cause toxic reactions when consumed (Benkerroum, 2016; Suzzi et al., 2022). These substances are formed during ripening by decarboxylase-producing bacteria from the amino acids histidine, tyrosine, phenylalanine, tryptophan, lysine, and ornithine.

The bacterial decarboxylation of tyrosine is one of the best studied. The amino acid is decarboxylated by the enzyme tyrosine decarboxylase (TDC, EC 4.1.1.25), producing tyramine as a product. Genes encoding this enzyme have been identified in enterococci, *Levilactobacillus brevis*, and *Latilactobacillus curvatus* (Aymerich et al., 2006; Connil et al., 2002; Moreno-Arribas and Lonvaud-Funel, 2001).

The decarboxylation of phenylalanine leads to β-phenylethylamine. This substance is often found together with tyramine in cheese, and the structural similarity of phenylalanine and tyrosine suggests that TDC also plays a role in the formation of β-phenylethylamine. In detail, however, the matter is not so clear. For example, a TDC purified from *L. brevis* IOEB 9809 did not decarboxylate phenylalanine (Moreno-Arribas and Lonvaud-Funel, 2001). In contrast, enterococci possess a TDC that accepts both phenylalanine and tyrosine as substrates (Liu et al., 2014; Marcobal et al., 2006).

Tryptamine can also be found in cheese, although less frequently than tyramine and β-phenylethylamine (EFSA Panel on Biological Hazards, 2011). There is evidence in the literature that individual strains of *Lactobacillus bulgaricus* and *L. curvatus* have the ability to produce tryptamine (Aymerich et al., 2006; Chander et al., 1989). However, little is known about the metabolic pathway and it is thought that tryptamine is formed by the decarboxylation of tryptophan.

There are very few reports describing bacterial tryptophan decarboxylases. Analyses of the gut microbiome found two bacterial tryptophan decarboxylases (EC 4.1.1.105) belonging to *Clostridium sporogenes* and *Ruminococcus gnavus* (Williams et al., 2014). While the decarboxylase from *C. sporogenes* decarboxylated tryptophan and tyrosine, but not phenylalanine, the decarboxylase from *R. gnavus* decarboxylated all three amino acids. Recently, an aromatic amino acid decarboxylase (EC

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https://doi.org/10.1016/j.fm.2023.104343
Received 31 May 2023; Received in revised form 16 July 2023; Accepted 17 July 2023
Available online 1 August 2023

4.1.1.28) from *Bacillus atrophaeus* was described to decarboxylate several amino acids, including phenylalanine, tyrosine, and tryptophan (Choi et al., 2021). A comparison of the amino acid sequences of these three bacterial tryptophan decarboxylating enzymes reveals that they share less than 25% identity, suggesting that these enzymes cannot be uniquely identified based on sequences.

In this study, *L. curvatus* isolated from cheese was screened for its ability to produce biogenic amines. It was found that the strain FAM25164 produced tyramine and tryptamine. The genome of FAM25164 was sequenced and analyzed to find and clone genes encoding aromatic amino acid decarboxylases.

## 2. Materials and methods

### 2.1. Bacterial strains and cultivation

*L. curvatus* strains were cultivated in MRS medium and stored in sterile skim milk at −80 °C. The species identity was determined using a MALDI Biotyper (Bruker Daltonics, Bremen, Germany) according to the manufacturer's protocols. To determine the ability of *L. curvatus* to decarboxylate aromatic amino acids, the strains were incubated in MRS medium supplemented with 1 g $L^{-1}$ of L-tyrosine, 1 g $L^{-1}$ of L-tryptophan, and 1 g $L^{-1}$ of L-phenylalanine at 37 °C for 2 days under anaerobic conditions.

*Escherichia coli* TOP10 and BL21(DE3) (both from Thermo Fischer Scientific, Reinach, Switzerland), harboring one of the plasmids listed in Table 1, were grown in LB medium (Sambrook et al., 1989) supplemented with ampicillin (0.1 mg $mL^{-1}$) at 37 °C at 220 rpm. The *E. coli* strains were stored in 30% (v/v) glycerol at −80 °C.

The decarboxylation activity of recombinant *E. coli* BL21(DE3) strains was determined in minimal medium M9 (Miller, 1972) supplemented with ampicillin (0.1 mg $mL^{-1}$), 0.2% (w/v) isopropyl beta-D-1-thiogalactoside, 5 mM L-tyrosine, 5 mM L-phenylalanine, and 5 mM L-tryptophan. For this purpose, the bacteria were incubated for 3 days at room temperature at 220 rpm.

### 2.2. Detection and quantification of aromatic monoamines

Culture supernatants were analyzed for the presence of biogenic amines using high-performance thin-layer chromatography, as previously described (Berthoud et al., 2022). High-performance liquid chromatography (HPLC) was used to quantitate the aromatic monoamines tyramine, tryptamine, dopamine, and *β*-phenylethylamine (Williams et al., 2014). Methanol was added 1:1 to the culture supernatants and filtered (0.45-μm nylon membrane, Millipore Millex-HN, Macherey-Nagel GmbH & Co. KG, Düren, Germany). The filtrate (5 μL) was separated on a Hypercarb™ column (100 × 4.6 mm, 5-μm particle size) at 30 °C and a flow rate of 1.0 mL $min^{-1}$. Eluent A consisted of 2% (v/v) methanol and 0.1% (v/v) trifluoroacetic acid. Eluent B consisted of 49% (v/v) acetonitrile, 49% (v/v) 2-propanol, 2% (v/v) methanol, and 0.1% (v/v) trifluoroacetic acid. The gradient was as follows: from 11.0% to 62.6% solvent B linearly over 14 min, then increasing to 99.0% solvent B within 3 min, then returning to 11.0% solvent B for 3 min, and finally equilibrating for 3 min before the next sample injection. The absorption was monitored at 220 nm and 280 nm. Data acquisition and analysis were performed using Chromeleon software (v7.2.10, Thermo Fisher Scientific). L-tyrosine, tyramine, L-phenylalanine, β-phenylethylamine, tryptophan, tryptamine, L-3,4-dihydroxyphenylalanine (L-DOPA), and dopamine dissolved in 50% (v/v) of methanol were used as reference substances.

### 2.3. DNA sequencing

Genomic DNA (gDNA) was extracted from *L. curvatus* FAM25164 using an EZ1 DNA tissue kit (Qiagen, Hombrechtikon, Switzerland), as previously described (Berthoud et al., 2017). The DNA quantity was determined using a Qubit DNA assay kit (Thermo Fisher Scientific).

The gDNA from the strain FAM25164 was sequenced at the Next Generation Sequencing Platform of the University of Bern, Switzerland. The library was prepared using the "TruSeq DNA PCR-free Library Prep" kit (Illumina), which was then paired-end sequenced in a fraction of a lane on an Illumina NovaSeq 6000 instrument. Sanger sequencing was performed at Microsynth AG (Balgach, Switzerland).

### 2.4. Bioinformatic analysis

Reads were trimmed using Trimmomatic (version 0.38, options: phred33 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:28 MINLEN:36) (Bolger et al., 2014) and were then assembled using SPAdes (version 3.14.1, options: k 21,33,55,77 –isolate) (Bankevich et al., 2012). Contigs with a size of less than 500 bp were removed from the assembly. The final assembly was annotated with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). The assembly obtained from the strain FAM25164 has been deposited in GenBank under accession number GCA_019926945.1.

To analyze the genomic regions of interest, the genome sequences of *L. curvatus* strain DSM20019 (GenBank NZ_CP026116.1), strain NFH-Km12 (GenBank AP018699.1), and strain HFS9 (GenBank JAMOHH010000041) were aligned using progressiveMauve (snapshot_2015-02-13) (Darling et al., 2010). BLAST was used to compare sequences and to calculate nucleotide and amino acid identities (Altschul et al., 1990). The DNA Features Viewer (version 3.1.2) was used to

**Table 1**
Primers and plasmids used in this study.

| Primer | Primer (5′ to 3′) | Usage |
|---|---|---|
| tdc1_F | ATGAGTAACACTAGTTTTAGTGCA | Cloning of *tdc1* |
| tdc1_R | gtttaaactcaatggtgatggtgatgatgaccctTTTACGATCTTCGTAAATTGCTTCT | Cloning of *tdc1* |
| tdc2_F | ATGACAGAAGAATTCAAAAAAAATGATACCGA | Cloning of *tdc2* |
| tdc2_R | ttaaactcaatggtgatggtgatgatgacccttAATTATTTTTTCATTTCTAGCAATGGT | Cloning of *tdc2* |
| vector_F | aagggtcatcatcaccatcacca | Cloning of *tdc1* and *tdc2* |
| vector_tdc1_R | AAATCTGTATCTTTTGCACTAAAACTAGTGTTACTCATaagggtatctccttcttaaagt | Cloning of *tdc1* |
| vector_tdc2_R | GATAAATCGGTATCATTTTTTTTTGAATTCTTCTGTCATaagggtatctccttcttaaagt | Cloning of *tdc2* |
| tnp256_F1 | ACACCTTCACTAACTCCAGC | Sequencing |
| tnp256_R1 | CTTCTTAGATGCCACTTACTTGC | Sequencing |
| tnp256_F2 | GGTAGTTCAGCACTTCTTTCG | Sequencing |
| tnp256_R2 | ACGTGAAGTTAAAGAAGCCATG | Sequencing |
| **Plasmids** | **Usage and properties** | **Source** |
| pEXP5-NT/CALML3 | Expression plasmid, ampR, inducible lac promoter | Thermo Fisher Scientific |
| pEXP5-CT/cadA | Expression plasmid used as PCR template, ampR, inducible lac promoter | Unpublished expression plasmid |
| pEXP5-CT/tdc1 | Expression plasmid, ampR, inducible lac promoter | This study |
| pEXP5-CT/tdc2 | Expression plasmid, ampR, inducible lac promoter | This study |

*Note:* Nucleotide sequences in lowercase belong to pEXP5-CT/TOPO; ampR: ampicillin resistance gene.

visualize gene annotations (Zulkower and Rosser, 2020).

### 2.5. Cloning of tdc1 and tdc2

The DNA sequences encoding Tdc1 (GenBank WP_148484661.1) and Tdc2 (GenBank WP_223315860.1) were cloned into the expression plasmid pEXP5-CT, which enabled the fusion of a His-tag coding region downstream to the gene of interest. The plasmids were created using Gibson Assembly cloning. The primers used for cloning are listed in Table 1.

For the cloning of *tdc1*, the DNA sequence was amplified by PCR with the primer pair tdc1_F/tdc1_R from the genomic DNA of the strain FAM25164. The vector pEXP5-CT was amplified with the primer pair vector_F/vector_tdc1_R using an existing pEXP5-CT expression plasmid (Table 1). The gene encoding *tdc2* was amplified with the primer pair tdc2_F/tdc2_R, and the plasmid backbone was amplified using the primer pair vector_F/vector_tdc2_R.

The PCR assays (final volume 50 μL) were digested with *Dpn*I to remove the template DNA. DNA was then extracted with phenol–-chloroform–isoamyl alcohol (25:24:1), precipitated with isopropanol (Sambrook et al., 1989), dissolved in nuclease-free water, and quantified using a Nanodrop device (Thermo Fisher Scientific). The Gibson Assembly reaction was carried out using the GeneArt Gibson Assembly HiFi Master Mix (Thermo Fisher Scientific) according to the manufacturer's instructions. The Gibson Assembly reaction was then transformed into chemically competent *E. coli* TOP10 (Thermo Fisher Scientific) according to the manufacturer's instructions.

The transformed colonies were propagated in LB medium, and plasmids were isolated using the Zyppy Plasmid Miniprep Kit (Lucerna Chem AG, Lucerne, Switzerland). The nucleotide sequence of the inserts was verified using Sanger sequencing. The plasmids containing *tdc1* and *tdc2* were named pEXP5-CT/tdc1 and pEXP5-CT/tdc2, respectively. The plasmids were transformed into OneShot BL21 Star (DE3) Chemically Competent *E. coli* (Thermo Fisher Scientific) for heterologous expression.

### 2.6. Heterologous expression and protein purification

An overnight culture (4 mL) of the *E. coli* BL21 harboring either pEXP5-CT/tdc1 or pEXP5-CT/tdc2 was added to 200 mL of LB medium supplemented with 1% (w/v) of D-glucose and 0.1 mg $mL^{-1}$ of ampicillin. The culture was incubated at 37 °C and 220 rpm until it reached an $OD_{600}$ of 0.4–0.5. Then, 80 μL of 0.5 M isopropyl beta-D-1-thio-galactoside was added to induce gene expression. The culture was further incubated at room temperature for 6 h at 220 rpm. The bacteria were harvested by centrifugation (3000*g*, 10 min), washed with 20 mM sodium phosphate buffer (pH 7.4), and frozen at −20 °C.

The His-tagged recombinant proteins were purified using Protino Ni-TED 1000 Kit (Machery-Nagel, Oensingen, Switzerland) and desalting size-exclusion chromatography, as described previously (Berthoud et al., 2022). The fractions containing the purified protein were analyzed by denaturing gel electrophoresis and Coomassie Blue staining. Protein concentrations were determined using the Qubit Protein Assay Kit (Thermo Fisher Scientific).

### 2.7. Amino acid decarboxylase assay

The enzyme reaction was performed in 200 μL containing 200 mM sodium acetate (pH 5.5), 0.1 mM pyridoxal 5′-phosphate, and either 0.05 μg of Tdc1 or 0.6 μg of Tdc2. To investigate substrate specificity, L-phenylalanine, L-tyrosine, L-3,4-dihydroxyphenylalanine (L-DOPA), and L-tryptophan were added at a final concentration of 5 mM. Different concentrations of amino acid substrates were used to determine the kinetic parameters. The assay was started by adding either Tdc1 or Tdc2. After incubation at 37 °C for 60 min, the reaction was stopped by adding 200 μL of methanol. The sample was then filtered using a 0.45-μm nylon membrane (Millipore Millex-HN), and the filtrate was subsequently analyzed for the presence of monoamines by HPLC. To calculate the Michaelis–Menten constant, the experimental data were fitted to the Michaelis–Menten equation using SciPy (Virtanen et al., 2020).

## 3. Results

### 3.1. Detection of tryptamine-producing L. curvatus strains

In a study on microbial and chemical characterization of Valais Raclette cheeses, *L. curvatus* was found in 19 of 21 cheeses (Wechsler et al., 2021). Since it has been reported several times in the literature that this species is aminogenic and may cause cheese defects, the isolates from this study were tested for their ability to form biogenic amines. In this screening, a strain was found that formed tryptamine in addition to tyramine (Suppl. Fig. S1). Since we had not previously observed bacterial formation of tryptamine in our laboratory, we were interested in identifying the enzymes that play a role in this metabolism.

To confirm the identity of both aromatic monoamines, the culture supernatant of this strain, FAM25164, was also analyzed using HPLC. The HPLC chromatogram of the culture supernatant of FAM25164 showed two peaks that eluted with the retention times of tyramine and tryptamine compared with the medium (Fig. 1). Furthermore, in the chromatogram of FAM25164, the peak area of tryptophan was significantly smaller and that of tyrosine disappeared compared to the uninoculated medium (Fig. 1).

### 3.2. Identification of aromatic amino acid decarboxylases

The genome of the strain FAM25164 was sequenced to search the genome data for coding sequences (CDSs) encoding aromatic amino acid decarboxylases. The final genome assembly of FAM25164 contained 88 contigs with a size of 1.97 MB and an average GC content of 41.5%. When the first sequence annotated by GenBank (acc. no. JAFJMA000000000) was searched using the keyword "decarboxylase", two CDSs were found to be annotated as tyrosine decarboxylases. The first CDS, encoding protein WP_148484661.1, was located on contig_4; the other, encoding WP_223315860.1, was located on contig_14. Sequence-based analyses showed that both protein sequences had 51% identity, and a BLAST search against the SwissProt database (March 2023) revealed that WP_148484661 and WP_223315860 shared approximately 76% and 50% identity, respectively, with the TDC of *Enterococcus faecalis* (SwissProt Q838D6) and of *Levilactobacillus brevis* (SwissProt P0DTQ4). The lower sequence identity of WP_223315860 raised doubts that it was really a tyrosine decarboxylase. For reasons of readability, the two proteins WP_148484661 and WP_223315860 will be referred to as Tdc1 and Tdc2 in the remainder of this report.

Regarding the genes surrounding *tdc1*, upstream is a gene encoding a tyrosine–tRNA ligase (tyrS), and downstream are genes encoding an amino acid transporter (tyrP), a Na+/H+ antiporter (nhaC), and a major facilitator superfamily transporter (data not shown). The entire region had a 99% nucleotide identity with the *tdc* clusters described for both *L. curvatus* strains Lbc1 and Lbc2 (Stroman et al., 2018).

To compare the genetic environment of *tdc2,* the gene was searched in the genomes of *L. curvatus* deposited in the GenBank database (December 2022). There, it was detected in only 2 of 70 genome sequences, namely in the genome of the strain NFH-Km12 (GenBank AP018699) and of HFS9 (GenBank JAMOHH010000041), where the corresponding gene in NFH-Km12 and HFS9 showed 99.9% and 99.8% nucleotide sequence identity, respectively. When the gene context of *tdc2* was compared with the reference genome of DSM 20019 (GenBank CP026116), it was found that the locus_tag LCU_07340 of DSM 20019, encoding a DUF916 and DUF3224 domain-containing protein, was replaced by a gene cluster (Fig. 2). The gene cluster comprised genes encoding Tdc2, insertion elements, a DUF554 domain-containing protein, a TetR/AcrR family transcriptional regulator, and a

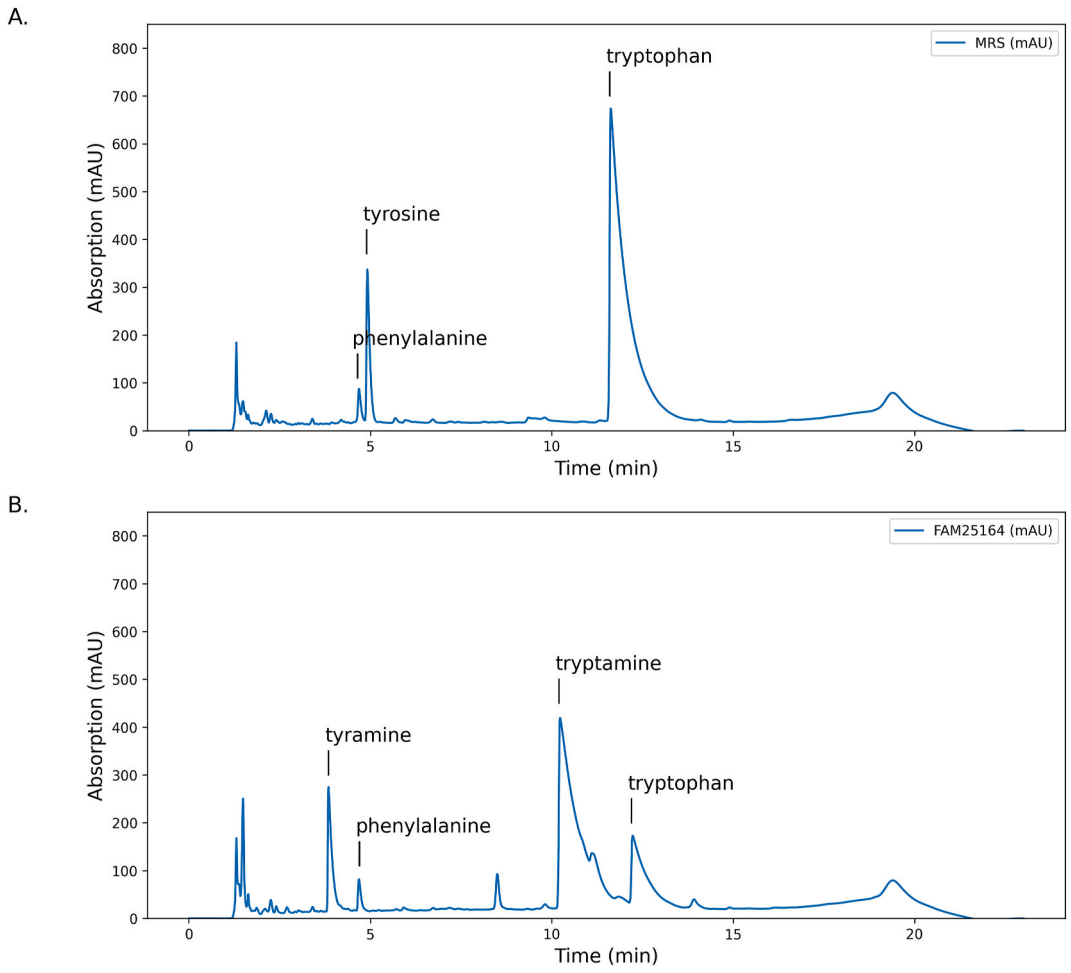


**Fig. 1.** Tyramine and tryptamine formation by *Latilactobacillus curvatus* FAM25164.
HPLC chromatograms showing the MRS medium supplemented with L-tryptophan, L-phenylalanine, and L-tyrosine before (A) and after (B) fermentation with *L. curvatus* FAM25164.

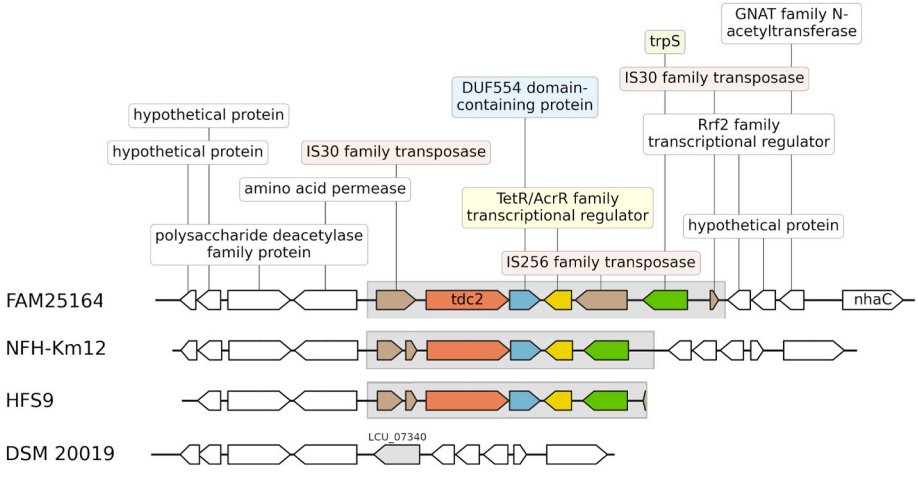


**Fig. 2.** Gene context analysis of the region containing *tdc2*.
Comparison of the genetic region surrounding the *tdc2* gene on contig_14 of the strain FAM25164 (GenBank JAFJMA010000014) with the chromosome of the strain NFH-Km12 (GenBank AP018699), scaffold41 of the strain HFS9 (GenBank JAMOHH010000041), and DSM 20019 (GenBank CP026116). The arrows represent coding sequences (CDSs). The gray rectangle illustrates the CDSs that replaced LCU_07340 in DSM 20019.

tryptophan–tRNA ligase (trpS).

Regarding the insertion sequences, all three strains, FAM25164, NFH-Km12, and HFS9, possess an IS30 family transposase upstream of the *tdc2* gene (Fig. 2), which carries an internal stop codon in all three strains, indicating that the transposase is nonfunctional. In addition, a 189-bp sequence upstream of *trpS* in FAM25164 was annotated as a partial sequence of an IS30 family transposase (Fig. 2). The sequence was also found in the genome of NFH-Km12, but it was not annotated there. On scaffold41 of HFS9, the sequence was absent. Finally, an IS256 family transposase lay between the TetR/AcrR family transcriptional regulator and the tryptophan–tRNA ligase genes in FAM25164 (Fig. 2). This transposase was not present in HFS9 or NFH-Km12. To exclude an

assembly error, the region containing this transposase was amplified by PCR with the primer pairs tnp256_F1/tnp256_R1 and tnp256_F2/tnp256_R2 (Table 1). Sanger sequencing of the PCR products confirmed the presence of the IS256 family transposase in the *tdc2* gene cluster in FAM25164. The region of the tdc2 gene cluster downstream of the IS256 element of FAM25164 shared 99.9% and 99.8% nucleotide identity with NFH-Km12 and HF9, respectively. The region upstream showed 100% nucleotide identity.

### 3.3. Amine formation by recombinant *E. coli*

In order to study the substrate specificity of the decarboxylases, *tdc1* and *tdc2* were cloned and expressed in *E. coli* in the presence of phenylalanine, tyrosine, and tryptophan. Since wild-type *E. coli* does not form tyramine, β-phenylethylamine, or tryptamine, it can be used to determine the substrate specificity of aromatic amino acid decarboxylase activity by expressing the corresponding genes in this host (Williams et al., 2014).

When *E. coli* BL21(DE3) strains harboring either the pEXP5-CT/tdc1 or pEXP5-CT/tdc2 expression plasmid were cultivated in minimal medium supplemented with precursor amino acids, it was observed that *E. coli* with *tdc1* produced tyramine (Fig. 3B) and *E. coli* with *tdc2* formed tryptamine (Fig. 3C). No aromatic monoamines were detected in the culture supernatant of the control strain transformed with the control plasmid pEXP5-CT/CALML3 (Fig. 3A). It is noteworthy that β-phenylethylamine was not detected in any of the samples.

### 3.4. In vitro decarboxylation activity of recombinant His-tagged decarboxylases

To facilitate protein purification, a His-tag was added to the C-terminus of *tdc1* and *tdc2* during cloning. When gene expression was induced in *E. coli* using LB medium, only low concentrations of soluble Tdc1 (usually less than 0.05 μg $\mu L^{-1}$) and no soluble Tdc2 protein were obtained. The addition of 1% D-glucose to the LB medium improved the yield, and approximately 0.6 mg of soluble Tdc1 (0.2 mg $mL^{-1}$ in 3 mL) and 0.3 mg of Tdc2 (0.1 μg $\mu L^{-1}$ in 3 mL) were obtained from a 200-mL culture. Gel electrophoretic analysis of the purified protein fractions showed that the enzyme preparations contained a protein with a high degree of purity (Suppl. Fig. S2). The purified proteins were used for enzymatic assays to assess their ability to decarboxylate amino acids.

The purified Tdc1 was found to decarboxylate tyrosine and L-DOPA

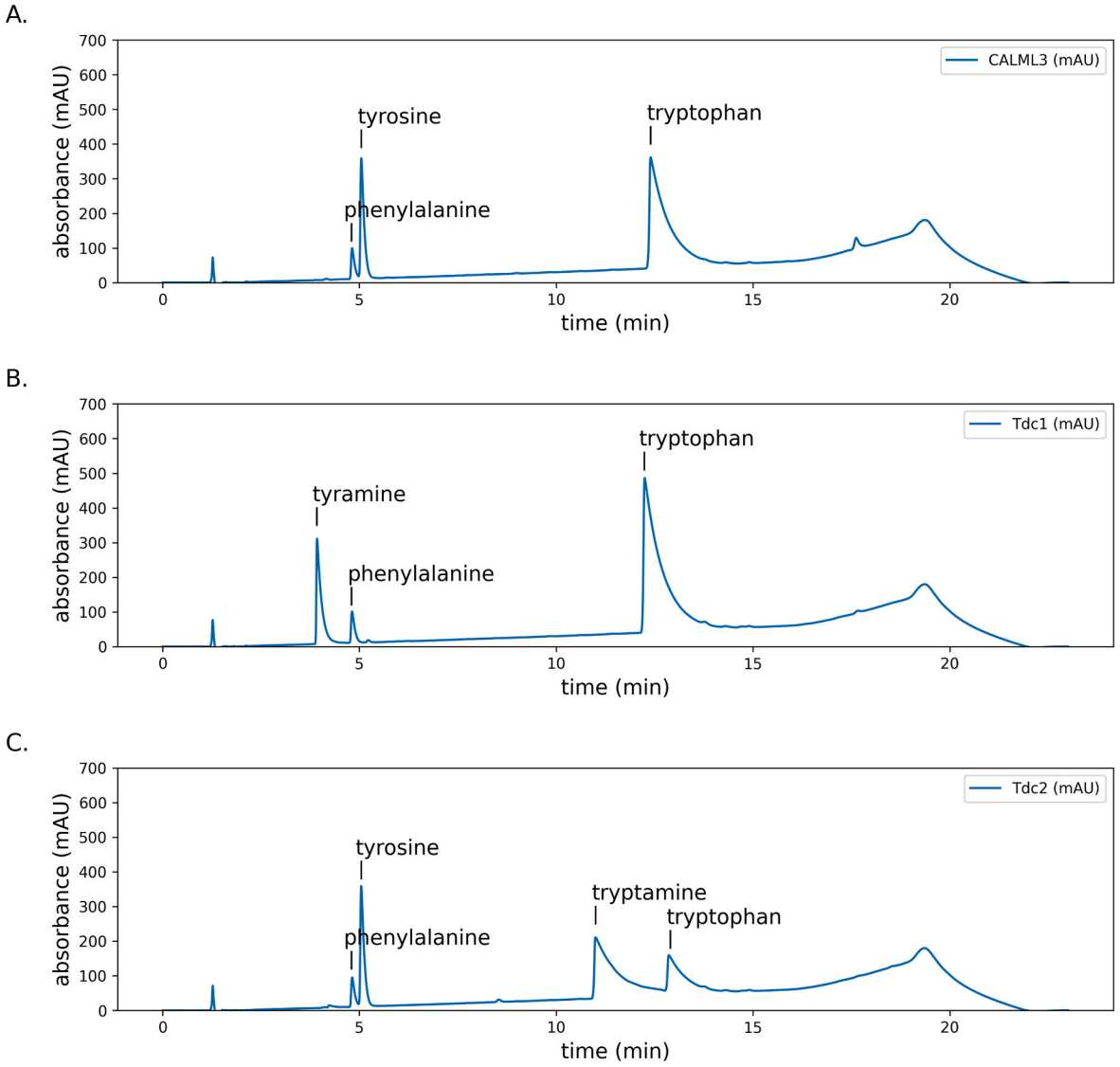


**Fig. 3.** Production of tyramine and tryptamine by recombinant *Escherichia coli*.
*E. coli* harboring pEXP5-NT/CALML3 (A), pEXP5-CT/tdc1 (B), or pEXP5-CT/tdc2 (C) were incubated in M9 medium supplemented with L-phenylalanine, L-tyrosine, and L-tryptophan. The HPLC chromatograms of the culture supernatants after 3 days of incubation are shown.

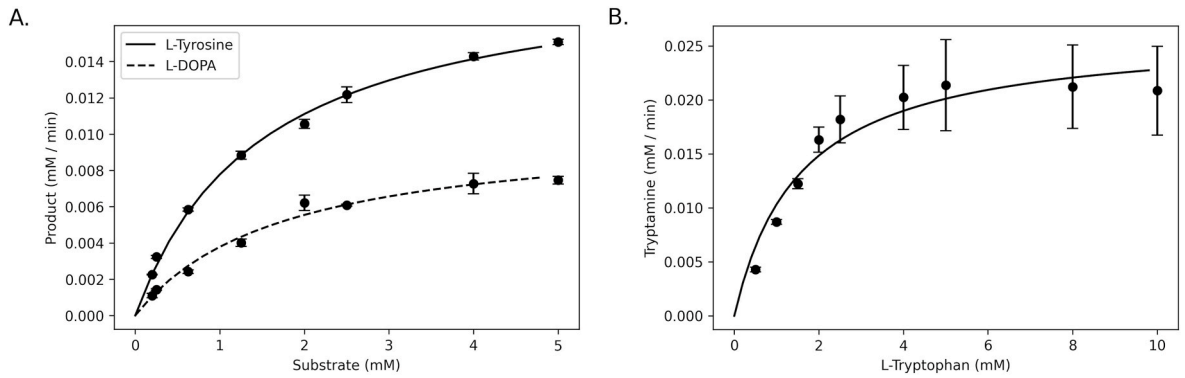


**Fig. 4.** Kinetic parameters of recombinant Tdc1 and Tdc2.
The graphs show the relationship between substrate concentration and reaction rate for Tdc1 (A) with L-tyrosine and L-DOPA and Tdc2 (B) with L-tryptophan. Error bars represent the standard deviation of three independently performed decarboxylation assays.

(Fig. 4A). No activity was observed toward phenylalanine or tryptophan. The $K_m$ and $k_{cat}$ values for L-tyrosine were 1.5 ± 0.1 mM and 92.1 ± 2.2 ($s^{-1}$) and for L-DOPA 1.7 ± 0.3 mM and 49.9 ± 2.2 ($s^{-1}$).

In the case of Tdc2, the protein clearly decarboxylated tryptophan with a $K_m$ of 1.6 ± 0.4 mM and a $k_{cat}$ of 10.3 ± 2.0 ($s^{-1}$) (Fig. 4B). The enzyme showed no decarboxylating activity toward phenylalanine, tyrosine, and L-DOPA.

## 4. Discussion

The bacterium *Latilactobacillus curvatus* is found in plant-, dairy-, and meat-fermented food (Aymerich et al., 2006; Beresford and Williams, 2004; Gobbetti et al., 2016; Yang et al., 2014). On the one hand, the bacterium is intentionally added as part of starter cultures, e.g., in the fermentation of meat. On the other hand, it may develop from raw food or the environment during fermentation.

For food quality and safety reasons, it is important to understand the genetic and biochemical properties of bacteria present in food. Regarding *L. curvatus,* there are several reports showing that it has aminogenic activity, with tyramine formation being the most common (Aymerich et al., 2006; Latorre-Moratalla et al., 2010; Li et al., 2018; Pircher et al., 2007). Furthermore, it has also been observed that the presence of *L. curvatus* in cheese can lead to excess gas production, causing cracks and splits (Porcellato et al., 2015).

In this study, *L. curvatus* isolated from cheese was screened for its ability to form biogenic amines. Thereby, it was found that most of the isolates formed tyramine and one strain additionally produced tryptamine. In a study by Aymerich et al. (2006), *L. curvatus* strains isolated from fermented sausages were examined for their ability to form biogenic amines. It was found that 67.9% of the strains analyzed produced tyramine and 15.1% formed tryptamine. Two conclusions can be drawn from these observations. First, the formation of tyramine is not linked with the formation of tryptamine, so there should be two different metabolic pathways. Second, the ability to synthesize tyramine is widespread within *L. curvatus* strains, suggesting that this is a species-level characteristic, as is the case with enterococci (Ladero et al., 2012).

Tryptamine biosynthesis by bacteria has rarely been reported. From the Rhea reaction database (Bansal et al., 2021), it can be concluded that the compound is produced by the decarboxylation of tryptophan by the action of aromatic amino acid decarboxylases (EC 4.1.1.28) and tryptophan decarboxylases (EC 4.1.1.105). Bacterial tryptophan decarboxylases have been found in *Clostridium sporogenes* and *Ruminococcus gnavus* (Williams et al., 2014). In addition, an aromatic amino acid decarboxylase from *Bacillus atrophaeus* was found to decarboxylate tryptophan and several other substrates (Choi et al., 2021). The protein sequences of these three decarboxylases shared less than 25% sequence identity, indicating that bacterial tryptophan decarboxylases cannot be easily identified by sequence comparisons and that functional screenings are required for clear identification.

Genome sequencing was performed with the aim of identifying and cloning amino acid decarboxylases. The analysis of the genomic data revealed that the strain FAM25164 possesses two amino acid decarboxylases, *tdc1* and *tdc2,* which exhibited an identity of 50% and more to the biochemically characterized TDCs from *L. brevis* and *E. faecium* (Moreno-Arribas and Lonvaud-Funel, 2001; van Kessel et al., 2019). While knockout studies have already shown that *tdc1* is responsible for tyramine formation in *L. curvatus* (Stroman et al., 2018), no conclusions could be drawn for *tdc2* from the literature.

The first annotation performed by GenBank (acc. no. JAFJMA00000000) assigned *tdc2* the function of a tyrosine decarboxylase. However, the presence of a tryptophan–tRNA ligase gene in the vicinity of *tdc2* questioned this functional assignment (Fig. 2). The genes for biogenic amine formation are often arranged in clusters that usually contain a gene encoding a tRNA ligase (Benkerroum, 2016). For example, the HDC cluster has a histidine–tRNA ligase, the TDC cluster has a tyrosine–tRNA ligase, and the CAD cluster has a lysine–tRNA ligase. The presence of a tryptophan–tRNA ligase suggests that *tdc2* actually encodes a tryptophan decarboxylase.

To verify that *tdc2* really encodes a tryptophan decarboxylase, the gene was cloned. In addition, *tdc1* was cloned to compare the decarboxylase activities with each other and with published activities. It has been described that the heterologous expression of aromatic amino acid decarboxylases often leads to poor yields (Han and Shin, 2022). In this study, it was also observed that the protein yield of *tdc1* and *tdc2* expressed in *E. coli* was weak and could be improved by adding glucose, as previously described by Zhang and Ni (2014). Tdc1 accepted tyrosine and L-DOPA as substrates, which has also been shown for the TDCs of *E. faecalis, E. faecium*, and *L. brevis* (van Kessel et al., 2019; Zhang and Ni, 2014).

Since phenylalanine and tyrosine are structurally similar, it can be argued that the same enzyme is responsible for the production of β-phenylethylamine as for tyramine. However, in this study, it was observed that neither the tyramine-forming cheese isolates nor the *E. coli* strain expressing *tdc1* or the recombinant Tdc1 produced β-phenylethylamine. The publication of Aymerich et al. (2006) describes that the percentage of tyramine-forming *L. curvatus* strains is not identical to that of the β-phenylethylamine-forming strains. These observations suggest that a metabolic pathway separate from tyramine is present in β-phenylethylamine-forming *L. curvatus* strains. The characterization of β-phenylethylamine-forming strains could investigate this hypothesis.

The recombinant Tdc2 accepted only tryptophan as a substrate. In contrast, the tryptophan decarboxylase from *R. gnavus* decarboxylated tryptophan, tyrosine, and phenylalanine, and that from *C. sporogenes*

decarboxylated tryptophan and tyrosine. The limited substrate spectrum of Tdc2 suggests that it is a tryptophan decarboxylase (EC 4.1.1.105) rather than an aromatic amino acid decarboxylase (EC 4.1.1.28).

The *tdc2* gene was also found in the genomes of *L. curvatus* NFH-Km12 and HFS9. The status of the NFH-Km12 genome is complete, showing that the *tdc2* gene is located on the chromosome. When the gene context of *tdc2* in FAM25164 was compared with other *L. curvatus* genomes, three additional CDSs encoding a DUF544 domain-containing protein, a TetR/AcrR family transcriptional regulator, and a tryptophan–tRNA ligase were found to be present only in the strains possessing *tdc2* (Fig. 2). Other amino acid decarboxylases, such as histidine decarboxylase, TDC, and lysine decarboxylase, are, as mentioned above, also organized in gene clusters (Benkerroum, 2016). Thus, it is proposed that the four CDSs form a tryptophan decarboxylase cluster responsible for tryptamine biosynthesis. The role of the DUF544 domain-containing protein could be evaluated by knocking out this gene.

The presence of transposases indicates that the *tdc2* cluster is a mobile genetic element. This idea is supported by the GC content, which is 35.0%, 35.5%, 25.8%, and 33.8% for the decarboxylase, the DUF554 domain-containing protein, the TetR/AcrR transcriptional regulator, and tryptophan–tRNA ligase, respectively, and is lower than the average GC content of the assembly (41.5%). The reason or the influence of the IS256 element in FAM25164 could not be determined with the sequence comparisons performed. It is conceivable that the presence of the transposase has an effect on the transcription of the TetR/AcrR family transcriptional regulator (Fig. 2) and thus perhaps on the expression of the entire gene cluster. This hypothesis could be investigated with comparative gene expression analyses of FAM25164 with a strain possessing the *tdc2* gene cluster without the IS256 element.

## 5. Conclusion

The present study describes the identification a novel bacterial tryptophan decarboxylase (EC 4.1.1.105) in *Latilactobacillus curvatus*. The protein sequence showed less than 25% identity with three other enzymatically characterized bacterial decarboxylases that decarboxylate tryptophan. Gene context analysis showed that the tryptophan decarboxylase gene is part of a gene cluster likely acquired by horizontal gene transfer, as transposases are present and the GC content is well below the average GC content of the assembled genome. These findings suggest that *L. curvatus*, possessing the tryptophan decarboxylase gene cluster, can synthesize tryptamine in cheese. Cheese trials, in which strain FAM25164 is used as an adjunct culture, will show whether the strain can actually form tryptamine in cheese.

## Declaration of competing interest

none.

## Acknowledgments

We thank the Next Generation Sequencing Platform of the University of Bern for supporting us with Illumina sequencing.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2023.104343.

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