Curr Microbiol (2007) 55:205–210
DOI 10.1007/s00284-006-0647-8

Characterization of a *Lactobacillus plantarum* Strain Able to Produce Tyramine and Partial Cloning of a Putative Tyrosine Decarboxylase Gene

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Received: 22 December 2006 / Accepted: 19 March 2007

Abstract The aim of this article was to analyze the ability of wine *Lactobacillus plantarum* strains to form tyramine. Preliminary identification of *L. plantarum* strains was performed by amplification of the *recA* gene. Primers pREV and PlanF, ParaF and PentF were used respectively as reverse and forward primers in the polymerase chain reaction tests as previously reported. Furthermore, the gene encoding for the tyrosine decarboxylase (TDC) was partially cloned from one strain identified as *L. plantarum*. The strain was further analyzed by 16S rDNA sequence and confirmed as belonging to *L. plantarum* species. The tyrosine decarboxylase activity was investigated and tyramine was determined by the high-performance liquid chromatography method. Moreover, a negative effect of sugars such as glucose and fructose and L-malic acid on tyrosine decarboxylase activity was observed. The results suggest that, occasionally, *L. plantarum* is able to produce tyramine in wine and this ability is apparently confined only to *L. plantarum* strains harboring the *tdc* gene.

Keywords Wine · TDC · *Lactobacillus plantarum* · Tyramine

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Introduction

Biogenic amines (BAs) are produced by lactic acid bacteria (LAB) during the process of fermentation of foods and beverages by amino acid decarboxylation [9, 30]. In wine, several amino acids can be decarboxylated, and as a result of this process, histamine, tyramine, putrescine, cadaverin and phenylethylamine are usually found, the first three being the most frequent [3, 8, 12, 13, 15, 20] and histamine the main amino acid responsible of health disturbances [11].

Tyrosine decarboxylase (TDC) converts tyrosine to tyramine and its purification and characterization has been reported for *Enterococcus faecalis* and *Lactobacillus brevis* species [5, 22]. Tyrosine decarboxylase (*tdc*) gene has been identified in several LAB, and primers for the detection of tyramine-producing LAB were developed [5–7, 17, 18]. Moreover, it has been suggested that bacterial tyrosine decarboxylases are encoded in an operon containing four genes [18]. LAB vary in their ability to form tyramine. Several strains of *Leuconostoc mesenteroides* isolated from wine [24], *E. faecalis* and *Enterococcus faecium* identified in fermented sausages are able to produce tyramine [4]. Among *Lactobacillus* species, several strains of *L. curvatus, L. brevis*, *L. paracasei*, and *L. sakei* have been report to produce tyramine [3, 4, 15, 24]. Most of the tyramine produced in fermented sausages is probably due to

L. curvatus [4], whereas *L. brevis* and *L. hilgardii* appear to be the main tyramine producer in wine [13–15, 23].

Lactobacillus plantarum is frequently isolated from red wine undergoing malolactic fermentation (MFL) and sterilized with sulfite [16, 28] and it usually contributes to production of undesirable products such as histamine and precursors of ethyl carbammate [16, 25, 26]. Therefore, *L. plantarum* is of general concern because of its spoilage nature. There are few reports concerning the ability of *L. plantarum* to produce tyramine in fermented food [21]. In this article, we report the identification and characterization of a tyramine-producing *L. plantarum* strain. Our findings suggest that some *L. plantarum* strains are able to decarboxylase tyrosine in wine. This ability is rare and apparently confined only to *L. plantarum* strains harboring the *tdc* gene.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

Escherichia coli JM109 High Efficiency Competent Cells (Promega) used for cloning procedures were grown in Luria-Bertani (LB) medium supplemented with ampicillin (50 μg/mL) when required. Plasmid DNA was purified with Wizard *Plus* SV Minipreps (Promega) and DNA sequencing was performed on both strands with universal primers (T7 and SP6) by MWG Biotech (Germany). The plasmid pGEM-T easy vector (Promega) was used as a general vector for cloning and sequencing. In order to identify *L. plantarum* strains able to produce tyramine, a total of 190 LAB were screened. One hundred fifteen LAB were previously isolated from red wine undergoing MFL in 2004, 2005, and 2006 [26–28], and 75 putative LAB were isolated from cheese [32]. The collection types *L. plantarum* ATCC 14917^T, *L. pentosus* ATCC 8041, and *L. paraplantarum* LMG 16673^T and the reference *L. plantarum* WCFS1 [10] were used as standard strains for molecular analysis. *L. brevis* strain 4258, able to degrade tyramine and harboring the *tdc* gene [14], and the *L. plantarum* WCFS1 [10] were used as positive and negative controls, respectively, for the analysis of the *tdc* gene in *L. plantarum*. Strains were grown without shaking at 28°C in a deMan Rogosa Sharpe (MRS) broth (Oxoid, Basingstoke, UK), pH 6.8.

Molecular Identification of *Lactobacillus plantarum* Strains

Preliminary identification of *L. plantarum* strains was performed by amplification of the *recA* gene in a multiplex polymerase chain reaction (PCR) reaction [31]. Primers pREV (5′-TCGGGATTACCAAACATCAC-3′) and PlanF (5′-CCGTTTATGCGGAACACCTA-3′), ParaF (5′-GTC ACAGGCATTACGAAAAC-3′), and PentF (5′-CAGTGG CGCGGTTGATAT-3′) were used respectively as reverse and forward primers in the PCR tests, as previously reported [25, 31]. Putative *L. plantarum* strains were furthermore confirmed by 16S rDNA gene amplification and sequence analysis. Amplification was performed as already reported [26] with primers designed on the 16S ribosomal gene (5′-GCGAACTGGTGAGTAACA-3′ and 5′-GGTTG CGCTCGTTGCGGG-3′, from nt 121 to nt 1321) of *L. plantarum* WCFS1 strain (EMBL database Accession No. 004567). The DNA sequence was analyzed and compared with the GenEMBL databases, using the FASTA program or the BLAST network service (NCBI).

Identification of Tyrosine Decarboxylase Gene

The genomic DNA of *L. plantarum* strains was isolated with the Microbial DNA extraction kit (CABRU, Milan, Italy) according to the manufacturer's procedure. For the PCR experiment, about 100 ng of genomic DNA was added to a 50-(L PCR mixture containing 1.25 U of *Taq* polymerase (Qiagen, Milan, Italy) 0.2 mM each of dATP, dTTP, dGTP, dCTP, 10 mM Tris-HCL, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.4 μM of primers p0303 [24] and P1-rev [23]. The reaction mix was cycled through the following temperature profile: 94°C, 5 min; 94°C, 1 min, 55°C, 1 min, 72°C, 1 min for the first 15 cycles, then 12 cycles at 58°C as the annealing temperature. The PCR reaction was terminated at 72°C for 5 min. The amplified products (with a size of ~370 bp) were purified with a Quantum Prep PCR Kleen Spin Columns (Bio-Rad) and cloned in a pGEM-T easy vector (Promega) as recommended by the manufacturer, and the DNA sequencing was performed with universal primers (T7 and SP6) by MWG Biotech (Germany). Analyses of DNA and amino acid sequences were carried out using programs accessible at the NCBI website (www.ncbi.nlm.nih.gov). The GenBank accession number of the tyrosine decarboxylase (*tdc*) nucleotide sequence is available at the follow accession number: EF178285.

Quantification of the Tyrosine Decarboxylase Activity

Lactobacillus plantarum strains were grown in the basal medium (BM) containing the following (in g/L): 5, peptone (Oxoid L37); 3, yeast extract (Oxoid L21); 1, glucose (Britania 046, Buenos Aires, Argentina) 1, tyrosine (Sigma), and 0.006 pyridoxal-5′-phosphate (Sigma). After incubation at 30°C for 48 h, the cells from the third subculture were harvested at the end of the logarithmic phase growth. Cells were harvested by centrifugation at 12,000

rpm for 15 min and the pellet was washed twice with 0.2 M sodium acetate buffer, pH 5.0. To prepare cell extracts, cells pellets were first resuspended in cold buffer acetate (4°C) at 30% (w/v) and then passed four through a French pressure [19]. Cell debris was removed by centrifugation at 15,000 rpm for 5 min, and the supernatant extract was used to determine tyrosine decarboxylase activity. The reaction mixture for tyrosine decarboxylase determination contained 0.2 mL of tyrosine (5 mM) adjusted to pH 5.0, 0.05 mL of pyridoxal-5′-phosphate buffer (0.4 mM), pH 5.0, 0.3 mL of cell free extract, and 0.2 mL of buffer acetate. The mixtures were incubated at 35°C and samples were taken every 10 min; the reaction was stopped by the addition of 0.2 mL of perchloric acid (70%) and centrifuged at 15,000 rpm for 1 min. The supernatant was then analyzed for tyramine concentration. Tyrosine decarboxylase activity was determined by measuring the tyramine released from tyrosine with high-performance liquid chromatography (HPLC) as described by Alberto et al. [1].

Results

Molecular Identification of *Lactobacillus plantarum* Strains and Partial Characterization of the *tdc* Gene

Preliminary identification of *L. plantarum* strains was performed by amplification of the *recA* gene in a multiplex PCR test using primers pREV and PlanF, ParaF, and PentF [31] as reverse and forward primers, respectively (Fig. 1). A single amplification fragment of 318 bp was obtained in 29 strains isolated from wine and 20 strains isolated from cheese, suggesting that the strains belonged to *L. plantarum* species (Fig. 1A). The PCR reaction was repeated twice and no amplified bands corresponding to *L. pentosus* or *L. paraplantarum* were observed. *L. plantarum* ATCC 14917^T and *L. plantarum* WCSI [10] were included as a positive control. In order to identify *L. plantarum* strains able to produce tyramine, primers p0303 [18] and P1-rev [17] were used in PCR experiments. This couple of primers has been shown to be the most suitable, as it is always able to detect the *tdc* gene on all of the strains analyzed [14]. As reported in Fig. 1B, only one strain isolated from red wine (named L65) yielded an amplification fragment of about 370 bp. As no *L. plantarum tdc* positive strain has been described to now, the DNA isolated from *L. brevis* strain 4258 was used as a positive control in the PCR test and a fragment of about 370 bp was also obtained for *L. brevis* strain 4258 (Fig. 1B). No amplified fragments were detected when DNA extracted from *L. plantarum* ATCC 14917^T and *L. plantarum* WCFS1 were used as the template. These results are in agreement with the published genome of *L. plantarum* WCFS1. Indeed, no *tdc* gene was detected when the entire genome of *L. plantarum* WCFS1 was sequenced [10]. The DNA fragment amplified from the L65 strain was cloned and sequenced. The homology between the amino acid sequence of this PCR product and the TDC proteins previously identified in *L. brevis* [17] and *L. curvatus* [2] confirmed that the fragment belonged to the internal portion of the *tdc* gene (Fig. 2). The identity of strain L65 was subsequently confirmed by amplification and sequence analysis of the 16S rDNA gene. Sequence of the 16S rRNA gene obtained for the *Lactobacillus plantarum* L65 strain was identical compared to those from

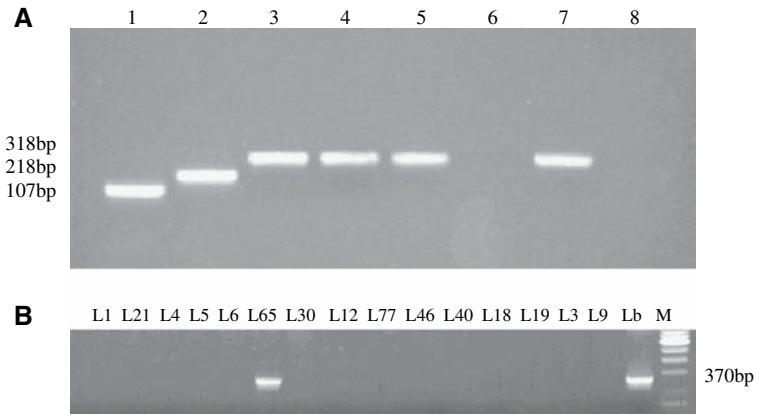

Fig. 1 **A** Example of identification of *L. plantarum* strains using the *recA* multiplex PCR assay. Lanes 1, 2, 3, and 4: PCR products from *L. paraplantarum* LMG 16673^T, *L. pentosus* ATCC 8041, *L. plantarum* ATCC 14917^T, and *L. plantarum* WCFS1, respectively; lanes 5 and 7: amplification products of putative *L. plantarum* strains isolated from red wine; lanes 6 and 8: negative results on DNA extracted from LAB isolated from red wine and amplified using the multiplex PCR assay. The size of the fragments is reported on the left. **B** PCR-based detection of *L. plantarum* strains containing a *tdc* gene. DNA isolated from *L. plantarum* strains previously characterized was amplified with primers p0303 and P1-rev and PCR fragments analyzed on 1.2% agarose gel. Lb: *L. brevis* strain 4258 (positive control); L65: *L. plantarum tdc* positive. M = 100-bp molecular marker (Promega)

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L. plantarum    TAASVWAAHKTLPLNVTGYGKL-GASIEGAHRFYNFLSGLKFKVGDKTIEVHPLTDPDFN 59
L. brevis       TAASVWAAHHTLPLNVTGYGKLEGASIEGAHRYYDFLKNLKFEVAGKRISVHPLISPDFN 60
L. curvatus     TAASVWAAHKTLPLNVTGYGKLVGASIEGARRFYNFLSGLEFKVGDKTIEVHPLTDPDFN 60
                ********* ************ ******* * * **  * * *  * * ****  ****

L. plantarum    METVDYVFQEKGNDDLVEMETNELNHEFYEQASVKG--SYGLEYLRHPT--FAPDY 111
L. brevis       METVDYVLKEDGNDDLIEMETNRLNHAFYEQASYVKGSLYGKEYIVSHTDFAIPDY 116
L. curvatus     METVDYVFQEKGNNNLVEMN--ELNHEFYNQA------SYELEHLRNPT------- 101
                *******  * **  * **    *** ** **       *  *     *
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Fig. 2 Sequence comparison between partial *L. plantarum* putative TDC protein (EMBL Accession No. ABM55261), *L. brevis* (EMBL Accession No. AF446085), and *L. curvatus* (EMBL Accession No.r Q2UZ89) TDC proteins. Identical amino acids are indicated by asterisks

L. plantarum strain WCFS1 [10], confirming that the isolated strain belonged to the *L. plantarum* species (data not shown).

Detection of Tyramine and Effect of Sugars and L-Malic Acid on Tyrosine Decarboxylase Activity in *L. plantarum*

In order to test the ability of wine *L. plantarum* L65 to degrade tyramine and to check whether tyramine might be produced by strains resulting as negative in the PCR test, the tyramine-forming capacity was also investigated in *L. plantarum* strains L61, L60, L21, and L11 (*tdc* negative). The results presented in Table 1 show that only *L. plantarum* strain L65 was able to produce tyramine and no appreciable enzymatic activity was detect for the remnant strains. These results suggest that TDC activity is apparently confined only to *L. plantarum* strains harboring the *tdc* gene. In addition to the presence of microorganisms, the influence of other factors such as nutrients, pH, and alcohol content might affect tyramine production [3, 28]. The results reported in Table 1 show that all of the substrates tested produce an inhibitory effect on the tyramine formation. The inhibition increases as the substrate concentration increases. The biogenic amine production diminishes by 35%, 56%, and 91% in the presence of 1, 2, and 3 g/L of glucose; 38%, 61%, and 93% in the presence of 1, 2, and 3 g/L of fructose, and 42%, 61%, and 86% in the presence of 1, 2, and 3 g/L of L-malic acid, respectively. Therefore, tyramine production from *L. plantarum* in wine seems to be strongly dependent on available precursors.

Table 1 Specific activities of TDC in strains of *L. plantarum* isolated from red wine and effect of sugars and L-malic on TDC activity in *L. plantarum* strain L65

L. plantarum (strains)	TDC activities (μmol product formed/ min/mg protein)
L65	257 ± 18
L61	ND
L60	ND
L21	ND
L11	ND
Substrate added to reaction mixture (g/L)	**TDC activities of strain L65 (μmol product formed/ min/mg protein)**
None (control)	254 ± 18
Glucose 1	165 ± 25
Glucose 2	113 ± 13
Glucose 3	21 ± 8
Fructose 1	157 ± 23
Fructose 2	98 ± 19
Fructose 3	16 ± 5
L-Malic acid 1	147 ± 27
L-Malic acid 2	100 ± 19
L-Malic acid 3	36 ± 10

The data presented are the means of three independent experiments with their standard deviations indicated. ND: not detected

Discussion

In this article, several strains of *L. plantarum* were characterized and screened with primers able to identify the *tdc* gene in several LAB and were already published [14, 17, 18]. None of the *L. plantarum* strains isolated from cheese were *tdc* positive and only one of the *L. plantarum* strains isolated from red wine resulted into a positive amplicon, as tested by PCR. The high sequence identity between TDC proteins from *L. curvatus* and *L. brevis* and that deduced from the sequence of the PCR product obtained from *L. plantarum* suggests that the putative gene cloned from *L. plantarum* strain L65 might be involved in tyramine formation in this microorganism. Although the highest homology was found with TDC previously identified in *Lactobacillus* species, similarities between tyrosine decarboxylase and glutamate decarboxylase proteins were observed. The similarity detected could be ascribed to the short sequences under examination.

The results presented in Table 1 lead to the conclusion that *L. plantarum* strain L65 is able to form tyramine and that tyramine production is affected by wine factors such as

sugars and L-malic acid. This result is in agreement with those reported by Soufleros et al. [29], which stated that the majority of biogenic amines (except putrescine) in wine is negatively correlated with malic acid and citric acid content [29]. We also found that tyrosine decarboxylase activity is only detectable on *L. plantarum* strains harboring the *tdc* gene, suggesting that the production of tyramine in *L. plantarum* is a strain-dependent characteristic, as previously reported for *L. hilgardii* and *Leuconostoc mesenteroides* [3, 13, 15, 24]. Although our results suggest that *L. plantarum*, in addition to *L. brevis*, *L. hilgardii*, and *Leuconostoc* strains previously described [3, 13, 14, 22–24], might be responsible for tyramine production in wine, this study also indicates that the ability of the *L. plantarum* tyramine-producer is not widespread in fermented food as already suggested [21] and it is confined only to *L. plantarum* strains harboring the *tdc* gene.

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