Production of Biogenic Amines by Lactic Acid Bacteria: Screening by PCR, Thin-Layer Chromatography, and High-Performance Liquid Chromatography of Strains Isolated from Wine and Must

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ABSTRACT

Biogenic amines are frequently found in wine and other fermented food. We investigated the ability of 133 strains of lactic acid bacteria isolated from musts and wines of different origins to produce histamine, tyramine, and putrescine. We detected the genes responsible for encoding the corresponding amino acid decarboxylases through PCR assays using two primer sets for every gene: histidine decarboxylase (hdc), tyrosine decarboxylase (tdc), and ornithine decarboxylase (odc); these primers were taken from the literature or designed by us. Only one strain of Lactobacillus hilgardii was shown to possess the hdc gene, whereas four strains of Lactobacillus brevis had the tdc gene. None of the Oenococcus oeni strains, the main agents of malolactic fermentation, was a biogenic amine producer. All PCR amplicon band-positive results were confirmed by thin-layer chromatography and high-performance liquid chromatography analyses.

Biogenic amines are natural compounds present in different types of food and beverage, such as cheese, fish, beer, and wine. Histamine and tyramine, when ingested, can have adverse reactions that affect the nervous and vascular systems (3, 28, 29). Putrescine is also potentially dangerous, because it can react with nitrites to form carcinogenic nitrosamines (16). Biogenic amines are mainly produced by decarboxylation of the precursor amino acid through the substrate-specific enzymes of microorganisms that are present in food (33). The enzymes on which most research has been focused are histidine decarboxylase (HDC), which catalyzes the formation of histamine (7); tyrosine decarboxylase (TDC), which is specific for tyramine formation; and ornithine decarboxylase (ODC), which catalyzes the formation of putrescine (14, 23).

HDC has been examined in Oenococcus oeni by Lonvaud-Funel and Joyeux (19), and Le Jeune et al. (17) developed a PCR method to detect the gene responsible for encoding HDC (hdc) in lactic acid bacteria. Coton et al. (6) found that, of the 118 wines they examined, half possessed bacteria that carried hdc, and they proposed a method for the rapid screening of amine-producing strains. These authors concluded that the ability to form histamine is strain-dependent and not related to the species.

TDC has been studied in Lactobacillus spp., particularly in Lactobacillus brevis strains (24). Structural and functional properties have been reported for Streptococcus faecalis (2, 4). Recently, TDC activity was studied in a tyramine-producing L. brevis isolated from wine (27), and its tdc gene was purified and characterized (25). In 2004, Fernandez et al. (10) sequenced the tdc gene in Lactobacillus lactis and developed a PCR method for detecting tyrosine decarboxylating lactic acid bacteria. A similar study was performed by Coton et al. (9) in Carnobacterium divergens.

ODC activity in lactic acid bacteria has been described in Lactobacillus 30a; the gene is activated by low pH and is enriched medium containing ornithine (11, 32). In 1999, O. oeni strain IOEB 8419, isolated from wine, was found to be able to produce putrescine (8) (this activity was measured by monitoring the production of CO2), and recently, Marcobal et al. (23) isolated a putrescine-producing strain of O. oeni and sequenced its odc gene.

The objective of this study was to detect the presence of genes that encode for amino acid decarboxylases (HDC, TDC, and ODC) in lactic acid bacteria that have been isolated from wine and must. Through various PCR assays, by means of both primers that have already been described as well as primers that were designed during this study, this goal was carried out. The biogenic amines produced by these strains were also confirmed by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). In total, 133 strains of lactic acid bacteria were tested. Because O. oeni is the most important malolactic fermentation bacterium, we primarily focused our examination on the strains of this species.

MATERIALS AND METHODS

Microorganisms and growth conditions. A total of 92 O. oeni strains, 1 Leuconostoc mesenteroides, 26 Lactobacillus strains, and 13 Pediococcus parvulus strains were studied. The strains belong to the collection of the Istituto Sperimentale per l’Enologia
TABLE 1. Lactic acid bacteria strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Species</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 a (ATCC 33222)</td>
<td>Lactobacillus sp.</td>
<td>ATCC</td>
</tr>
<tr>
<td>CECT 5354 (ATCC 367)</td>
<td>Lactobacillus brevis</td>
<td>CECT</td>
</tr>
<tr>
<td>ISE 5001, ISE 5002, ISE 5003, ISE 5004, ISE 5005, ISE 5006, ISE 5007, ISE 5008, ISE 5009, ISE 5010, ISE 5011, ISE 5012, ISE 5013, ISE 5014, ISE 5015, ISE 5016, ISE 5017, ISE 5018, ISE 5019, ISE 5020, ISE 5021, ISE 5022, ISE 5023, ISE 5024, ISE 5025, ISE 5026, ISE 5027, ISE 5028, ISE 5029, ISE 5030, ISE 5031, ISE 5038, ISE 5041, ISE 5043, ISE 5051, ISE 5056, ISE 5058, ISE 5060, ISE 5062, ISE 5063, ISE 5071, ISE 5081, ISE 5084, ISE 5089, ISE 5091, ISE 5096, ISE 5102, ISE 5106, ISE 5117, ISE 5159, ISE 5160, ISE 5161, ISE 5162, ISE 5163, ISE 5232, ISE 5233, ISE 5234, ISE 5235, ISE 5236, ISE 5237, ISE 5238, ISE 5239, ISE 5240, ISE 5241, ISE 5242, ISE 5243, ISE 5244, ISE 5245, ISE 5246, ISE 5247, ISE 5248</td>
<td>Oenococcus oeni</td>
<td>Wine/grape must</td>
</tr>
<tr>
<td>ISE 5033, ISE 5197, ISE 5198, ISE 5202</td>
<td>Lactobacillus parakeferi</td>
<td>Wine/grape must</td>
</tr>
<tr>
<td>ISE 5179, ISE 5180, ISE 5181, ISE 5182, ISE 5183, ISE 5184, ISE 5191</td>
<td>Lactobacillus casei</td>
<td>Wine/grape must</td>
</tr>
<tr>
<td>ISE 5032, ISE 5034, ISE 5097, ISE 5201, ISE 5204, ISE 5206, ISE 5211, ISE 5212, ISE 5213, ISE 5214, ISE 5215</td>
<td>Lactobacillus hilgardii</td>
<td>Wine/grape must</td>
</tr>
<tr>
<td>ISE 5199, ISE 5200, ISE 5203, ISE 5209, ISE 5210</td>
<td>Lactobacillus parakeferi</td>
<td>Wine/grape must</td>
</tr>
<tr>
<td>ISE 5218</td>
<td>Leuconostoc mesenteroides</td>
<td>Wine/grape must</td>
</tr>
<tr>
<td>ISE 5216, ISE 5217</td>
<td>Lactobacillus brevis</td>
<td>Wine/grape must</td>
</tr>
</tbody>
</table>

* ATCC, American Type Culture Collection; CECT, Collección Española de Cultivos Tipo; ISE, Istituto Sperimentale per l’Enologia.

I (ISE) at Asti, and they were originally isolated from musts or wines from various wine-producing areas in Italy and other countries. The *O. oeni* strains from 110/8 to 113/12 have recently been isolated from wine by us during this screening (Table 1). As positive controls, we used *Lactobacillus* 30a, a histamine- and putrescine-producing strain from the American Type Culture Collection in Manassas, Va. (ATCC 33222) and *L. brevis* CECT 5354 (ATCC 367), which produces tyramine.

*O. oeni* strains were cultured in deMan Rogosa Sharpe (MRS) broth (Merck, Darmstadt, Germany), pH 4.8, supplemented with 100 ml/liter of tomato juice. Strains of the genera *Lactobacillus* and *Pediococcus* were cultured in MRS broth, pH 6.3. All bacteria were incubated at 30°C.

**DNA extraction.** Total bacterial DNA was extracted as follows: 2 ml of culture in exponential phase was harvested by centrifugation (15 min, 13,400 x g). The pellet was then resuspended in 600 μl of TE (10 mM Tris-HCl and 1 mM EDTA) containing lysozyme (10 mg/ml) and incubated at 37°C for 30 min. One volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added, and the mix was centrifuged for 15 min at 13,400 × g. The upper phase was collected and precipitated with 99% ethanol. The dry pellet was resuspended in an appropriate volume of TE solution.

**PCR conditions.** To detect the hdc gene, we initially followed the protocol of Le Jeune et al. (17) with the primer pair CL1-JV17HC, which amplifies a 500-bp DNA fragment. We then designed a new primer pair using the hdc sequence of *O. oeni* (7), it is named PHDC1 (5’ CCGTGCCGAAACAAAAGAT 3’) and PHDC2 (5’ CCAAAACCCAGCATCTTCA 3’), and it amplifies a 497-bp fragment.

To detect the tdc gene, the primer pair P1 and P2 (20) was used to amplify an 800-bp fragment. Subsequently, we designed a primer pair based on *L. brevis* and *Enterococcus faecalis* nucleotide sequences; these primers were named Pt3 (5’ TACACGTAAGTGGCTGATATG 3’) and Pt4 (5’ ATGGTGCTAATGTTTTAAAAGAA 3’), and they amplify a fragment of ca. 560 bp.

According to our search of the literature (23), primers 3 and 16 are the only primers that detect the odc gene by PCR. We designed two new primers, which were chosen by aligning nucleotide sequences of odc from *Lactobacillus* 30a (U111816) and *O. oeni* (AJ461665) in two different conserved domains; they were named AOCD1 (5’ GTCGTGAAATYGAARCKG 3’) (shown in Fig. 1) and AOCD2 (5’ KGRGTTCMGCYGGRGTCAT 3’). PCR assays were performed with various combinations of all primers, various concentrations, and various annealing temperatures.

PCR amplifications were performed in a 20-μl mixture containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgCl₂, 2 μM deoxynucleotide triphosphate, and 0.5 U of Taq DNA polymerase (Sigma, St. Louis, Mo.), along with 0.4 μM of the primers (MWG, Ebersberg, Germany) for the tdc and hdc genes and 1 μM of primers for the odc gene.

Thirty cycles of PCRs were performed with an i-cycler (Bio-Rad Laboratories, Richmond, Calif.). The following conditions were used: DNA denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min. Amplification products were visualized on 1.2% agarose gel (Sigma) made with Tris-acetate buffer and stained with ethidium bromide.

**DNA sequencing.** PCR products were sequenced at the Chemical Laboratory of the Camera di Commercio in Turin, Italy. Sequencing was performed both to verify the identity of the amine-producing strains found (16S rDNA by means of the uni-
versal primers 63f and 1387r (22) and to confirm that the amplification products corresponded to the genes studied. Sequence similarity searches were carried out by means of the Basic Local Alignment Search Tool on the National Center for Biotechnology Information databases.

**TLC conditions.** In TLC assays, we used the method of García-Moruno et al. (12). *O. oeni* strains were grown at 30°C in MRS broth, pH 4.8, supplemented with 100 ml/liter of tomato juice, and *Lactobacillus* and *Pedococcus* were grown at 30°C in MRS broth, pH 6.3. The broths were supplemented with biogenic amine precursor amino acids such as histidine (5 mg/ml), ornithine (5 mg/ml), and tyrosine (5 mg/ml). Samples taken at 3, 6, 9, and 12 days of growth were analyzed. Amines were converted to their fluorescent dansyl derivatives as follows: 1 volume of 250 mM NaH₂PO₄, 0.1 volume of 4 N NaOH, and 2 volumes of dansyl chloride solution (5 mg/ml of dansyl chloride in acetone) were added to 1 volume of the sample. The mixture was vortexed and incubated at 55°C for 1 h in the dark. The amines were fractionated on silica gel plates (silica gel 60 F₂₅₄, Merck) with a solution of chloroform-triethylamine (4:1) as the mobile phase. The spots were visualized under UV light.

**HPLC conditions.** HPLC analysis was performed by a Hewlett-Packard model 1100 (Hewlett-Packard, Palo Alto, Calif.) with a fluorometric detector. We subjected the samples to an automatic precolumn derivatization procedure using o-phthalaldehyde (OPA Reagent, Agilent Technologies, Palo Alto, Calif.). All separations were performed on an Alltima C₁₈ column, with a 5-μm-thick film, measuring 200 by 4.6 mm (Alltech, Deerfield, Ill.), with a matching guard cartridge (7.5 by 4.6 mm). Mobile phases were 200 ml/liter of 0.1 M phosphate buffer (pH 7.0) and 200 ml/liter of methanol. A 1-h gradient program began with an initial concentration of 10% of eluent B at a flow of 0.450 ml/min and terminated with 100% of eluent B at a flow of 0.700 ml/min. Fluorescence wavelengths for excitation and emission were 340 and 450 nm, respectively. Quantification of the biogenic amines was performed with an internal standard of 15 mg/liter of norvaline solution.

**RESULTS**

**PCR detection of histamine-producing strains.** PCR was useful for rapidly identifying bacteria that carried the *hdc* gene. Two primer sets were used to amplify internal *hdc* sequences. To standardize the technique (to find the best temperature and primer concentration), we used the DNA extracted from *Lactobacillus* 30a. For the primer pair CL1JV17HC, only *Lactobacillus hilgardii* ISE 5211 was positive for the *hdc* gene, besides *Lactobacillus* 30a (positive control) (data not shown). All *O. oeni* strains studied were negative.

The study was repeated with the primer pair PHDC1 and PHDC2, which is more specific for *O. oeni* on the basis of its *hdc* nucleotide sequence. The results confirmed the preceding data: only *L. hilgardii* ISE 5211 was positive besides the positive control. Thus, of 133 strains of lactic acid bacteria isolated from wine, only ISE 5211 was positive for the *hdc* gene by PCR analysis (Fig. 2). Histamine production was confirmed through TLC analysis of the broth medium enriched with histidine (Fig. 3). HPLC determined that ISE 5211 produced 3.5 g/liter of histamine under the conditions described. Samples taken at no growth (*t = 0*) were analyzed by HPLC, and the resulting chromatogram shows the presence of histidine in the medium (Fig. 4a). An HPLC sample that was run on day 9 of growth (Fig. 4b) shows the presence of a histamine peak and the absence of a histidine peak. This proves that *L. hilgardii* ISE 5211 consumes histidine to produce histamine, which thus confirms the PCR results.

**PCR detection of tyramine-producing strains.** The same protocol was applied for detecting strains containing the *tdc* gene. We used the primer pairs P1 and P2 and P3 and P4 (the latter designed by us). The results obtained with the primer set P1-P2 were the same as those obtained with the primer set P3-P4.
with Pt3-Pt4; screening the same 133 strains of bacteria, only four yielded positive results: ISE 5033, ISE 5197, ISE 5198, and ISE 5202 (Fig. 2). All of these were strains of L. brevis, as confirmed by sequencing of the 16S gene.

The tyramine produced by these strains, grown in MRS with the precursor amino acid, was quantified by HPLC. We studied different primer combinations, evaluating band intensity and the absence of nonspecific fragments. Optimal results were obtained with the primer pair AODC1 and 16, which amplified a 1.7-kb fragment; the optimal concentration of these primers in the reaction mix was 1 μM.

The result of PCR screening of the 133 strains showed that none was odc positive. These results also were confirmed by TLC and HPLC analysis.

**DISCUSSION**

In this article, we have detailed our study of the production of biogenic amines by lactic acid bacteria that had been isolated from wine and must through molecular analysis. Every gene was examined with two primer sets, optimizing concentrations and temperatures for the primers. In addition, we analyzed the media broth by TLC and HPLC, with the goal of identifying possible gene products that were formed. In all cases, analysis of the broth confirmed the results obtained by PCR.

On the basis of our results, biogenic amine-producing strains. Gel electrophoresis did not show any band of the expected dimensions (1.5 kb), except for the positive control; however, nonspecific bands were observed and were eliminated only with higher annealing temperatures.

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strains isolated from red wine are very rare and seem to be exceptional. Furthermore, our results are based on their growth in broth media, and it should be confirmed that the few amine-producing strains we isolated can also produce biogenic amines when inoculated in sterile red wine.

With histamine—the most dangerous biogenic amine because of the toxicological effects derived from its vasoactive and psychoactive properties (16)—only L. hilgardii ISE 5211 possessed the hdc gene and could potentially produce histamine. No other Lactobacillus strain and no O. oeni strain among those tested could produce histamine. Our data agree with Straub et al. (31), who found no amine producers among 88 strains of O. oeni, and with Moreno-Arribas et al. (26), who screened 38 strains of O. oeni by HPLC and found no histamine production in their cultures. Recently, Lucas et al. (21) demonstrated that the hdc gene is located on an unstable plasmid and that lactic acid bacteria could lose the ability to produce histamine, depending on culture conditions. This may explain the negative results; however, L. hilgardii ISE 5211 hdc positive did not seem to lose this characteristic under our culture conditions or after storage.

With tyramine, we found that all L. brevis strains analyzed (ISE 5033, ISE 5197, ISE 5198, and ISE 5202) possessed the tdc gene and produced tyramine, but none of the other lactic acid bacteria analyzed possessed this gene. TDC activity was the focus of the study of L. brevis (IOEB 9808 and ATCC 367) by Moreno-Arribas and Lonvaud-Funel (24), and the tdc gene of L. brevis IOEB 9808 was sequenced by Lucas and Lonvaud-Funel (20). To determine whether tyramine might be produced by other lactic acid bacterial species, Moreno-Arribas et al. (27) investigated amine-forming capacity in a number of different strains belonging to the genera Oenococcus, Pediococcus, and Lactobacillus. From a total of 60 strains, these authors found that only three L. brevis (ATCC 367, IOEB 8511, and IOEB 8907) and one L. hilgardii strain possessed TDC activity.

Finally, no lactic acid bacteria among those studied possessed the odc gene. Guirard and Snell (14) studied ODC activity in Lactobacillus 30a, and Hackert et al. (15) studied its gene. In a recent article, Marcobal et al. (23) sequenced the odc gene in the O. oeni strain BIFI 83 and, after screening 42 strains, found that it was the only putrescine producer. To our knowledge, there are no other references to the presence of the odc gene in lactic acid bacteria, although Gloria et al. (13), Soufleers et al. (30), and Vazquez-Lasa et al. (35) reported that putrescine was the most prevalent amine in wines. They found it in almost 100% of the wines analyzed.

We conclude that there are very few amine-producing lactic acid bacteria and that, with respect to O. oeni, the prevalent species occurring in wines once malolactic fermentation is finished (18), none of the strains studied are producers of amine. We therefore believe that the problem with the production of biogenic amines in wine by ecological lactic acid bacterial strains may be associated with the geographical origin of these bacteria; in fact, most of the strains analyzed were isolated from Italian wines. It may also be possible that biogenic amine formation in wine is due to spoilage bacteria and is associated with a lack of hygiene during the winemaking process.

ACKNOWLEDGMENT

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REFERENCES


FIGURE 4. Example of an HPLC chromatogram of the MRS medium with the addition of the precursor amino acid histidine and inoculated with L. hilgardii ISE 5211. This chromatogram has been processed by the analytical procedure described in “Materials and Methods.” (a) Sample taken at no growth (t = 0): a histidine peak is present in the elution profile. (b) Sample taken on day 9 of growth at 30°C: the histidine peak is absent; however, a histamine peak is present in the elution profile.