Identification of the ornithine decarboxylase gene in the putrescine-producer *Oenococcus oeni* BIFI-83

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Abstract

We report here the identification of an ornithine decarboxylase (ODC) gene in the putrescine-producer *Oenococcus oeni* BIFI-83 strain. The gene contains a 2,235-nucleotide open reading frame encoding a 745-amino acid residues protein with a deduced molecular mass of 81 kDa. The primary structure of the ODC deduced from the nucleotide sequence has a consensus sequence containing the pyridoxal-5-phosphate (PLP) binding domain, and the critical amino acids residues involved in enzymatic activity are also conserved. As determined by BLAST analysis, the deduced amino acid sequence of the *odc* gene shares a 67% identity with the ODC protein from *Lactobacillus* 30a. The *odc* gene appears to be rarely present in the genome of *O. oeni*, since in a screening for the presence of this gene in 42 oenococcal strains none of the strains possessed an *odc* gene copy.

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1. Introduction

Biogenic amines have been implicated in food poisoning incidents, usually from the consumption of fermented foods like cheese, meat, fish products and wine [1]. Wines are very selective media, which can support growth of only few species of lactic acid bacteria, mainly *Oenococcus oeni* and several lactobacilli species. In wine, several amino acids can be decarboxylated and as a result biogenic amines are usually found. The presence of biogenic amines in wine is of interest from a toxicological point of view. It is assumed that histamine is the causative agent of physiological distress experienced by sensitive individuals following ingestion of red wine. The symptoms commonly reported include intense headache, rashes, nausea, facial flushing, thirst, sore throat, itching, swelling, diarrhoea, and vomiting. However, reports have also demonstrated that tyramine, tryptamine, and 2-phenylethylamine may play an important role as well as the simultaneous presence of potentiating compounds in wines, such as ethanol, and polyamines, such as putrescine [2]. In wines, putrescine is the most prevalent amine, being found in almost the 100% of the wines analysed [3–5]. Putrescine concentration in wine ranged from 0.8 to 200 mg/l [6].

Biogenic amines are mainly formed by decarboxylation of the corresponding amino acids by microorganisms through substrate-specific decarboxylase enzymes.
These enzymes are generally induced at acidic pH and hence have a possible role in maintaining pH homeostasis or extending the growth period by detoxification of the extracellular medium [7]. Amino acid sequence analysis suggested multiple evolutionary origins for four distinct groups of decarboxylases [8]. Group I consisted of the glycine decarboxylases; Group II the glutamic acid, histidine, and dopa-like decarboxylases; Group III the bacterial ornithine, lysine, and arginine decarboxylases; and Group IV the eukaryotic ornithine plus arginine and diaminopimelate decarboxylases. On the basis of sequence database [8].

Ornithine decarboxylase (ODC, EC 4.1.1.17) is a PLP-dependent enzyme which catalyses the conversion of ornithine to putrescine at the beginning of the polyamine pathway. Many bacteria contain two forms of ODC, a biosynthetic or constitutive, and a biodegradative or inducible form. In Escherichia coli, biosynthetic ODC is constitutively expressed by all strains of E. coli grown in minimal media at a neutral pH; in addition, a biodegradative form of ODC is induced in ≈10% of E. coli strains grown in rich media at low pH where it plays a role in regulating intracellular pH [9]. Among lactic acid bacteria, only a biodegradative ODC in Lactobacillus 30a had been described which is induced under low-pH conditions in rich media to compensate for the pH drop associated with lactic acid production [7].

It is generally assumed that the capability of biogenic amine formation seems to be strain dependent rather than being related to specific species [10,11]. In this sense, the isolation of the putrescine-producing O. oeni IOEB 8419 from a ropy red wine has been described [12]. This strain seems to possess ornithine decarboxylase activity and requires pyridoxal-phosphate as cofactor. Later, in a study to check the biogenic amine-producing capability of 44 O. oeni strains, Guerrini et al. [13] reported seven O. oeni strains able to produce putrescine in culture media. In a recent study in our laboratory (unpublished results), a putrescine-producing O. oeni strain was isolated from lees of a wine with high concentration of putrescine. In this work, we reported for the first time an odc gene present in a O. oeni strain. It need to keep on mind that high levels of putrescine in wine may be of health concern since it is well known that putrescine can potentiate the action of histamine.

2. Materials and methods

2.1. Bacterial strains and growth conditions

O. oeni BIFI-83 has been isolated from lees of a Spanish wine (unpublished results). The lees were grown in medium for Leuconostoc oenos (MLO) (ADSA, Spain) [14] containing 0.1% (w/v) of histidine, tyrosine and ornithine in order to supply the media with the corresponding amino acid for the formation of biogenic amines (histamine, tyramine and putrescine, respectively). Strains were incubated at 30 °C in a 5% CO2 atmosphere.

O. oeni BIFI-83 was isolated as the only putrescine-producer strain from the lees. Forty-two additional O. oeni strains obtained by the Spanish Type Culture Collection (CECT) and from the bacterial culture collection of the Instituto de Fermentaciones Industriales (IFI, CSIC, Spain) were tested for the presence of an odc gene.

2.2. Putrescine determination

Putrescine was determined by reverse-phase high performance liquid chromatography (RP-HPLC) as previously described [15]. A Waters (Waters, Milford, MA) liquid chromatograph consisting of a Waters 600 Controller programmable solvent module, a WISP 710B autosampler and a Waters 420 fluorescent detector was used. Chromatographic data were collected and analysed with the Millenium32 system (Waters, Milford, MA). Samples were submitted to an automatic precolumn derivatization with o-phthalaldehyde (OPA), prior to injection. The reaction solution consisted of 350 mg of OPA and 2.5 ml 2-mercaptoethanol in 47.5 ml methanol. All separations were performed on a Waters Nova-Pak C18 column (150 × 3.9 mm i.d., 60 Å, 4 μm) with a matching guard cartridge of the same type. Derivatized amines were detected using a fluorescence detector (excitation wavelength of 340 nm and emission wavelength of 425 nm). Samples were injected in duplicate onto the column after being filtered through a 0.45 μm filter (Millipore, Bedford, MA, USA).

2.3. DNA manipulations

Restriction endonucleases (Roche Diagnostics, GmbH), T4 DNA ligase (New England Biolabs, Inc.), and the Klenow fragment of DNA polymerase (New England Biolabs, Inc.) were obtained commercially and used according to the recommendations of the suppliers. Gel electrophoresis of restriction fragments and PCR products were carried out in agarose gels as described [16]. DNA was digoxigenin-labeled and chemiluminescently detected by using the DIG High Prime DNA labelling and detection Starter Kit (Roche Diagnostics, GmbH) according to the manufacturer’s instructions.

PCR amplifications were performed in 25-μl amplification reaction mixture containing 12.5 ng of template DNA, 20 mM Tris–HCl, pH 8.0, 50 mM KCl, 2.5 mM MgCl2, 200 of each dNTP, 1 μM of each primer, and 1 U of AmpliTaq Gold™ or Pfu DNA polym-
were: 3 (5′-GTNTTYAYACNGAYAACRACNTAYTTGTYG) and 16 (5′-AATCRAATATCTCGNNNGGRTANGG) (N = A, C, G or T; Y = C or T; R = A or G). Primers used for the PCR performed at annealing temperature of 36 °C were A22 (5′-ATGGACACCA) and OPA20 (5′-AGTTGCGATCC), previously described for RAPD analyses, [17] and the specific primer 125 (5′-CATTGAACCTCGTGCTGCATG). For inverse PCR we used: primer 111 (5′-CTTTGCAATCTTTTCGAAATAGAC) and primer 112 (5′-AAGAATATCAGAAGGAGATGTTCTTGG) for the 1.3-kb HaeIII DNA fragment.

To confirm the taxonomic position of the putrescine-producer BIFI-83 strain, the 16S rDNA was PCR amplified using the eubacterial universal pair of primers 63f (5′-CAGGCCTAAACATGCACCTGCAAGTC) and 1387r (5′-GGCCGGGTGTACAAAGGC) (W = A or T) previously described [18]. The 63f and 1387r primer combination generates an amplified DNA fragment of 1.3-kb.

To locate the gene involved in the putrescine production of O. oeni BIFI-83 an 1.4-kb internal odc fragment was amplified by PCR using primer 3 and 16. These primers were designed based on two conserved domains shown by the alignment of amino acid sequences of ODC proteins [Marcobal et al., unpublished]. The 1.4-kb DNA fragment was sequenced and similarity searches showed that it contains an incomplete odc gene sequence. To sequence the 5′ end and the 3′ end, this 1.4-kb internal DNA fragment was used as probe. Southern blot hybridizations (not shown) indicated that the 1.4-kb fragment was included in a 3.0-kb ClaI, in a 3.2-kb CfoI and in a 1.3-kb HaeIII DNA fragments. To amplify the ClaI, CfoI and HaeIII fragments, primers 111, close to the 5′ end of the 1.4-kb fragment and primer 112, located close to the 3′ end, were used. Attempts to amplify the 3.0-kb ClaI and 3.2-kb CfoI DNA fragments by inverse PCR were unsuccessful. However, the 1.3-kb HaeIII fragment gave a positive amplification. Sequence analysis indicated that this DNA fragment contained the 3′end of the gene. To clone the 5′end of the odc gene, several PCR experiments were performed by using unspecific conditions such as a low annealing temperature (36 °C) and the use of only one specific primer or a combination of an specific with an unspecific primer (A22 or OPA20). By using these conditions with the specific primer (primer 125) and the unspecific primer A22, a major PCR product of about 800-bp appeared, corresponding to the chromosomal region upstream of odc. A total of 2,372-bp DNA sequence from O. oeni BIFI-83 was obtained.

DNA sequencing was carried out by using an Applied Biosystems, Inc. mRNA sequencing was carried out using Basic local alignment search tool (BLAST) [19] on the EMBL/GenBank databases. Computer promoter predictions carried out at the internet site http://www.fruitfly.org/cgi-bin/seq_tools/promoter.html. Signatures, pI/MW, etc. were analysed on EXPASY (http://www.expasy.ch) site and multiple alignment was done using CLUSTAL W on EBI site (http://www.ebi.ac.uk) after retrieval of sequences from GenBank and Swiss-Prot. Phylogenetic trees and RNA secondary structure predictions were carried out by the GeneBee program (http://www.genebee.msu.su/genebee.html).

3. Results and discussion

3.1. Putrescine production by O. oeni BIFI-83

Our hypothesis for the research was that wine lees contains various kinds of bacteria that are capable of decarboxylate one or several amino acid. To check this hypothesis, isolated bacterial strains were cultivated in MLO media containing the amino acid precursors of the biogenic amines histamine, tyramine and putrescine. After five days incubation, the cultures were filtered through a 0.45 µm filter and analyzed for biogenic amines. A control corresponding to MLO medium without inoculation was also included. Results are shown in Fig. 1. A typical O. oeni strain, as O. oeni CECT 4028 (Fig. 1(d)), produced no amines, however, O. oeni BIFI-83 produced putrescine (Fig. 1(c)). Putrescine was the only amine produced by O. oeni BIFI-83 that did not possess other decarboxylase activity.

Since BIFI-83 was only presumptively identified as O. oeni by microscopic and biochemical test we confirm its taxonomical identity by sequencing a 1.3-kb DNA fragment coding for the 16S rRNA. The bacterial isolate identified as being putrescine-producer was then identified using sequence data from 1.3 kb of the 16S rRNA gene. The sequence obtained was identified by database comparison (BLAST search) using the GenBank nucleotide database [19]. This isolate contained a sequence identical to that of O. oeni strains present in the databases, including the type strain O. oeni ATCC 23279. Therefore, the putrescine-producer strain BIFI-83 unequivocally belongs to the O. oeni species.

3.2. Analysis of the ODC gene sequence

The complete odc gene was included in a 2,372-bp DNA fragment. Putative consensus regulatory elements were localized on the 5′ DNA sequence (Fig. 2). The
deduced product of *odc* is a protein of 745 amino acid residues. The TGA stop codon was followed by a putative transcription terminator since there is a possible stem-loop structure which begins 17 nucleotides downstream from the TGA stop codon which has a 10-base stem and a 9-base loop. This structure may serve as a terminator for transcription. Preliminary data clearly indicate that this 2,372-bp DNA fragment was capable of reverting an *E. coli* mutant which has an absolute putrescine requirement for growth (not shown). This result seems to confirm that an ornithine decarboxylase protein is encoded by this *odc* gene.

A gene responsible for product transport across the membrane was found adjacent to genes encoding ODCs

![Graph](image1.png)

**Fig. 1.** Putrescine production by *O. oeni* strains: (a) Chromatographic separation of seven biogenic amines in standard solution containing histamine, tyramine, phenylethylamine, methylamine, ethylamine, putrescine, and cadaverine. (b) Culture media chromatogram. (c) *O. oeni* BIFI-83. (d) *O. oeni* CECT 4028 grown in the culture media showed in (b).

![Graph](image2.png)

**Fig. 2.** Nucleotide sequence of 240-bp fragment of the putrescine-producer *O. oeni* BIFI-83, containing the 3’end of the *odc* gene. The strand corresponding to the mRNA sequence is shown. Nucleotides are numbered by taking the first nucleotide in the AJ746165 sequence as nt 1. Putative consensus regulatory elements are underlined and typed in boldface. The putative transcription start is shown on a black background. Dashed arrows represent a probable transcription terminator hairpin.
in *Lactobacillus johnsonii* (NC_005362) and in *Lactobacillus gasseri* (NZ_AAA02000021). In both strains, putative amino acid transporters were localized upstream and separated by \(250\)-bp from the gene coding for a putative ODC. In *L. johnsonii* and *L. gasseri*, both genes appear to be divergently transcribed. The nucleotide upstream and downstream sequences from the *O. oeni BIFI-83* *odc* gene were searched for a similar transport protein gene, but none was found over the range sequenced (151 bases 5' of *odc* and 134 bases 3' of *odc*). In a similar way, no amino acid transporter protein was found in *Lactobacillus 30a*.

### 3.3. Homology of the translated *O. oeni* ODC sequence with other selected ODCs

The deduced product of *O. oeni* ODC is a protein of 745 amino acid residues and 81 kDa and pI of 5.50.

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**wing domain**

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<th>LJO</th>
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<td><em>γT-DV</em></td>
<td>SSS<em><strong>D</strong></em></td>
<td>M<em><strong>F</strong></em></td>
<td>L<em><strong>I</strong></em></td>
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**PLP-binding domain**

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<td><em>γT-NN</em><strong>G</strong>*</td>
<td>M<em><strong>E</strong></em></td>
<td>L<em><strong>I</strong></em></td>
<td>L<em><strong>I</strong></em></td>
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**Fig. 3.** Comparison of protein sequence of ODC from *O. oeni* (OEN), *Lactobacillus 30a* (L30), *H. influenzae* (HIN), *L. gasseri* (LGA), and *L. johnsonii* (LJO). Clustal W program was used to compare predicted sequences. Residues of *Lactobacillus 30a* ODC involved in PLP-binding are typed in boldface and are underlined in the *O. oeni* sequence. Residues involved in the association of dimmers into dodecamers are typed in boldface. Vertical dashed lines indicated the boundaries of the different domains described in *Lactobacillus 30a* ODC. Asterisks, amino acid identity; dashed, gaps introduced to maximize similarities. The sequence from *O. oeni* BIFI-83 was deposited in GenBank under the Accession No. AJ746165.
BLAST databases searches of the translated O. oeni DNA sequence identified high-scoring similarities with a family of pyridoxal phosphate-dependent amino acid decarboxylase protein sequences that act on lysine, arginine or ornithine. The predicted sequence of the O. oeni BIFI-83 ODC protein was aligned with selected PLP-dependent decarboxylases in the databases. The alignment of some ODC protein sequences that had the highest overall identity with that of O. oeni is shown in Fig. 3. The highest sequence identity (67%) was shown between O. oeni and Lactobacillus 30a. Surprisingly, Lactobacillus 30a ODC showed lower identity with similar proteins found in other members of the Lactobacillus genera (44% with L. jhonsii and L. gasseri, respectively) than with enzymes from unrelated microorganisms such as Haemophilus influenzae (62%), Pasteurella multocida (59%), Shewanella oneidensis (59%), and also from E. coli (57%). Phylogenetic trees were constructed to precisethe evolutionary relationships between these proteins. As shown in Fig. 4, the distribution of ODC proteins did not follow the phylogeny of their hosts. These proteins made two clusters clearly separated suggesting that L. gasseri and L. jhonsii ODC proteins evolved together and separately from the other ODC proteins analysed, including O. oeni BIFI-83 and Lactobacillus 30a.

It is noteworthy, the extra 19-amino acid residues N-terminal tail present in the O. oeni ODC protein as compared with all the available ODC protein sequences.

Fig. 3 (continued)
tail had been removed. This might be due that the Met residue at position 21 had been considered the first amino acid of the protein. Similarly, in O. oeni, alternative ATG or GTG start codons are not available (20 amino acid residues per subunit[21]). The homodimers have one wall of a 27 Å deep channel at the dimmer interface leading to the active site [22]. As showed in Fig. 3 absolute conservation among substrate-binding domains and also in amino acid residues involved in PLP-binding and in the association of dimmers into decamers, were found for O. oeni BIFI-83 ODC and Lactobacillus 30a ODC.

3.4. Presence of an odc gene among oenococcal isolates

In a recent study, we have analysed the production of biogenic amines in a collection of lactic acid bacteria of various origins, including commercial malolactic starter cultures, type strains and 78 strains isolated from Spanish grape must and wine [15]. The presence of putrescine in a decarboxylase synthetic broth was determined by reverse-phase high performance liquid chromatography. In that previous study, no potential to form biogenic amines was observed in 42 O. oeni strains analyzed. Since, these oenococal non-producer strains could have a mutated version of the odc gene, we studied the presence of this gene among 42 oenococcal strains. To determine the odc gene presence we extracted chromosomal DNAs from the 42 strains and PCR amplified. A 1.1-kb gene fragment was PCR amplified using a pair of oligonucleotides designed on the basis of the O. oeni BIFI-83 odc gene sequence. We used O. oeni BIFI-83 DNA as positive control. None strain amplifies the 1.1-kb DNA fragment.

The synthesis of putrescine in E. coli involves either decarboxylation of (i) arginine by arginine decarboxylase to produce agmatine, agmatine is then hydrolized by agmatine ureohydrolase; or (ii) ornithine by ornithine decarboxylase. Amino acids are naturally present in grapes [23]. Arginine is the most abundant amino acid found in wines and its concentration decreases significantly during malolactic fermentation. However, ornithine is one of the less abundant amino acids in wine, and during malolactic fermentation an increase in the concentration of ornithine, a precursor of putrescine, is noted, along with a higher concentration of the latter substance [6]. This situation can be explained by the fact that an alternative pathway of synthesis exists for putrescine, resulting from arginine [24]. According to recent studies [25], O. oeni strains posses the arginine deiminase pathway which produces ornithine and ammonia from arginine. Therefore, it should be stated that arginine may be at the origin of ornithine and putrescine. As result of the metabolism of O. oeni possessing ODC activity, arginine could be converted to ornithine by the arginine deiminase pathway, and the latter substance metabolised into putrescine by the action of the ODC.
In summary, even though the toxicological significance of putrescine in wine is still not well established, it is prudent to prevent any avoidable formation and accumulation in wine. Therefore, the sources for amine formation during wine manufacture should be determined in order to limit its formation and accumulation. In this sense, we have identified a putrescine-producer *O. oeni* strain, belonging to a species that perform malolactic fermentation of wines, and sequenced its ornithine decarboxylase gene. We have also shown that the presence of a *odc* gene is a rare event in Spanish wine *O. oeni* strains.

Acknowledgements

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