

High Frequency of Histamine-Producing Bacteria in the Enological Environment and Instability of the Histidine Decarboxylase Production Phenotype[∇]

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Lactic acid bacteria contribute to wine transformation during malolactic fermentation. They generally improve the sensorial properties of wine, but some strains produce histamine, a toxic substance that causes health issues. Histamine-producing strains belong to species of the genera *Oenococcus*, *Lactobacillus*, and *Pediococcus*. All carry an *hdcA* gene coding for a histidine decarboxylase that converts histidine into histamine. For this study, a method based on quantitative PCR and targeting *hdcA* was developed to enumerate these bacteria in wine. This method was efficient for determining populations of 1 to 10⁷ CFU per ml. An analysis of 264 samples collected from 116 wineries of the same region during malolactic fermentation revealed that these bacteria were present in almost all wines and at important levels, exceeding 10³ CFU per ml in 70% of the samples. Histamine occurred at an often important level in wines containing populations of the above-mentioned bacteria. Fifty-four colonies of histamine producers isolated from four wines were characterized at the genetic level. All were strains of *Oenococcus oeni* that grouped into eight strain types by randomly amplified polymorphic DNA analysis. Some strains were isolated from wines collected in distant wineries. Moreover, *hdcA* was detected on a large and possibly unstable plasmid in these strains of *O. oeni*. Taken together, the results suggest that the risk of histamine production exists in almost all wines and is important when the population of histamine-producing bacteria exceeds 10³ per ml. Strains of *O. oeni* producing histamine are frequent in wine during malolactic fermentation, but they may lose this capacity during subcultures in the laboratory.

Lactic acid bacteria (LAB) used in food fermentation may produce histamine, a biogenic amine that accumulates in foods and causes diverse health issues when large quantities are ingested (28, 30, 31). Histamine results from the decarboxylation of the natural amino acid histidine catalyzed by a specific enzyme, the histidine decarboxylase (HDC). The gene *hdcA* coding for HDC is associated with other genes coding for a histidine/histamine exchanger and accessory proteins (14, 16). The coupled reactions of HDC and the exchanger promote the uptake of extracellular histidine, its conversion into histamine, the release of histamine and CO₂ out of the cell, and importantly, the consumption of a cytoplasmic proton. The HDC pathway might help bacteria to regulate their cytoplasmic pH in acid environments or provide metabolic energy via the activity of the ATP synthase (19). LAB producing histamine (HDC⁺ LAB) belong to diverse species, but these species comprise both HDC⁺ and HDC⁻ strains. In the HDC⁺ strain *Lactobacillus hilgardii* IOEB 0006, *hdcA* was detected on an unstable plasmid, which could explain the erratic phylogenetic distribution of HDC⁺ strains (14).

During winemaking, LAB are used to improve the organoleptic properties of wine, mainly by converting the malic acid that is present in grape juice and characterized by a strong acidic taste into the softer lactic acid (11). The conversion occurs during a phase of winemaking named malolactic fer-

mentation, when the population of LAB in wine exceeds 10⁶ CFU · ml⁻¹. LAB growing in wine belong to the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Oenococcus*. The species *Oenococcus oeni* is generally predominant in wines undergoing spontaneous malolactic fermentation, i.e., fermentation performed by LAB naturally present in wine. *O. oeni* is also the species that is used industrially to inoculate wine in order to perform this fermentation under controlled conditions (11). Histamine is often detected in wines but at a rather low concentration compared to that of other fermented foods, such as cheese (29). However, wine contains alcohol and other substances that increase the toxicity of histamine. Therefore, the recommended upper limits of histamine in wine range from 1 to 10 mg · liter⁻¹ (8). It is well established that histamine is produced by LAB during malolactic fermentation and the ensuing storage period (12, 14). Many HDC⁺ strains of the genera *Oenococcus*, *Lactobacillus*, and *Pediococcus* were isolated from wine, but it is still not well determined whether these bacteria are frequent and abundant or not during winemaking (2, 7, 9, 13, 20). The role of *O. oeni* in histamine formation is also unclear. Independent works suggest that HDC⁺ strains of this species are frequent or rare in wine, and they are considered either weak or strong histamine producers (2, 6, 7, 13, 20).

Real-time quantitative PCR (QPCR) is an efficient technique used to detect and count microorganisms in foods (for a review, see reference 27). During the past few years, diverse methods based on QPCR were proposed to determine populations of yeasts and bacteria in wine (3, 18, 21, 23, 24). Interestingly, a method was recently proposed to determine HDC⁺ LAB in cheese (4). The present study was initiated with the

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aim of analyzing the populations of HDC⁺ LAB present in wine during malolactic fermentation. A method involving QPCR was developed. It was used to analyze 264 samples collected in numerous wineries at the end of malolactic fermentation. Unexpectedly important populations of these undesirable bacteria, comprising between 10³ and 10⁷ · ml⁻¹, were often detected. The involved bacteria were characterized at the species and strain levels and a correlation was made between populations of HDC⁺ LAB and concentrations of histamine in wine.

MATERIALS AND METHODS

Bacteria, growth conditions, and identification. LAB were isolated on agar-solidified de Man, Rogosa, and Sharpe (MRS) broth supplemented with 100 mg · liter⁻¹ pimaricin at 25°C in an anaerobic atmosphere. LAB were propagated in liquid MRS broth. LAB species were identified by species-specific PCR tests using primers matching the *rhoB* gene (26). *O. oeni* strain typing was carried out by using multiplex randomly amplified polymorphic DNA (RAPD)-PCR (25). Eight HDC⁺ *O. oeni* strains isolated during this work, designated strains A to H, were stored in the IOEB collection under reference numbers IOEB 0605 to IOEB 0612, respectively.

Wine samples. All of the 264 samples used in this study were red wines produced in 116 wineries of the Bordeaux, France, area during the 2005 vintage. They were collected at or close to the end of the malolactic fermentation, before SO₂ was added. Usually commercial starters are not used for malolactic fermentation in these wines, but this information was not known for some wines.

DNA purification. Genomic DNA of bacteria grown in MRS broth was prepared by using the Wizard genomic DNA purification kit (Promega) according to the manufacturer's instructions. DNA of microorganisms present in wine was purified by a previously reported method (5) that was modified as follows. Microorganisms of a 10-ml sample of wine were collected by centrifugation at 5,300 × g for 15 min. The pellet was washed once with 1 ml of Tris-EDTA buffer (10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA) and resuspended in 300 μl of the same buffer. Cells were disrupted with 200 μl of 0.1-mm-diameter glass beads in a FastPrep FP120 instrument (MP Biomedicals) used at a power level of 6.5 during five cycles of a 45-s disruption interspaced with 1 min of cooling at 4°C. The cell lysate was mixed with 300 μl of nucleus lysis solution and 200 μl of protein precipitation solution from the Wizard genomic DNA purification kit (Promega) and kept on ice for 5 min. Cell debris and proteins were precipitated by centrifugation for 3 min at 10,000 × g. A volume of 600 μl of supernatant was mixed with 100 μl of 10% polyvinylpyrrolidone molecular-grade solution (Sigma) and centrifuged for 10 min at 10,000 × g. The supernatant was collected and nucleic acids were precipitated in the presence of isopropanol. The DNA pellet was washed once with 70% ethanol, dried, and dissolved in 20 μl of sterile water.

QPCR assay. Primers hdcAf (5'-ATGAAGCCAGGACAAGTTGG) and hdcAr (5'-AATTGAGCCACCTGGAATTG) were designed on the basis of the sequences of *hdcA* genes from *O. oeni* IOEB 9204, *Lactobacillus hilgardii* IOEB 0006, *Lactobacillus sakei* LTH 2076, *Lactobacillus* strain 30A, *Lactobacillus buchneri* DSM 5987, and *Tetragenococcus muraticus* LMG 18498 that were available from databases. This primer set amplifies an 84-bp internal region of *hdcA*. Amplification and detection were performed with 20-μl reaction mixtures containing 10 pmol of each primer, 1 μl of DNA template prepared as mentioned above, and 10 μl of a 2× concentration of iQ Sybr green supermix (Bio-Rad) using an iCycler system (Bio-Rad). Amplification conditions were 5 min at 95°C, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The baseline and cycle threshold (C_T) were automatically calculated. A melt curve analysis was performed with the same equipment after the completion of QPCR. Samples were heated from 65°C to 95°C in 0.5°C increments. The melting temperature of the samples was automatically determined.

Colony hybridization. Detection of HDC⁺ LAB colonies was carried out by colony hybridization as previously described (10). The probe used for hybridization was a mixture of equivalent amounts of PCR products obtained using primers hdc3 and hdc4 specific for a 437-bp fragment of the *hdcA* gene (14) and genomic DNA of the HDC⁺ strains *O. oeni* IOEB 9204, *L. hilgardii* IOEB 0006, *Lactobacillus buchneri* DSM 5987, and *Lactobacillus* strain 30A. The probe was labeled with digoxigenin-11-UTP by using the DIG DNA labeling kit (Roche), and detection was performed by chemiluminescence with an antidigoxigenin antibody and CDP-Star reagent (Roche).

PfGE and Southern blotting. Bacteria grown in MRS broth up to the exponential phase were collected by centrifugation, washed twice in Tris-EDTA

buffer, resuspended in the same buffer adjusted to 100 mM EDTA (T₁₀₀E), and embedded in 1% agarose slices. The bacteria were incubated at 37°C for 8 h in T₁₀₀E containing 10 mg · ml⁻¹ lysozyme and incubated for 16 h in T₁₀₀E supplemented with 1.5% *N*-lauryl sarcosine and 2 mg · ml⁻¹ pronase. NotI digestions were performed by incubating agarose slices at 25°C for 16 h in the presence of 30 U of the enzyme (New England Biolabs). Pulsed-field gel electrophoresis (PFGE) was carried out in a 1% agarose gel with the CHEF-DR III system (Bio-Rad) with pulse times of 1 to 25 s for 20 h at 6 V/cm and 15°C in 45 mM Tris, 45 mM boric acid, and 1 mM EDTA (pH 8). DNA fragments were transferred onto a Hybond-N+ membrane and hybridized with an *hdcA* probe as described previously.

Determination of biogenic amines. Aliquots of wines were centrifuged for 10 min at 10,000 × g. Supernatants were collected, filtered through a 0.2-μm filter (Millipore), derivatized on a precolumn with *o*-phthalaldehyde, and separated by reverse-phase high-performance liquid chromatography (HPLC) on a Waters Nova-Pak C₁₈ column according to reference 22.

RESULTS

QPCR method for determination of HDC⁺ LAB in wine.

The nucleotide sequences of *hdcA* genes from diverse LAB were aligned to identify conserved regions and to design PCR primers specific for these genes. The alignment included *hdcA* genes of two strains isolated from wine, *O. oeni* IOEB 9204 and *L. hilgardii* IOEB 0006, and *hdcA* genes from the following strains originating from cheese, sauerkraut, squid liver sauce, and horse intestines: *Lactobacillus buchneri* DSM 5987, *Lactobacillus sakei* LTH 2076, *Tetragenococcus muraticus* LMG 18498, and *Lactobacillus* strain 30A, respectively. The selected primers bordered an 84-bp DNA region that was the same for *L. hilgardii*, *O. oeni*, *L. sakei*, and *T. muraticus* and slightly divergent for the two other species. Preliminary QPCR assays were carried out using genomic DNA of *L. hilgardii* IOEB 0006 as a template. Optimal QPCR conditions allowed amplification of a PCR product with a melting temperature of 80.5°C ± 0.5°C. The same product was obtained using genomic DNA of other HDC⁺ strains, while no unspecific amplification was detected in the presence of genomic DNA from HDC⁻ bacteria (not shown).

A previously described procedure was used to prepare DNA of microorganisms present in wine (5). Some modifications were made in order to purify the microbial DNA from a 10-ml sample of wine and to obtain a DNA fraction free of wine compounds inhibiting PCR, such as polyphenols. The sensitivity and the efficiency of the QPCR assay were determined by using DNA extracted from nine freshly prepared samples containing 10 ml of filter-sterilized wine, 10⁸ · ml⁻¹ cells of the yeast *Saccharomyces cerevisiae*, and serial dilutions of cells of HDC⁺ *L. hilgardii* IOEB 0006 (10⁷ · ml⁻¹ to 1 · ml⁻¹, plus a control without HDC⁺ cells). The addition of yeasts was used not only to mimic yeast lees present in wine during malolactic fermentation but also to help precipitate the bacteria and to provide a large amount of DNA acting as a carrier during bacterial DNA preparation. Microbial DNA of the nine samples was purified and tested in QPCR. The eight reaction mixtures corresponding to samples containing HDC⁺ cells produced detectable amplification products and measurable C_Ts (Fig. 1). The presence of as few as 1 HDC⁺ cell per ml of wine was detected. In contrast, no amplification occurred in the control reaction performed without HDC⁺ cells. To determine the efficiency of the method, the measured C_T values were plotted against the concentrations of HDC⁺ bacteria initially present (Fig. 1). A linear relationship was obtained for

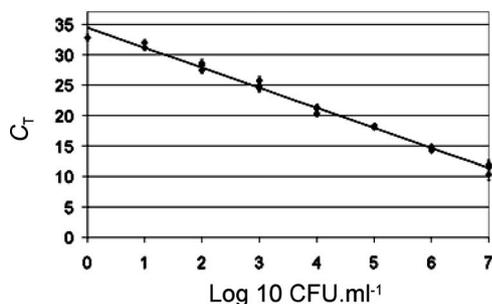


FIG. 1. QPCR standard curve obtained with DNA of HDC⁺ cells serially diluted in sterile wine supplemented with yeasts. C_t values are averages of results from three replicates. The coefficient of correlation (R^2) was 0.996.

LAB populations ranging from 1 to $10^7 \cdot \text{ml}^{-1}$. Therefore, coupling the QPCR assay with the optimized DNA extraction procedure allowed us to determine efficiently populations of HDC⁺ LAB.

Validation of the method with environmental samples. To validate the QPCR method, the populations of HDC⁺ LAB in wines were determined and the results were compared with enumerations performed by colony hybridization. The analyzed samples were red wines collected from several wineries during malolactic fermentation when the population of LAB is above $10^6 \cdot \text{ml}^{-1}$. Determinations carried out by QPCR disclosed some wines containing apparently more than $10^5 \cdot \text{ml}^{-1}$ histamine-producing bacteria (see below). Those wines were preferred for colony hybridization experiments given that the sensibility of this technique requires at least $10^4 \cdot \text{ml}^{-1}$ target bacteria to be present in samples containing $10^6 \cdot \text{ml}^{-1}$ total LAB (at least 1 positive colony on a plate containing 100 colonies of LAB). Aliquots of four wines were spread on plates to grow LAB. Plates containing 100 to 300 colonies were used for colony hybridization with a DNA probe specific for the gene *hdcA*. The populations of HDC⁺ LAB determined by this method and by QPCR were compared (Fig. 2). Both strategies disclosed very similar populations of HDC⁺ LAB, ranging from $3 \times 10^5 \cdot \text{ml}^{-1}$ in wine 1 up to $8 \times 10^6 \cdot \text{ml}^{-1}$ in wine 4, indicating that the method based on QPCR could be employed to determine the presence of HDC⁺ LAB in wines.

Presence of HDC⁺ LAB in wines. The QPCR method was used to analyze 264 wines of the 2005 vintage. All were red wines collected during malolactic fermentation. They origi-

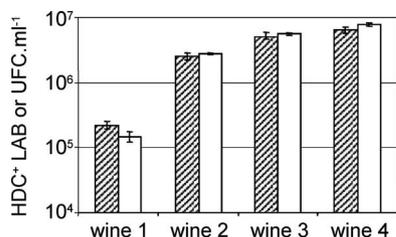


FIG. 2. Comparison of results obtained by QPCR and colony hybridization. The populations of HDC⁺ LAB present in four wines were determined by QPCR (hatched bars) and colony hybridization (empty bars) using a DNA probe specific for the gene *hdcA*. QPCR data are the means of three replicates.

TABLE 1. Populations of HDC⁺ LAB detected in 264 wines by QPCR

No. of HDC ⁺ LAB/ml	Wines with indicated HDC ⁺ LAB population	
	No.	% of total
Undetected	7	2.7
1–10	23	8.7
10^1 – 10^2	21	8.0
10^2 – 10^3	30	11.4
10^3 – 10^4	66	25.0
10^4 – 10^5	69	26.1
10^5 – 10^6	35	13.3
> 10^6	13	4.9

nated from 116 wineries distributed in a 3,500-km² area around Bordeaux. Up to 20 samples were from the same winery, but they all originated from different tanks. Microbial DNA was purified and the presence of HDC⁺ LAB was investigated by QPCR. Unexpectedly, HDC⁺ bacteria were detected in more than 97% of the samples at concentrations ranging from 1 to more than $10^6 \cdot \text{ml}^{-1}$ (Table 1). Only 7 out of the 264 samples were apparently free of these bacteria, or at least they contained less than 1 HDC⁺ cell per ml. Important HDC⁺ LAB populations above $10^3 \cdot \text{ml}^{-1}$ were found in 70% of the wines, which include very important populations exceeding $10^5 \cdot \text{ml}^{-1}$ in 18% of the wines. The total population of LAB in some samples was determined by plating or by QPCR using the WLAB primers (21). QPCR revealed generally larger LAB populations than did plating, but with both methods they were in the same range of 10^7 to $10^8 \cdot \text{ml}^{-1}$ (not shown). This allowed us to calculate that histamine producers represented up to 13% or 34% of the LAB detected during malolactic fermentation (as determined by QPCR or plating, respectively).

The measured HDC⁺ LAB populations were plotted on a map to compare them based on the geographic origins of the wines (Fig. 3). In this representation, values corresponding to wines of the same local site were grouped together. In some production sites, the populations of HDC⁺ bacteria in all the wines were quite similar, but in other sites they varied considerably. Therefore, there was no obvious correlation between the production areas and the populations of HDC⁺ LAB in wines.

Identification of HDC⁺ LAB. To characterize the HDC⁺ LAB detected by QPCR, aliquots of wines were plated on a medium to grow LAB colonies, and the histamine producers were detected by colony hybridization. Four wines originating from four distant wineries were analyzed. All the colonies that appeared on the plates had a similar aspect. Detection of those corresponding to HDC⁺ LAB was performed by DNA hybridization with the *hdcA* probe. A total of 54 HDC⁺ colonies, representing 1 to 23 HDC⁺ colonies per wine, were analyzed. The presence of *hdcA* in these bacteria was confirmed by PCR (not shown). The 54 colonies were analyzed by a PCR-based method to determine the species (26). All were *O. oeni* (not shown). A multiplex RAPD-PCR strategy was used to determine if they represented only one or several strains. Eight different patterns were detected, indicating the presence of eight different strains, named A to H (Fig. 4A). Strain A was

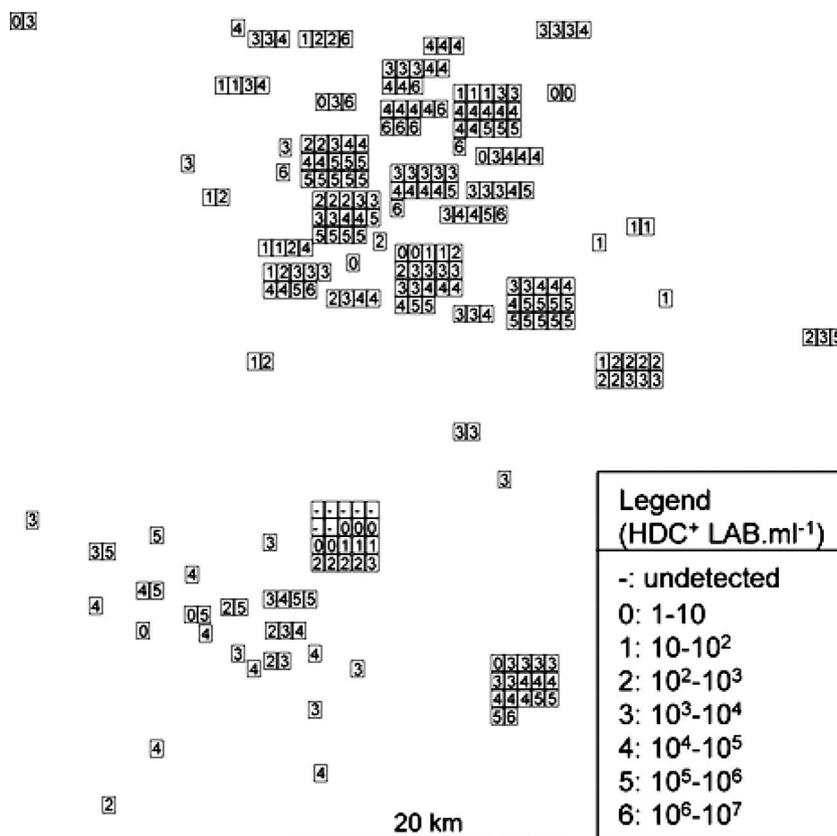


FIG. 3. Populations of HDC⁺ LAB determined by QPCR for 264 wines collected during malolactic fermentation in 116 wineries of the Bordeaux area. Each number represents the HDC⁺ LAB population measured in a wine as described on the figure (“Legend”). They were positioned according to their site of production and grouped together when they were from the same local site.

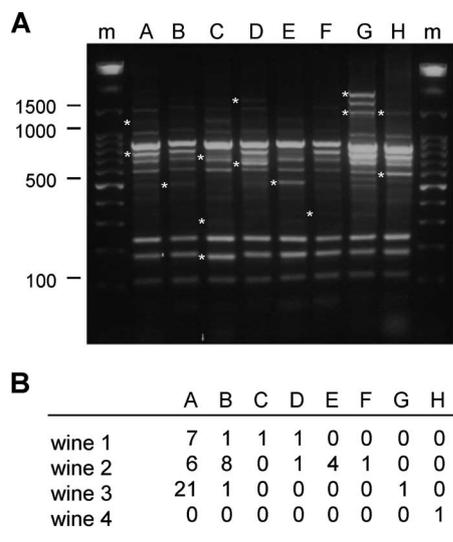


FIG. 4. Identification of eight HDC⁺ *O. oeni* strains by RAPD analysis (A) and frequency of these strains in four wines (B). Fifty-four bacterial colonies that hybridized with an *hdcA* probe were analyzed by RAPD analysis. The eight different RAPD patterns (named A to H) obtained for the 54 colonies are shown. Stars indicate some specific bands. The DNA marker was a 1-kb ladder (Promega) (lanes m).

the most abundant. It was detected in three wines and comprised more than 52% of all the HDC⁺ colonies (Fig. 4B). Strains B and D were detected in three and two wines, respectively, whereas each of the five other strains was associated with only one wine. Each wine contained one to five different HDC⁺ strains.

Genetic localization of *hdcA*. HDC⁺ *O. oeni* strains were analyzed to determine the genomic localization of their *hdcA* gene. The strains were grown in MRS medium and incorporated into agarose plugs that were divided into two equivalent parts. One part of each plug was submitted to DNA digestion by NotI, while the other part was kept undigested. The obtained DNA fragments were separated by PFGE, transferred onto a nitrocellulose filter, and hybridized with a probe specific for *hdcA*. No signal of hybridization was detected with five of the eight strains analyzed (not shown). In contrast, the *hdcA* probe hybridized with the DNA of strains A, B, and C (Fig. 5B). The same hybridization pattern was obtained in all three cases. In lanes containing undigested DNA, a band of 100 kb was hybridized but not the band corresponding to the native chromosome (Fig. 5A and B). After NotI digestion, the probe revealed a smaller band of less than 20 kb (Fig. 5B). These hybridization patterns indicated that *hdcA* was not located on the chromosome of the strains but rather on a large plasmid.

HDC⁺ LAB populations and histamine concentrations. Histamine concentrations were determined by HPLC in 12 wines

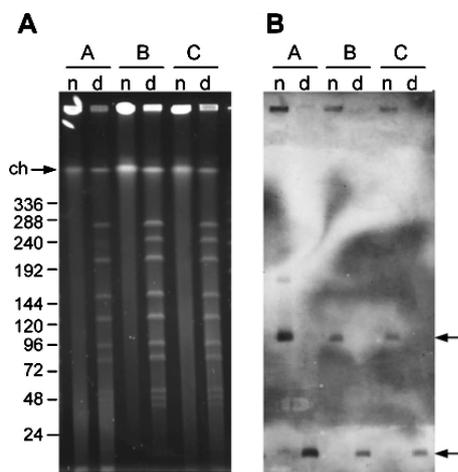


FIG. 5. Determination of the genetic localization of the gene *hdcA* in three strains of *O. oeni* (A, B, and C) by PFGE (A) and Southern blotting (B). Native (n) and NotI-digested (d) genomic DNA of three HDC⁺ *O. oeni* strains was separated by PFGE prior to transfer to a nitrocellulose filter and hybridization with a probe specific for *hdcA*. The sizes of the DNA standards are shown on the left of the figure. Arrows indicate the positions of native chromosomal DNA (ch) and hybridization signals of 100 kb and less than 20 kb.

at the end of malolactic fermentation. According to plate counts, they had similar populations of LAB, ranging from $6.4 \times 10^6 \cdot \text{ml}^{-1}$ to $9.9 \times 10^7 \cdot \text{ml}^{-1}$. In contrast, populations of HDC⁺ LAB determined by QPCR varied from 13 to $6.3 \times 10^6 \cdot \text{ml}^{-1}$ in these wines (Fig. 6). Levels of histamine in the range of 1 to $18 \text{ mg} \cdot \text{liter}^{-1}$ were detected in seven wines; all contained more than $10^3 \cdot \text{ml}^{-1}$ HDC⁺ LAB (Fig. 6). The other wines in which HDC⁺ LAB ranged from 13 to $10^3 \cdot \text{ml}^{-1}$ contained very low or undetectable levels of histamine (Fig. 6). Interestingly, wine 12, characterized by the highest HDC⁺ population, contained a rather low concentration of histamine ($2.7 \text{ mg} \cdot \text{liter}^{-1}$). These results suggested that histamine levels above the recommended limit of $1 \text{ mg} \cdot \text{liter}^{-1}$ could appear when the population of HDC⁺ LAB exceeded $10^3 \cdot \text{ml}^{-1}$ and that higher populations of histamine producers were not directly related to higher levels of histamine.

DISCUSSION

Enumeration of HDC⁺ LAB in wine to evaluate the risk of histamine spoilage. A method based on QPCR was developed to detect and count HDC⁺ LAB in wine. This method makes it possible to detect as few as 1 HDC⁺ cell per ml of wine, even in the presence of polyphenols or of a large excess of yeasts in wine. Although the method was based on a standard curve made with *L. hilgardii* DNA, it is assumed that it was efficient to enumerate HDC⁺ *O. oeni* cells given that (i) the DNA regions targeted by the selected primers were identical in these LAB, (ii) determination of HDC⁺ *O. oeni* cells in four samples by QPCR and colony hybridization provided identical counts (Fig. 2), and (iii) standard curves made using a dilution of DNA prepared from HDC⁺ *O. oeni* or *L. hilgardii* were very similar (not shown). Previous QPCR methods used to enumerate LAB in wine were significantly less sensitive (3, 21). It is likely that optimizing the procedure used to prepare microbial

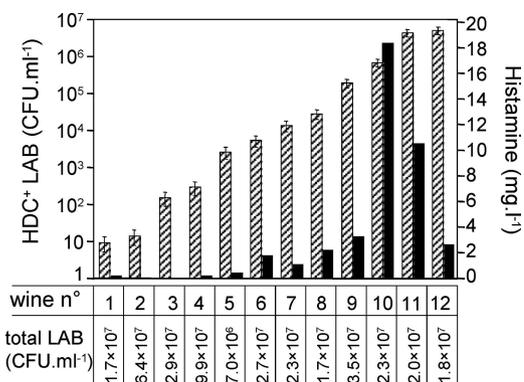


FIG. 6. Comparison of histamine concentrations (filled bars) and HDC⁺ LAB populations (hatched bars) determined in 12 wines by HPLC analysis and QPCR, respectively. Populations of total LAB were determined by enumeration on plates.

DNA from wine samples helped to lower the detection limit of the method. The C_T values obtained with standard samples correlated well with populations of HDC⁺ LAB in the range of 1 to $10^7 \text{ CFU} \cdot \text{ml}^{-1}$. Given that the maximum population of LAB expected in wine is 10^6 to $10^7 \text{ cells} \cdot \text{ml}^{-1}$ during malolactic fermentation, the method could be employed to count HDC⁺ LAB at any stage of winemaking.

An analysis of 264 wines collected in numerous wineries of the Bordeaux area during malolactic fermentation revealed that almost all wines were contaminated by HDC⁺ LAB. Previous work performed by PCR to detect these bacteria in a collection of 118 wines suggested that they were present in approximately 50% of the samples (2). It is possible that this proportion was underestimated given that PCR is generally much less sensitive than QPCR. Nevertheless, both studies are consistent and indicate that HDC⁺ LAB are very frequent in wines. In fact, according to QPCR, they are present in almost all wines during malolactic fermentation. However, populations of HDC⁺ LAB vary considerably. They represent only a few cells per ml in some samples and more than 10^6 per ml in others. It is usually considered that undesirable LAB may produce alterations in wine when their population reaches $10^3 \cdot \text{ml}^{-1}$ or more (11). QPCR revealed that nearly 70% of the wines contained HDC⁺ populations above this level during malolactic fermentation. Determination of histamine concentrations in some wines showed that almost all of those containing more than $10^3 \cdot \text{ml}^{-1}$ HDC⁺ LAB also contained more than $1 \text{ mg} \cdot \text{liter}^{-1}$ histamine. In contrast, histamine levels were negligible in wines with lower HDC⁺ populations. Therefore, $10^3 \text{ HDC}^+ \text{ LAB} \cdot \text{ml}^{-1}$ could be considered the upper limit above which the risk of histamine accumulation becomes important.

Interestingly, in wines containing more than $10^3 \cdot \text{ml}^{-1}$ HDC⁺ LAB, the production of histamine is not proportional to the population of HDC⁺ LAB. For instance, $6.5 \times 10^6 \text{ HDC}^+ \text{ LAB}$ per ml produced only $2.5 \text{ mg} \cdot \text{liter}^{-1}$ histamine, compared to the $18.2 \text{ mg} \cdot \text{liter}^{-1}$ produced in another wine containing $8.7 \times 10^5 \text{ HDC}^+ \text{ LAB}$ per ml. It is important to note that the QPCR assay proposed here does not discriminate between live and dead cells nor between functional genes and pseudogenes. The results suggest that the limiting factor for

histamine production in most wines is not the population of HDC⁺ LAB. Therefore, the determination of the HDC⁺ LAB population would not allow the prediction of the final concentration of histamine in wine. However, it could help to predict the risk of histamine spoilage given that populations of HDC⁺ LAB only above 10³ · ml⁻¹ produce concentrations of histamine exceeding the recommended upper limit in wine. Among the other factors possibly involved in the accumulation of histamine, the concentration of the amino acid precursor, histidine, is probably important (15). Histamine production seems to increase with the use of winemaking techniques such as maceration with grape skins or yeast lees (13, 17). It is likely that these techniques contribute to an increase in the concentration of histidine in wine, which can in turn be converted to histamine by HDC⁺ LAB.

Recurrence of HDC⁺ *O. oeni* strains during malolactic fermentation. The HDC⁺ bacteria isolated from wine until now belong to many species, including *O. oeni*, *L. hilgardii*, *Lactobacillus mali*, *Leuconostoc mesenteroides*, and *Pediococcus parvulus* (7, 8, 14, 20). Therefore, it was unexpected to find that all of the 54 HDC⁺ colonies analyzed in this study were strains of the same species, *O. oeni*. This result suggests that HDC⁺ *O. oeni* bacteria are frequently present at high populations during malolactic fermentation. The predominance of this species is well supported by previous findings: (i) *O. oeni* becomes predominant during spontaneous malolactic fermentation and is often the only species detected at this stage (11), (ii) a large proportion (25% to 60%) of strains with the capacity to produce histamine exist in the species *O. oeni* (6, 7), and (iii) the dominance of HDC⁺ bacteria of the species *O. oeni* during malolactic fermentation is a possibility (2). Interestingly, this study revealed that a few strains of HDC⁺ *O. oeni* were present in diverse wines produced at distant sites. These strains were probably propagated in the vineyard before that grape was harvested or in the wine at the very beginning of winemaking. Dissemination of a limited number of strains in the wineries of a region followed by spontaneous malolactic fermentations could explain why these strains are frequent and abundant in wines during malolactic fermentation. However, it must be noted that these results were obtained for wines of the same region and collected during the same vintage. It remains to be determined whether the results repeat for other regions and vintages.

Localization of *hdcA* and instability of the HDC⁺ phenotype. The detection of many HDC⁺ *O. oeni* strains in environmental samples contrasts with the low abundance of these strains in laboratory collections of wine LAB (1, 20). In our collection, which comprises several hundred *O. oeni* strains, only two scored positive in a PCR test specific for *hdcA* (unpublished results). The results reported here suggest that this situation results from the genetic localization of *hdcA*. Indeed, in three *O. oeni* strains, the gene was detected on a large plasmid of a size supposed to be 100 kb. In a previous work, *hdcA* was detected on an 80-kb plasmid in the HDC⁺ strain *L. hilgardii* IOEB 0006 isolated from wine (14). This plasmid was unstable and was rapidly lost during bacterial cultures. Similarly, if the *O. oeni* plasmid containing *hdcA* was unstable, then HDC⁺ strains could be easily converted into HDC⁻ strains during subcultures of the bacteria in laboratory. This could explain some surprising experimental results, such as the low abun-

dance of HDC⁺ strains in laboratory collections in contrast to the high frequency of these strains in environmental samples (2, 13, 20). Interestingly, a previous work has reported that HDC⁺ *O. oeni* strains were unstable during subcultures in laboratory-defined media (13). It remains to be determined if this instability actually results from the loss of the plasmid carrying *hdcA*.

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REFERENCES

- Costantini, A., M. Cersosimo, V. Del Prete, and E. Garcia-Moruno. 2006. 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. *J. Food Prot.* **69**:391–396.
- Coton, E., G. Rollan, A. Bertrand, and A. Lonvaud-Funel. 1998. Histamine-producing lactic acid bacteria in wines: early detection, frequency, and distribution. *Am. J. Enol. Vitic.* **49**:199–204.
- Delaherche, A., O. Claisse, and A. Lonvaud-Funel. 2004. Detection and quantification of *Bretanomyces bruxellensis* and 'ropy' *Pediococcus damnosus* strains in wine by real-time polymerase chain reaction. *J. Appl. Microbiol.* **97**:910–915.
- Fernández, M., B. del Río, D. M. Linares, M. C. Martín, and M. A. Alvarez. 2006. Real-time polymerase chain reaction for quantitative detection of histamine-producing bacteria: use in cheese production. *J. Dairy Sci.* **89**:3763–3769.
- Gindreau, E., E. Walling, and A. Lonvaud-Funel. 2001. Direct polymerase chain reaction detection of ropy *Pediococcus damnosus* strains in wine. *J. Appl. Microbiol.* **90**:535–542.
- Guerrini, S., S. Mangani, L. Granchi, and M. Vincenzini. 2002. Biogenic amine production by *Oenococcus oeni*. *Curr. Microbiol.* **44**:374–378.
- Landete, J. M., S. Ferrer, and I. Pardo. 2005. Which lactic acid bacteria are responsible for histamine production in wine? *J. Appl. Microbiol.* **99**:580–586.
- Lehtonen, P. 1996. Determination of amines and amino acids in wine—a review. *Am. J. Enol. Vitic.* **47**:127–133.
- Le Jeune, C., A. Lonvaud-Funel, B. ten Brink, H. Hofstra, and J. M. B. M. van der Vossen. 1995. Development of a detection system for histidine decarboxylating lactic acid bacteria based on DNA probes, PCR and activity test. *J. Appl. Bacteriol.* **78**:316–326.
- Lonvaud-Funel, A., C. Fremaux, N. Biteau, and A. Joyeux. 1991. Speciation of lactic acid bacteria from wines by hybridization with DNA probes. *Food Microbiol.* **8**:215–222.
- Lonvaud-Funel, A. 1999. Lactic acid bacteria in the quality improvement and depreciation of wine. *Antonie van Leeuwenhoek* **76**:317–331.
- Lonvaud-Funel, A. 2001. Biogenic amines in wines: role of lactic acid bacteria. *FEMS Microbiol. Rev.* **199**:9–13.
- Lonvaud-Funel, A., and A. Joyeux. 1994. Histamine production by wine lactic acid bacteria: isolation of a histamine-producing strain of *Leuconostoc oenos*. *J. Appl. Bacteriol.* **77**:401–407.
- Lucas, P. M., W. A. Wolken, O. Claisse, J. S. Lolkema, and A. Lonvaud-Funel. 2005. Histamine-producing pathway encoded on an unstable plasmid in *Lactobacillus hilgardii* 0006. *Appl. Environ. Microbiol.* **71**:1417–1424.
- Marcobal, A., P. J. Martin-Alvarez, M. C. Polo, R. Munoz, and M. V. Moreno-Arribas. 2005. Formation of biogenic amines throughout industrial red wine manufacture. *J. Food Prot.* **69**:397–404.
- Martin, M. C., M. Fernandez, D. M. Linares, and M. A. Alvarez. 2005. Sequencing, characterization and transcriptional analysis of the histidine decarboxylase operon of *Lactobacillus buchneri*. *Microbiology* **151**:1219–1228.
- Martin-Alvarez, P. J., A. Marcobal, C. Polo, and M. V. Moreno-Arribas. 2006. Influence of technological practices on biogenic amine contents in red wines. *Eur. Food Res. Technol.* **222**:420–424.
- Martorell, P., J. S. Bosscher, B. ten Brink, A. J. M. Driessen, and W. N. Konings. 1993. Generation of a proton motive force by histidine decarboxylation and electrogenic histidine/histamine antiport in *Lactobacillus buchneri*. *J. Bacteriol.* **175**:2864–2870.
- Moreno-Arribas, M. V., M. C. Polo, F. Jorganes, and R. Munoz. 2003. Screening of biogenic amine production by lactic acid bacteria isolated from grape must and wine. *Int. J. Food Microbiol.* **84**:117–123.

21. Neeley, E. T., T. G. Phister, and D. A. Mills. 2005. Differential real-time PCR assay for enumeration of lactic acid bacteria in wine. *Appl. Environ. Microbiol.* **71**:8954–8957.
22. Pereira Monteiro, M. J., and A. Bertrand. 1994. Validation d'une méthode de dosage. Application à l'analyse des amines biogènes du vin. *Bull. Int. Org. Vine Wine* **765**:916–962.
23. Phister, T. G., and D. A. Mills. 2003. Real-time PCR assay for detection and enumeration of *Dekkera bruxellensis* in wine. *Appl. Environ. Microbiol.* **69**:7430–7434.
24. Pinzani, P., L. Bonciani, M. Pazzagli, C. Orlando, S. Guerrini, and L. Granchi. 2004. Rapid detection of *Oenococcus oeni* in wine by real-time quantitative PCR. *Lett. Appl. Microbiol.* **38**:118–124.
25. Reguant, C., and A. Bordons. 2003. Typification of *Oenococcus oeni* strains by multiplex RAPD-PCR and study of population dynamics during malolactic fermentation. *J. Appl. Microbiol.* **95**:344–353.
26. Renouf, V., O. Claisse, and A. Lonvaud-Funel. 2006. *rpoB* gene: a target for identification of LAB cocci by PCR-DGGE and melting curves analyses in real time PCR. *J. Microbiol. Methods* **67**:162–170.
27. Rudi, K., H. K. Nørga, B. Moen, H. Nissen, S. Bredholt, T. Mørtrø, K. Naterstad, and A. Holck. 2002. Development and application of new nucleic acid-based technologies for microbial community analyses in foods. *Int. J. Food Microbiol.* **78**:171–180.
28. Silla Santos, M. H. 1996. Biogenic amines: their importance in foods. *Int. J. Food Microbiol.* **29**:213–231.
29. Stratton, J. E., R. W. Hutkins, and S. L. Taylor. 1991. Biogenic amines in cheese and other fermented foods: a review. *J. Food Prot.* **54**:460–470.
30. Sumner, S. S., M. W. Speckhard, E. B. Somers, and S. L. Taylor. 1985. Isolation of histamine-producing *Lactobacillus buchneri* from Swiss cheese implicated in a food poisoning outbreak. *Appl. Environ. Microbiol.* **50**:1094–1096.
31. ten Brink, B., C. Damink, H. M. L. J. Joosten, and J. H. J. Huis in't Veld. 1990. Occurrence and formation of biologically active amines in foods. *Int. J. Food Microbiol.* **11**:73–84.