Degradation of vanillic acid and production of guaiacol by microorganisms isolated from cork samples

María Luisa Álvarez-Rodríguez a, Carmela Belloch b, Mercedes Villa b, Federico Uruburu b, Germán Larriba a, Juan-José R. Coque a,*

a Departamento de Microbiología, Facultad de Ciencias, Universidad de Extremadura, Avenida de Elvas s/n, 06071 Badajoz, Spain
b Colección Española de Cultivos Tipo (CECT), Universidad de Valencia, 46100 Burjassot, Valencia, Spain

Received 4 November 2002; received in revised form 7 January 2003; accepted 13 January 2003
First published online 2 February 2003

Abstract

The presence of guaiacol in cork stoppers is responsible for some cases of cork taint causing unpleasant alterations to wine. We have performed a characterization of the cork-associated microbiota by isolating 55 different microorganisms: eight yeast, 14 filamentous fungi or molds, 13 actinomycetes and 20 non-filamentous bacteria. A screening for degradation of vanillic acid and guaiacol production showed that none of the filamentous fungi could achieve any of these processes. By contrast, five of the eight yeast strains isolated were able to degrade vanillic acid, although it was not converted to guaiacol. Guaiacol production was only detected in four bacterial strains: one isolate of \textit{Bacillus subtilis} and three actinomycetes, \textit{Streptomyces} sp. A3, \textit{Streptomyces} sp. A5 and \textit{Streptomyces} sp. A13, were able to accumulate this compound in both liquid media and cultures over cork. These results suggest that guaiacol-mediated cork taint should be attributed to the degradative action of vanillic acid by bacterial strains growing on cork.

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Keywords: Cork taint; Vanillic acid; Guaiacol; Wine aroma

1. Introduction

Cork is the bark of the cork oak (\textit{Quercus suber}). Its main application is in the manufacture of cork stoppers for wine bottles. In fact, cork is recognized as an unsurpassed material to ensure the safe and effective closing of bottles of wine [1]. Unfortunately, some wines (2–7%) suffer from a defect, commonly known as cork taint, that is attributed to the cork stopper [2,3]. This serious off-flavor problem in the wine industry is usually perceived as a moldy, musty, muddy, smoky, phenolic, medicinal and/or earthy flavor that masks the natural wine aroma, wreaking its quality and precluding its commercialization. Nevertheless, it should be emphasized that some authors indicate that the term cork taint should only be applicable to the contamination of wine with a musty or moldy aroma [3]. Several chemical compounds including anisoles, guaiacol, geosmine, 2-methylisoborneol, pyrazines, sesqui-terpenes and several aliphatic compounds have been related to this problem [1]. Of these, guaiacol has been identified as the compound giving the wine a typical phenolic, medicinal or smoky aroma. Wine is not the only food tainted by this compound, since guaiacol contamination has also been detected in fruit juice and fruit juice-containing drinks [4,5], and in chocolate milk [6].

Cork is a very complex medium which can support the development of large microbial populations [7,8]. In Spain several studies have been carried out analyzing the cork-associated microbiota isolated from Catalonian oak forests [9,10], and also from the national park of cork oaks of Cádiz [11]. Consequently, the appearance of these compounds on cork has been traditionally attributed to the metabolic action of its endogenous microbiota [3]. In fact, recently the ability of several fungal strains to produce 2,4,6-trichloroanisole by \textit{O}-methylation of 2,4,6-trichlorophenol when growing directly on cork has been reported [8,12]. The precise origin of guaiacol on the cork stopper remains unclear. It has been proposed that its formation may require the previous breakdown of lignin by the action of saprophytic microorganisms [13].
specifically, guaiacol has been identified as an intermediate in the degradation of vanillic acid, via catechol [14,15], as a result of a non-oxidative decarboxylation reaction (Fig. 1) achieved by the vanillate decarboxylase enzymatic complex [16]. Guaiacol production has been detected in Bacillus megaterium and Streptomyces strains [17], the yeast Rhodotorula rubra [18], a Nocardia sp. [15], and a Streptomyces sp. D7 strain. In this last microorganism the vdc genes encoding the vanillate decarboxylase complex have recently been cloned and characterized [16]. In the present work we have performed a survey of the production of guaiacol by microorganisms isolated on cork.

2. Materials and methods

2.1. Isolation of microorganisms from cork samples

Microorganisms were isolated from different cork samples throughout the manufacturing process of agglomerated cork stoppers and maintained as previously described [8]. The yeast strains have been deposited in the Spanish Type Culture Collection (CECT) under the following strain numbers (the GenBank accession numbers of the partial sequences of the 28S rDNA genes determined for their identification are also showed between parentheses): the black yeast Aureobasidium sp. Y11 CECT 11965 (GenBank accession number AY167611), Cryptococcus sp. Y1 CECT 11955 (AY167602), Debaryomyces hansenii var. fabryi CECT 11957 (AY167604), Rhodospiridium kratochvilovae CECT 11956 (AY167603), Rhodotorula slooffiae CECT 11959 (AY167606), Sporidiobolus Johnsonii CECT 11961 (AY167608), Sporabolomyces nylundii CECT 11958 (AY167605), and Sporabolomyces salmonicolor CECT 11960 (AY167607).

2.2. Screening procedures for vanillic acid degradation

Microorganisms with the ability to metabolize vanillic acid were selected on plates of agar minimal medium containing vanillic acid and bromothymol blue [16].

2.3. Bioconversion of vanillic acid to guaiacol in liquid cultures

Guaiacol production was assessed in 50-ml cultures (in a 250-ml Erlenmeyer flask) in mineral salts medium supplemented with 0.5% yeast extract containing vanillic acid (0.84 g l⁻¹) [16]. For the actinomycetes the cultures were inoculated with 10 ml of a preinoculum grown in yeast extract–malt extract medium supplemented with sucrose (34%) [19], whereas for Bacillus subtilis and yeast strains the preinoculum consisted of 5 ml of a culture of OD₆₀₀nm = 1 developed in yeast extract–peptone–dextrose broth (YPD) (Sigma, Alcobendas, Spain). The flask were incubated at 25°C (yeast), 28°C (actinomycetes) or 37°C (for the B. subtilis strain) with agitation at 200 rpm in an orbital incubator (Sanyo Gallemkamp, Loughborough, UK). The supernatants of cultures were analyzed by high-performance liquid chromatography using a Zorbax SB-C8 (4.5 U 150mm) column (Agilent Technologies, Madrid, Spain) with acetonitrile–water–formic acid (20:80:1) at a flow rate of 1 ml min⁻¹. Eluted peaks were detected at 275 nm in a 1100 Series liquid chromatograph (Hewlett Packard, Wilmington, DE, USA). Quantification was achieved by peak area determination.

2.4. Production of guaiacol by microorganisms growing on cork

Cultures on granulated cork were performed as previously described [8] with minor modifications. Briefly, 5 g of cork was inoculated with 1 × 10⁵ CFU in a 250-ml flask containing 10 ml distilled water, 20 mM ammonium acetate and 100 µg vanillic acid. Cultures were incubated for 20 days at 25°C. Afterwards, the culture was extracted with 50 ml methanol and the solution filtered through a filter of glass fiber. The alcoholic solution was extracted with pentane and concentrated under vacuum. Samples were analyzed by gas chromatography/mass spectrometry as reported before [8].

2.5. Characterization and identification of microorganisms

The filamentous fungi isolated were identified previously [8]. Yeast strains were identified by polymerase chain reaction (PCR) amplification and sequencing of the D1-D2 regions of their 28S rDNA genes using primers NL-1 and NL-4 [8]. The PCR reaction was as follows: a first step at 95°C/5 min, then 40 cycles of 94°C/40 s, 52°C/40 s and 72°C/30 s, and an extension of 10 min at 72°C.Automatic DNA sequencing was achieved by Sistemas Genómicos (Paterna, Spain). The identity of a species was confirmed when sequence homology was higher than 98%.
Non-filamentous bacteria (designated B1–B20) were isolated on the basis of several morphological characteristics such as cell shape, Gram staining, colony shape and color on plate count agar plates (Microkit Laboratories, Madrid, Spain) and endospore formation, physiological (growth at 30 or 37°C) or biochemical traits (catalase, oxidation/fermentation tests and citrate utilization).

Actinomycetes-like bacteria (designated A1–A13) were initially selected according to their mycelial structure, and differentiated according to several macroscopic traits such as color of aerial and substrate mycelium, pigment and exudate production, and sporulation ability on maltose–yeast extract plates [19] and pigment production in YPD broth.

The identification of the bacterial strains of interest was carried out by sequencing their PCR-amplified 16S rDNAs using the primers and PCR conditions described by Chow et al. [16].

2.6. Molecular biological methods

Chromosomal DNA from actinomycetes and B. subtilis strains was isolated by the Smith method [19]. Amplification of vdc genes from Streptomyces sp. D7 was achieved using pKC1 as template [16]. The vdcB gene was amplified using primers VDCB1 (5'-ATGCCGATCCATGCGGTTGTCGTGGGAAT-3') and VDCB2 (5'-ATGCCGATCCCTCAGCCCTATGACGACT-3'). Primers VDCD1 (5'-ATGCCGATCCATGCGGTTGTCGTGGGAAT-3') and VDCD2 (5'-ATGCCGATCCCTCAGCCCTATGACGACT-3') were designed for amplification of vdcC, and primers VDCD1 (5'-ATGCCGATCCCTCAGCCCTATGACGACT-3') and VDCD2 (5'-ATGCCGATCCCTCAGCCCTATGACGACT-3') for the amplification of vdcD. The reactions were subjected to a denaturation step of 95°C/2 min, followed by 30 cycles of 94°C/1 min, 55°C/1 min and 72°C/2 min, with a final extension of 72°C/5 min. Southern blots and hybridizations at 42°C were performed according to standard protocols [20].

3. Results and discussion

3.1. Evolution of the microbiota associated with cork throughout the manufacture of an agglomerated cork stopper

In order to characterize the microbial population of cork we grouped the isolated microorganisms into four groups: filamentous fungi or molds, yeast, actinomycetes and non-filamentous bacteria. The evolution of the microbial population was followed throughout the manufacturing process of an agglomerated cork stopper (data not shown). The microbial content of the initial sample A (raw cork) was rather high, 3.57(±1.21)×10^5 microorganisms per gram of cork, with a predominance of non-filamentous bacteria, 2.44(±1.46)×10^5. As expected, the immersion of cork in boiling water for 30 min (sample B) did not result in a significant reduction of their microbial content, 1.17(±0.45)×10^5, in agreement with the fact that cork is a poor transmitter of heat. The microbial population reached maximum levels, 6.91(±2.22)×10^6, during the stacking of the cork slabs in the molding room (sample C), the filamentous fungi being the predominant microorganisms at the end of this stage, 6.19(±2.36)×10^6. In addition, we also observed at this stage a notable increase of actinomycetes on cork, 6.90(±3.25)×10^5, which became predominant over other bacterial groups, 2.23(±0.61)×10^6. The size of the microbial population was rather stable or decreased slightly, 6.67(±2.34)×10^6, during the storage of planks at the factory exposed to ambient environmental conditions (sample D). As expected, the size of the population decreased drastically during the next stages of the manufacturing process, sample E (granulated cork), 1.35(±0.41)×10^5, and sample F (glued agglomerated cork stopper), 1.55(±0.68)×10^4, probably as a consequence of the chemical and mechanical treatment undergone by the cork. Finally, the microbial content of the end product (sample G or SO_2-treated cork stopper) was as low as 4 microorganisms per gram of cork, and yeast and actinomycetes were never isolated from this sample, in accordance with the observations of other authors indicating that non-filamentous bacteria and filamentous fungi are the predominant microorganisms at this end stage of stopper production [7–10]. Our results, as also indicated by others, confirm that cork can harbor very complex microbial populations [3,7,11,21–23]. However, they are difficult to characterize because they are not static or constant over time, and besides they can vary depending on the nature, origin and conditions of cork transportation and storage [3]. In the current work we found that a gram of cork may contain up to 10^6 microorganisms, although the microbial content of the final product is insignificant. Except for sample C, bacteria are the predominant populations throughout the manufacturing process. This contrasts with previous reports which had indicated a predominance of fungal populations [3,7,9,10].

3.2. Isolation and characterization of microorganisms isolated from cork

A total of 55 different microorganisms were isolated from cork samples. As filamentous fungi had been postulated to play an important role in the quality of cork [3] we identified the 14 filamentous fungi isolated in a previous work [8].

Our current knowledge on the yeast populations on cork is scarce. Therefore, next we turned our attention to the characterization of the yeast isolates, six of which were identified at the species level: they were the basidio-
mycetes R. kratochvilovae (99% homology with sequence AF444778), S. nylandii (99% homology with AF387123), R. slooffiae (99% homology with AF444722), S. salmonicolor (100% homology with AF189979), and S. johnsonii (100% homology with AF189976), and the ascomycete D. hansenii var. fabryi (100% homology with U94927). Besides, two strains were identified solely at the genus level: they were the black yeast, Aureobasidium sp. Y1 (91% homology with several species of this genus), and the basidiomycete Cryptococcus sp. Y1 (99% homology with AF444742). They probably represent new species, although their unambiguous identification will require further work.

Most of the previous reports aimed at characterizing the cork-associated microbiota were particularly concerned with populations of filamentous fungi, since these microorganisms have been blamed for producing 2,4,6-trichloroanisole, the major agent causing cork taint [7,8,12,21]. Unfortunately, no similar efforts have been carried out to characterize the yeast strains present on cork, and to analyze their putative role in cork taint. As a consequence, with the exception of S. johnsonii [21], all the yeast isolated in this work have been detected for the first time on cork.

We also isolated 20 different strains of non-filamentous bacteria and 13 actinomycetes, although identification in these cases was carried out solely for vanillic acid-degrading microorganisms. One non-filamentous bacterium (B2) was identified as B. subtilis, on the basis of its 16S rDNA sequence, which was 100% identical to those of several strains of this species deposited in databases. We also identified three actinomycetes of interest: the first was identified as Streptomyces sp. A3, since its 16S rDNA (accession number AY154478) showed 97% homology with its counterparts from S. galilaeus (AB045878), S. bohili (AB045876), S. peruviensis (AJ310924) and S. panayensis (AB045860), and slightly lower levels of homology with other Streptomyces strains. Although macroscopically very different, the 16S rDNA sequences of the strains identified as Streptomyces sp. A5 and Streptomyces sp. A13 (accession number AY154479 with two entries) were 99% homologous. The same level of homology was found when they were compared to sequences of Streptomyces sp. YIM8 (AF389344), Streptomyces sp. VTT E-99-1334 (AF429399), Streptomyces sp. AA8321 (AF221837) and S. griseus (AB030572).

### 3.3. Screening for the identification of vanillic acid-degrading microorganisms

Of the 55 microbial isolates tested for vanillic acid degradation only nine were able to change the color, from green to blue, of the selection agar minimal medium containing vanillate as the sole carbon source. Remarkably, none of the 14 filamentous fungi isolated was able to degrade this compound; by contrast, this ability appeared to be quite common among the yeast strains, since five (R. kratochvilovae, R. slooffiae, S. nylandii, S. salmonicolor and S. johnsonii) out of the eight isolates induced a significant change in the color of the medium, and also showed a good capability to grow on the selection plates.

With regard to the 33 bacterial strains tested, only the non-filamentous bacteria identified as B. subtilis, and three actinomycetes (Streptomyces sp. A3, Streptomyces sp. A5 and Streptomyces sp. A13) were able to degrade vanillic acid.

### 3.4. Analysis of guaiacol production by vanillic acid-degrading microorganisms

Next, we checked the production of guaiacol by those microorganisms previously selected on plates as vanillic acid degraders when grown in liquid cultures. Guaiacol was not detected in culture supernatants of any of the yeast strains tested, in spite of the fact that time course

### Table 1

<table>
<thead>
<tr>
<th>Microorganism added</th>
<th>Guaiacol production (ng g⁻¹ cork)ᵃ</th>
<th>Bioconversion of vanillic acid to guaiacol (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (non-inoculated cork sample)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Aureobasidium sp. Y11</td>
<td>5.4</td>
<td>4.3</td>
</tr>
<tr>
<td>Cryptococcus sp. Y1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>D. hansenii var. fabryi</td>
<td>6.5</td>
<td>6.9</td>
</tr>
<tr>
<td>R. kratochvilovae</td>
<td>ND</td>
<td>3.9</td>
</tr>
<tr>
<td>R. slooffiae</td>
<td>7.1</td>
<td>6.6</td>
</tr>
<tr>
<td>S. johnsonii</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>S. nylandii</td>
<td>4.0</td>
<td>ND</td>
</tr>
<tr>
<td>S. salmonicolor</td>
<td>5.2</td>
<td>ND</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>ND</td>
<td>1329.3</td>
</tr>
<tr>
<td>Streptomyces sp. A3</td>
<td>ND</td>
<td>257.6</td>
</tr>
<tr>
<td>Streptomyces sp. A5</td>
<td>ND</td>
<td>380.0</td>
</tr>
<tr>
<td>Streptomyces sp. A13</td>
<td>ND</td>
<td>524.6</td>
</tr>
</tbody>
</table>

ND: not detected.

ᵃ Values shown are the averages for duplicate estimates obtained in two independent experiments.
experiments indicated that vanillic acid was readily metabolized and disappeared almost completely (>95% degradation) from the media after 24–30 h of incubation; as shown for R. kratochvilovae (Fig. 2), vanillic acid was almost undetectable in a 24-h culture. Similar results were obtained for all the yeast isolated (data not shown).

In contrast, all the bacterial strains selected were able to degrade vanillic acid with the concomitant accumulation of guaiacol in the supernatants of liquid cultures (Fig. 3). The highest production was accomplished by the B. subtilis strain (Fig. 3A) which showed an overall efficiency of biotransformation into guaiacol after 33 h of incubation of 55.4% (±3.6). Most of this transformation occurred during the first 12 h of incubation (50% and 90% of degradation of vanillic acid after 5 and 12 h respectively). The maximum levels of guaiacol detected at 33 h remained almost unchanged until the end of the fermentation, indicating that this compound did not undergo further degradation.

The efficiency of the catabolism of vanillic acid among the actinomycete strains was variable, although its degradation was not completed in any case: the maximum degradation levels detected were 34.4% (±1.8), 40.4% (±2.7) and 53.3% (±3.7) for Streptomyces sp. A3 (Fig. 3B), Streptomyces sp. A5 (Fig. 3C), and Streptomyces sp. A13 (Fig. 3D) respectively, whereas the detected maximum levels of accumulated guaiacol were 14.3% (±3.0), 20.0% (±2.3) and 27.3% (±5.1) respectively.Remarkably, the four bacterial strains analyzed could accumulate guaiacol in liquid cultures, which suggests that the catechol branch of the β-ketoadipate pathway was truncated (see Fig. 1).

The calculated rate of bioconversion of vanillic acid into guaiacol in liquid medium was around 50% in all cases (note in Fig. 3 that the maximum amounts of guaiacol detected in all cultures are approximately half of the quantities of vanillic acid degraded, with small variations attributed to the experimental handling). This suggests that vanillate was simultaneously decarboxylated to guaiacol and degraded through the protocatechuate branch of the pathway practically in an equimolecular ratio. In fact, all these strains could grow efficiently on a minimal medium containing protocatechuate as the sole carbon source (data not shown).

In order to determine whether the production of guaiacol could occur under natural conditions we tested the ability of several bacterial and yeast isolates to produce guaiacol when growing directly over granulated cork. As shown in Table 1, again solely the bacterial strains were able to produce guaiacol in the presence of the precursor vanillic acid under these conditions, in accordance with previous observations in liquid media. Nevertheless, the efficiencies of bioconversion obtained were significantly lower than those in liquid cultures, perhaps due to a lower ability of the strains to grow on cork.

The origin of guaiacol on cork remains unclear. It has been proposed that, in materials of vegetable origin, guai-
col could originate from the chemical breakdown of lignin [13]. The absence of traces of guaiacol in the cultures over cork developed in the absence of vanillic acid suggests that these microorganisms cannot produce guaiacol by performing a chemical breakdown of lignin. Nevertheless, it is quite feasible that in such a complex microbial ecosystem as cork, some lignin-degrading microorganisms, acting as a consortium, could supply the precursor vanillic acid. It is well known that guaiacol is formed from decarboxylation of vanillic acid, which is an abundant component of solubilized lignin biomass [13^17]. Our screening boxylation of vanillic acid, which is an abundant component of cork developed in the absence of vanillic acid, suggests that the appearance of guaiacol on cork is due, with the great difference in the G+C content between Streptomyces and Bacillus genomes, since homologues to these genes exist in the B. subtilis genome, and they are clustered in the same order as Streptomyces sp. D7 [16]. In contrast, a search of the databases did not yield homologous vdc genes in other Streptomyces strains, including a search against the complete genome of S. coelicolor.

**Acknowledgements**

We thank Dr. Julian Davies for providing plasmid pKCE1. We also are grateful to SANVICORK S.A. (San Vicente de Alcántara, Badajoz, Spain) and IPROCOR (Merida, Badajoz, Spain) by the cork samples and technical support, and Lecocia Franco for technical assistance. This work was supported by the European Community and the Ministerio de Educacion y Cultura of Spain (Grant 1FD97-1172) and also by the Junta de Extremadura (Grants IPR00C004 and 2PR01A009).

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