JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. **2010,** *58,* 12481–12490 **12481**
DOI:10.1021/jf102874f

Origin and Incidence of 2-Methoxy-3,5-dimethylpyrazine, a Compound with a "Fungal" and "Corky" Aroma Found in Cork Stoppers and Oak Chips in Contact with Wines

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This study identifies a previously isolated bacterium as *Rhizobium excellensis*, a new species of proteobacteria able to form a large quantity of 2-methoxy-3,5-dimethylpyrazine (MDMP). *R. excellensis* actively synthesizes MDMP from L-alanine and L-leucine and, to a lesser extent, from L-phenylalanine and L-valine. MDMP is a volatile, strong-smelling substance detected in wines with cork stoppers that have an unpleasant "corky", "herbaceous" (potato, green hazelnut), or "dusty" odor that is very different from the typical "fungal" nose of a "corked" wine that is generally due to 2,4,6-trichloroanisole (TCA). The contamination of cork by MDMP is not correlated with the presence of TCA. It appears possible that *R. excellensis* is the microorganism mainly responsible for the presence of this molecule in cork bark. However, other observations suggest that MDMP might taint wine through other ways. Oak wood can also be contaminated and affect wines with which it comes into contact. Nevertheless, because 93% of the MDMP content in wood is destroyed after 10 min at 220 °C, sufficiently toasted oak barrels or alternatives probably do not represent a major source of MDMP in most of the cases. Due to MDMP's relatively low detection threshold estimated at 2.1 ng/L, its presence in about 40% of the untreated natural cork stoppers sampled at concentrations above 10 ng/cork suggests that this compound, if extracted from the stoppers, may pose a risk for wine producers.

KEYWORDS: 2-Methoxy-3,5-dimethylpyrazine; cork stopper; cork; oak wood; wine; *Rhizobium excellensis*

INTRODUCTION

Among the most common and unpleasant alterations found in wines sealed with cork stoppers, a "corked taste" usually corresponds to a "fungal" or "moldy" odor primarily caused by 2,4,6-trichloroanisole (TCA) (*1*,*2*). However, it cannot be said that one and only one molecule is responsible for defects transmitted by corks to bottled wine. Other molecules have also been identified in corks that can cause, albeit to a lesser degree than TCA, complementary alterations (*3*). The most noteworthy of these compounds include geosmin with an "earthy" aroma, 1-octen-3-one with a "mushroom" aroma, and 2-methoxy-3,5-dimethylpyrazine (MDMP) (**1**) (**Figure 1**), which confers a more complex odor simultaneously reminiscent of "wet cork" (or a "corky" character), "fresh hazelnut" (or "herbaceous" character), and a frankly "moldy" or "earthy" aroma (potato) when the concentration is high (*4*). MDMP was clearly isolated and identified in cork by Simpson et al. (*4*) but was characterized for the first time by Mottram et al. (*5*) in a very different environment. These latter authors detected the presence of MDMP in liquid emulsions used to lubricate cutting tools and isolated an aerobic bacterial strain responsible for forming this volatile, strong-smelling substance, although they were unable to identify it. With regard to wine, the detection threshold of MDMP has been estimated at 2.1 ng/L (*4*). This molecule has also been identified in coffee (*6*) and might also contribute to the positive or negative aroma of a number of food items. Indeed, alkyl- and alkoxypyrazines are aromatic molecules widely found in nature. A fairly large number of organisms such as bacteria (*7*−*10*), insects (*11*), plants (*12*, *13*), and even *Vitis vinifera* (*14*) are able to synthesize alkylmethoxypyrazines similar to MDMP. Cai et al. (*15*) also identified an isomer of MDMP that interested us in this study, 3-methoxy-2,5-dimethylpyrazine, in *Harmonia axyridis* (multicolored Asian lady beetle).

The works presented in this paper have various objectives. First of all, we fully identify the microorganism responsible for MDMP isolated by Mottram et al. (*5*), which was unknown until now. Then, the influence of the nitrogen source on the biosynthesis of MDMP by *Rhizobium excellensis* is presented. Using concrete examples from the wine industry, various cases of contamination of wines by MDMP from cork stopper, as well as wines aged with oak wood chips, are reported. These data lead to a new evaluation of the risks associated with this particular molecule in the wine industry.

MATERIALS AND METHODS

Materials. All compounds, even the deuterated one, were obtained from Sigma-Aldrich and were of the highest available purity. All solvents were Sigma Adrich Puriss grade and were systematically checked for purity by gas chromatography/mass spectrometry (GC/MS) prior to use.

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Published on Web 11/08/2010

Permuted distilled water was purified with a Milli-Q system (Millipore, USA). Ready-to-use culture media were obtained from Sigma Aldrich (L'Isle d'Abeau, France).

Synthesis of 2-Methoxy-3,5-dimethylpyrazine and [$^2H_3$]-2-Methoxy-3,5-dimethylpyrazine. We used the protocol previously described in detail by Simpson et al. (*4*) to prepare MDMP and its deuterated analogue. NMR spectra (data not shown) and MS spectra (**Figures 2** and **3**) conformed to expected values. The purity thus obtained, evaluated with GC/MS on a DBWAX polar column (Supelco, USA), 30 × 0.25 mm, and an HP5-MS apolar column (Agilent, USA), 30 m × 0.25 mm, in full scan mode (40−300 uma), was greater than 99%.

Analysis by HSSPME/GC/MS in Wine and Liquid Culture Media. Gas chromatography/mass spectrometry was carried out with a Hewlett-Packard 6890N (Agilent, USA) fitted with a Gerstel MPS2 autosampler (Mulheim an der Ruhr, Germany) operated in headspace (HS) solid-phase microextraction (SPME) with a DVB/PDMS/Carboxen fiber (Supelco, USA) and connected to an HP5975 inert source mass spectrometer (Agilent) working by electronic impact as previously described in detail by Boutou and Chatonnet (*16*). In light of its physicochemical characteristics (K_a and K_b), the alkylmethoxypyrazines are organic bases which are protonated at low pH to form nonvolatile quaternary ions ($pK_a < 1.5$). Alkylpyrazine basic ionization constants (pK_b) increase as alkyl substituents are added, but alkoxypyrazines are potentially more strongly stabilized by the electron-donating methoxy group. MDMP assay is facilitated when the medium's pH increased over 10 (*16*). However, in order not to affect the quantification of the other molecules evaluated at the same time, the pH of the liquid media was systematically adjusted to 7.0 ± 0.02.

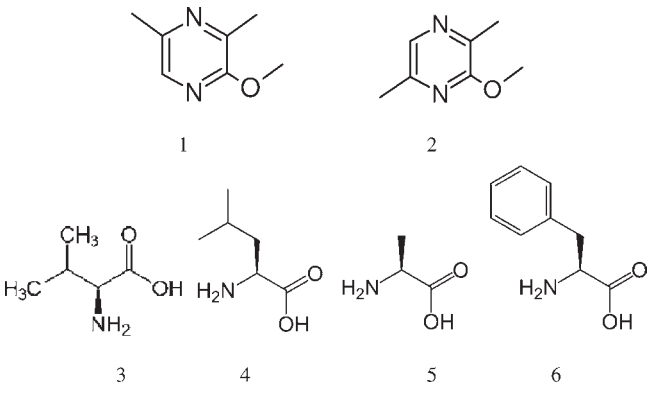

Figure 1. 2-Methoxy-3,5-dimethylpyrazine (**1**), 2-methoxy-3,6-dimethylpyrazine (**2**), L-valine (**3**), L-leucine (**4**), L-alanine (**5**), and L-phenylalanine (**6**).

Fifty milliliters of the liquid was adjusted with a pH meter to pH 6.0 with a solution of 36 M NaOH and then to pH 7.0 with a 0.5 M solution; 5 ± 0.01 mL of sample was placed in a 20 mL SPME vial with 5 mL of Milli-Q water and 3 ± 0.1 g of NaCl. We added 10 μL of an internal standard solution (deuterated analogues) at 5 μg/L in absolute ethanol, and the vial was sealed with a silicone/Teflon joint (Supelco, USA). The vial was extracted in headspace mode for 90 min at 45 °C with agitation (250 rpm) in the presence of a DVB/CARB/PDMS SPME fiber (ref 57329-U; Supelco, USA), preheated to 270 °C for 1 h as per the manufacturer's recommendations, and then directly desorbed from the head of the column in a splitless injector at 270 °C for 5 min.

The GC/MS analysis was done on a Varian FactorFour VF-5 ms column (Palo Alto, USA), 30 m × 0.25 mm i.d., with a phase thickness of 0.25 μm and a 10 m guard column made of deactivated silica. The gas vector (helium N55; AirProduct, Toulouse, France) was programmed at a steady flow of 45 cm/s (1.5 mL/min). The injector functioned in splitless mode at 270 °C (split at 5.90 min at 15 mL/min) with a borosilicate glass insert having an internal diameter of 0.75 mm (Supelco, USA). The chromatography oven was set at 50 °C for 2 min, then raised until 190 °C at 3 °C/min, and finally 320 °C at 50 °C/min with an isotherm at 320 °C of 1 min. Measurement was ensured by an HP5975 mass detector (source

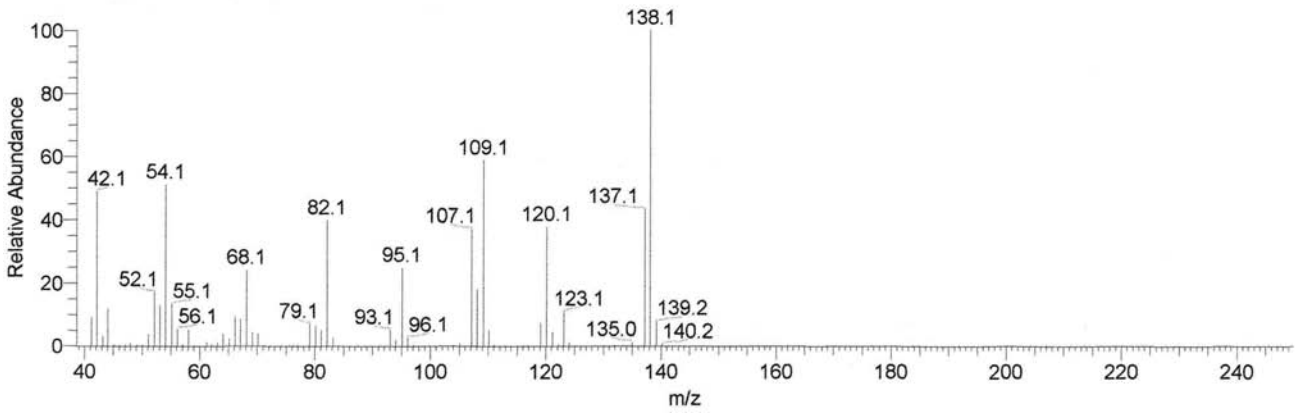

Figure 2. EI mass spectra of synthetic 2-methoxy-3,5-dimethylpyrazine (**1**) (MDMP).

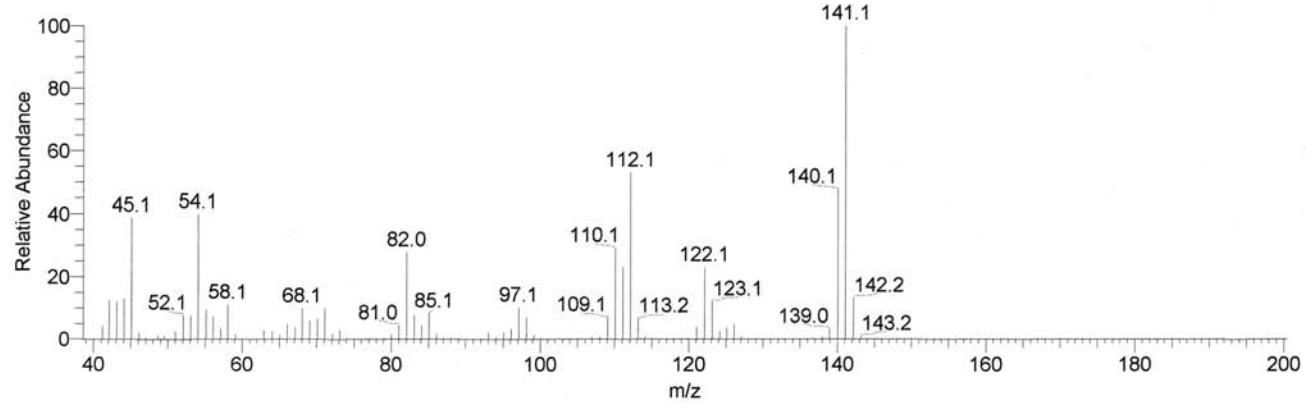

Figure 3. EI mass spectra of synthetic [$^2H_3$]-2-methoxy-3,5-dimethylpyrazine (MDMP−OCD_3).

temperature of 230 °C, quadrupole temperature 150 °C, energy 70 keV, electron multiplier 1600 V) and operated in SIM mode on selected ion characteristics of each molecule (*14*). (TCA, 197, 210, 212; 2,3,4,6-tetrachloroanisole (TeCA), 244, 246; pentachloroanisole (PCA), 278, 280; 2,4,6-tribromoanisole (TBA), 344.346; MDMP, 138, 120, 109; dwell time, 100 ms). The following ratios between the original compound and its deuterated analogue on the methoxy function ($-OCD_3$) were used for quantification: *m*/*z* 212/213 (TCA/TCA$-OCD_3$); 246/249 (TeCA/TeCA$-OCD_3$); 280/283 (PCA/PCA$-OCD_3$); 329/331 (TBA/TBA$-OCD_3$); and 138/141 (MDMP/MDMP$-OCD_3$). To optimize the detection of MDMP in wine, the electron multiplier that normally operates at 1600 V was boosted by +200 V 1 min before and after the retention time of the molecule.

Assay of MDMP in Solid Objects. *Assay in the Extractable Fraction of Corks of Doubtful Quality*. Corks from bottles of wine that were tasted and found both altered and not altered were placed in 50 mL of a hydroalcoholic solution with 12% alcohol by volume and containing 5 g/L tartaric acid adjusted to pH 3.50 for 72 h, totally immersed in a glass flask. In order to limit sources of outside contamination, care was taken to remove 10 mm of the upper part of the cork in contact with the outside atmosphere. The extractable contaminant content was then measured according to the protocol used for wines described above.

Assay by Total Extraction. Samples of weighed new corks (50 units) or oak chips were first crushed in a cutting mill with an interchangeable stainless steel bowl cleaned with ethanol and then dried in an autoclave at 105 °C between each crushing. This yielded a rough powder (1.5−3 mm) that was kept in aluminum foil until analysis. The powder (5 ± 0.1 g) was put into a purified cellulose nacelle in a Soxhlet extractor of 50 mL capacity, to which was added 100 μL of an internal standard solution with 100 μg/L deuterated analogues in absolute ethanol. The evaporator was filled with 70 mL of dichloromethane heated to a temperature of (45 ± 5 °C) to produce a reflux of 1 drop per second for 2 h with a refrigerant maintained at 10 ± 1 °C. The extract thus obtained was concentrated to 1 mL in a Turbovap II automatic evaporator (Zymark, Maryland, USA) under nitrogen flux (100 mL/min) at 25 °C and maintained at +4 ± 2 °C until injection. In this way, we extracted more than 98.5% of all contaminants in the oak or cork.

The extract (1 μL) was injected in splitless liquid mode with the help of a MPS2 Gerstel autosampler working in fast liquid injection mode with a 10 μL syringe (SGE, Melbourne, Australia) and analyzed using the same chromatographic conditions described above for liquid solutions but with an insert linear of 2.5 mm diameter (Supelco, USA). The quantitative assay was compared to a calibration line between 0 and 50 ng/g in 10 ng/g increments according to the same conditions of extraction and using solid cork or oak previously decontaminated by back-washing (2 h) in a Soxhlet extractor. The characteristics of assaying MDMP were as follows: limit of detection (LOD) = 0.6 ng/g, limit of quantification (LOQ) = 2.0 ng/g, and RSD% = 4.

Cultured Bacteria. The strain of bacteria originally recorded with the National Collection of Industrial Bacteria under the number 118020 by Mottram et al. (*5*) was obtained from NCIMB Ltd. (National Collection of Industrial, Food and Marine Bacteria, Aberdeen, Scotland, U.K.). The strain was reactivated in a classic (tryptone soya agar) medium. After subculturing three times, a well-isolated colony was used for the following stages of culturing and identification.

The liquid medium used to study the influence of the source of nitrogen on the biosynthesis of MDMP (**1**) by *R. excellensis* consisted of NaCl (0.50%), K_2HPO_4 (0.25%), and $MgSO_4$ (0.05%) in distilled water, with pH adjusted to 7.0 by NaOH. This was sterilized in an autoclave at 120 °C for 15 min, after which 0.1% of amino acids, yeast extract (Y1625; Sigma), or $(NH_4)_2SO_4$ in a concentrated solution (10%) in Milli-Q (Millipore) water previously sterilized with sterile 0.22 μm filtration (Z370541; Nalgen) was added. Culture was performed in a dark environment in aerobiosis at 28 ± 1 °C for 2 weeks in 100 mL Erlenmeyer flasks, after which a sample aliquot was taken and centrifuged for 15 min at 5000 rpm. The supernatant was analyzed with HSSPME/GC/MS using the protocol described above.

The other control microorganisms used in this study were obtained from BCCM/LMG (Ghent, Belgium), DSMZ (German Collection of Microororganisms, Braunschweig, Germany), BCCM/MUCL (Fungi and Yeast Collection of the Catholic University of Louvain-La-Neuve, Belgium), and ATCC (LGC Standards, Molsheim, France).

Various culture media, adapted to each type of microorganism in keeping with the recommendations of the reference collections, were used to obtain a significant quantity of biomass with the same easy-to-assimilate source of complex amino nitrogen (yeast extract) quickly and easily. The media were seeded in a sterile manner with a platinum inoculation loop from a culture reactivated from the control strain kept in the same type of medium. The cultures were performed in aerobiosis at 28 ± 1 °C for 2 weeks in the dark. After that time, a sample aliquot was taken and centrifuged for 15 min at 5000 rpm before analysis in order to assay the MDMP by HSSPME/GC/MS as described above.

Medium 1 (*Pseudomonales* and *Rhizobiales*): beef extract 1 g, yeast extract 2 g, peptone 5 g, NaCl 5 g, K_2HPO_4 0.45 g, $Na_2HPO_4 \cdot 12H_2O$ 2.39 g, pH 6.8, adjusted with NaOH 6 M until 1000 mL with distilled water.

Medium 2 (*Enterobacteriales*): beef extract 1 g, yeast extract 2 g, peptone 5 g, NaCl 5 g, H_2O qsp 1000 mL, pH 7.4, NaOH 6 M, sterilization in an autoclave at 120 °C for 15 min.

Medium 3 (*Deuteromycetes*): malt extract 20 g, yeast extract 1 g, H_2O qsp 1000 mL, pH 7.0, NaOH 6 M, sterilization in an autoclave at 120 °C for 15 min.

Milieu 4 (*Mixococcales*): yeast extract 1 g, $CaCl_2 \cdot 2H_2O$ 1.36 g, H_2O qsp 1000 mL, NaOH 6 M, vitamin B_{12} 0.5 mg/L added after heating in an autoclave at 120 °C for 15 min in a 1% aqueous solution passed through a sterile 0.22 μm filter.

Identification of the Bacterium. Identification of the bacterium previously isolated by Mottram et al. (*5*) was done by the BCCM/LMG (Bacteria Collection Laboratorium voor Microbiologie, University of Ghent, Belgium) using classic bacterial tests (cell morphology, Gram stain, oxidase and catalase reactions), fatty acid analysis done using the MIDI (Microbial Identification System Inc., Delaware, USA) commercial identification system, and research done on TSBA50 rev. 5.0 and LMG databases. Partial 16S rDNA sequence analysis was performed according to the protocol of Niemann et al. (*17*) to extract total DNA. A fragment of the 16S rDNA gene, corresponding to positions 8−1522 in the *Escherichia coli* numbering system and coding for ribosomal deoxyribonucleic acid (rDNA), was amplified by polymerase chain reaction (PCR) using conserved primers. The PCR product was purified using the Nucleofast 96 PCR cleanup kit (Macherey and Nagel, Germany). Sequencing reactions were performed using the BigDye Terminator Cycle sequencing kit (Applied Biosystems, USA) and purified using the Montage SEQ96 sequencing reaction cleanup kit (Millipore, USA). Sequencing was performed with an ABI Prism 3130 XL genetic analyzer (Applied Biosystems, USA). Sequence assembly was performed using the program Autoassembler (Applied Biosytems, USA), and phylogenic analysis was performed using the Bionumerics (Applied Matchs, Belgium) software package after including the consensus sequence in an alignment of small ribosomal subunit sequences collected from the International Nucleotide Sequence Library of the European Molecular Biology Laboratory (Grenoble, France). A similarity index was created by homology calculation with a gap penalty of 0% (unknown bases were discarded). In this way, a similarity equal to or greater than 97% was significant for possible species identification.

Scanning Electron Microscope. A bacterial colony was diluted in an isotonic solution of NaCl at 0.1% on a glass slide cleaned with ethanol. The dehydrated plating was then metallized in a vacuum with a combination of platinum and palladium and covered with a layer of carbone on a CRESSINGTON 108 sputter coater (Cressington Scientific Instruments, Watford, England (U.K.)) before observation under a JEOL 6490LA microscope (JEOL Europe, Croissy-sur Seine, France) with a voltage of 5−15 kV.

Thermal Stability of MDMP. Fifty grams of oak in the form of chips 2.5−3.5 mm thick was impregnated with a MDMP and alcohol solution to obtain approximately 30 ng/g of MDMP oak. After drying and evaporation of the ethanol (24 h in the open air, under a hood), an oak chip aliquot (about 10 g) was put into a single aluminum capsule and heated in a ventilated oven at 220 °C for 0, 5, 10, and 20 min. After cooling in the open air, the oak's MDMP content was then assayed using the total extraction procedure. This test was repeated three times.

RESULTS AND DISCUSSION

Identification of the Bacterium That Causes the Synthesis of MDMP. The bacterial strain able to biosynthesize MDMP isolated by Mottram et al. (*5*) and registered with the National

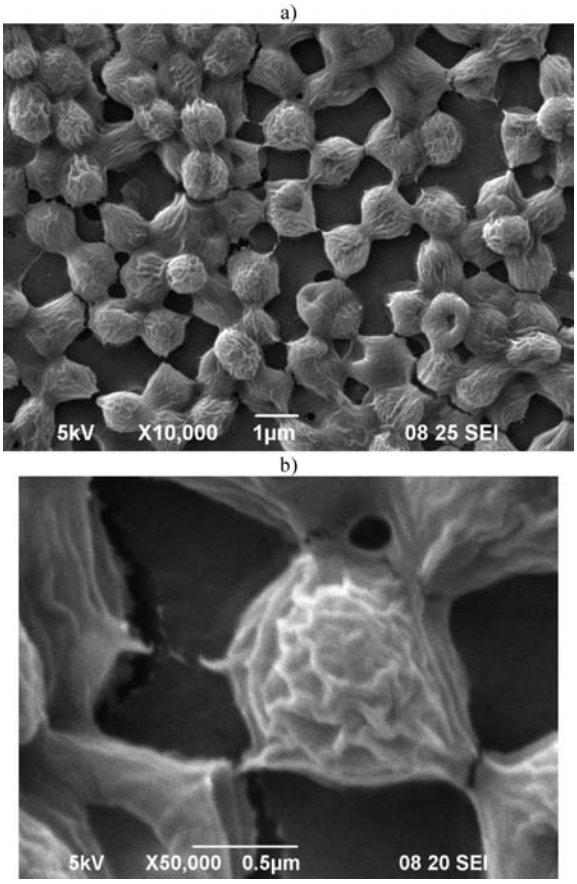

Figure 4. *R. excellensis* view by scanning electronic microscopy.

Collection of Industrial Bacteria under the no. 118020 has a Gram-negative nonmoving single or double shell morphology ($1.1 \times 1.2-2$ μm). **Figure 4** shows cells of *R. excellensis* and an exocellular secretion that tends to amalgamate bacterial cells as observed with a scanning electron microscope. The bacteria react negatively to oxidase and positively to catalase testings. Analysis of the composition of fatty acids and comparing this to control profiles using the MIDI protocol did not permit direct identification. The best comparison was obtained with *Phyllobacterium rubiacearum*, although the correspondence index (0.225) was too low. Comparing with LMG's database showed that, *a priori*, the culture can be classified in the genus *Rhizobium* sp.

Neither did analysis by partial sequencing of 16S rDNA allow immediate identification of the species when compared with profiles available in the EMBL database, which showed that the bacterial isolate in question belonged to a new species. The greatest similarity was found with several members of the *Bradyrhizobiaceae* (*Afipia*, *Bosea*, *Bradyrhizobium*, *Nitrobacter*, and *Rhodopseudomonas*) and *Methylobactoriaceae* (*Methylbacterium*) families from the order *Rhizobiales* (**Figure 5**). However, it was not possible to define exactly what genus or what family the isolated strain belonged to based solely on this pyhlogenetic analysis. That having been said, it was definitely a species not as yet formally classified. *Rhizobium*, *Agrobacterium*, *Allorhizobium*, and *Sinorhizobium* are all different genera in the family *Rhizobiaceae*. Based on their 16S rDNA profiles and phenotypes, these various genera can be included in a single genus: *Rhizobium* (*18*). We therefore identified the unknown bacteria NCIMB 11802 as *R. excellensis* and have registered it in the BCCM/LMG public collection under the reference number LMG 24089.

Various bacteria are able to synthesize alkylmethoxypyrazines. *Pseudomonas perolens* (a group of gammaproteobacteria belonging to the *Pseudomonadales* order) is able to synthesize 2-isopropyl-3-methoxypyrazine (IPMP) and, to a lesser extent, 2-*sec*-butyl-3-methoxypyrazine in meat and fish, producing odors reminiscent of moldy potatoes (*7*). *Pseudomonas taetrolens* was identified as likely to form IPMP in milk (*8*). *Serratia* sp. and *Cedecea* sp. are known to produce these same alkylmethoxypyrazines (*9*). Myxobacteria (belonging to the deltaproteobacteria group, *Mixococcales* order) such as *Chondromyces crocatus* produce a fairly large number of hydroxyalkylmethoxypyrazines, as well as MDMP (**1**) and its isomer 2-methoxy-3,6-dimethylpyrazine (**2**) (*10*). Prat et al. (*19*) pointed out that *Pseudomonas fluorescens* is thought to be incapable of forming TCA but able to synthesize MDMP in cork. However, these authors were unable to clearly characterize the presence of this latter substance (detection by GC/olfactometry analysis but unconfirmed by GC/MS). Prat et al. (*19*) deduced their conclusion from the study by Mottram et al. (*5*) reporting that other *Pseudomonas* also produce this molecule, which is not accurate. On the one hand, studies cited by these authors concerning *Pseudomonas* sp. were relative to IPMP and not MDMP, and on the other hand, the bacterium isolated by Mottram et al. (*5*) belongs to the *Rhizobium* rather than the *Pseudomonas* genus, as we have shown. However, in light of the study of restriction profiles of the gene sequence coding for ribosomal ribonucleic acid (16S rDNA), many bacteria previously classified as *Pseudomonas* do not, in fact, belong to this genus (*20*). The modern phylogeny of the genus *Pseudomonas* is not clear, and many intersections with *Rhizobium* (belonging to a group of alphaproteobacteria, *Rihizobiales*) have been noted (*20*, *21*).

The ability of several microorganisms thought to be capable of synthesizing alkylmethoxypyraziness, including MDMP, was confirmed under laboratory conditions (**Table 1**). Among the various proteobacteria studied and compared to a deuteromycota (*Penicilium glabrum*) traditionally identified in cork and able to form TCA (*19*), only *Serratia odorifera* (from the Enterobacteriales order in the gammaproteobacteria group), *C. crocatus*, and *R. excellensis* proved to be able to form MDMP in the presence of a complex source of amino acids (yeast extract). The *P. fluorescens* strain studied under our laboratory conditions did not form MDMP at a concentration detectable by coupling GC/MS to analyze the media (LD = 0. 6 ng/L). To our knowledge, the formation of MDMP by *Serratia* sp. had not been previously reported. Gallois and Grimont (*9*) found that *Serratia rubidaea*, *S. odorifera*, and *Serratia ficaria* mainly produced 3-isopropyl-2-methoxy-5-methylpyrazine and also named 3-isopropyl-2-methoxypyrazine, 3-isobutyl-2-methoxypyrazine, 3-*sec*-butyl-2-methoxypyrazine, and 3-isobutyl-2-methoxy-6-methylpyrazine among other biosynthesized pyrazines, but not MDMP.

The results presented in this study identify for the first time a bacterium of the *Rhizobium* genus, initially isolated from a medium having nothing to do with cork and capable of synthesizing large quantities of MDMP. This kind of microorganism is especially widespread in the soil and could perfectly well contaminate sheets of harvested cork bark, at a stage and under conditions that have yet to be determined. It is also possible that this bacterium may contaminate cork when hollow metal tubes are punched through it due to the lubrication system in the punching machines.

Influence of the Nitrogen Source on the Biosynthesis of MDMP by *R. excellensis*. The way in which alkylmethoxypyrazines are biosynthesized is not well-known. The cause may be the condensation on an α-amino acid with an α,β-carbonal compound such as glyoxal or methylglyoxal (*12*, *13*). Gallois et al. (*7*) contested this pathway for the formation of IPMP by *P. taetrolens*. They suggested instead that pyrazines are formed by the condensation of

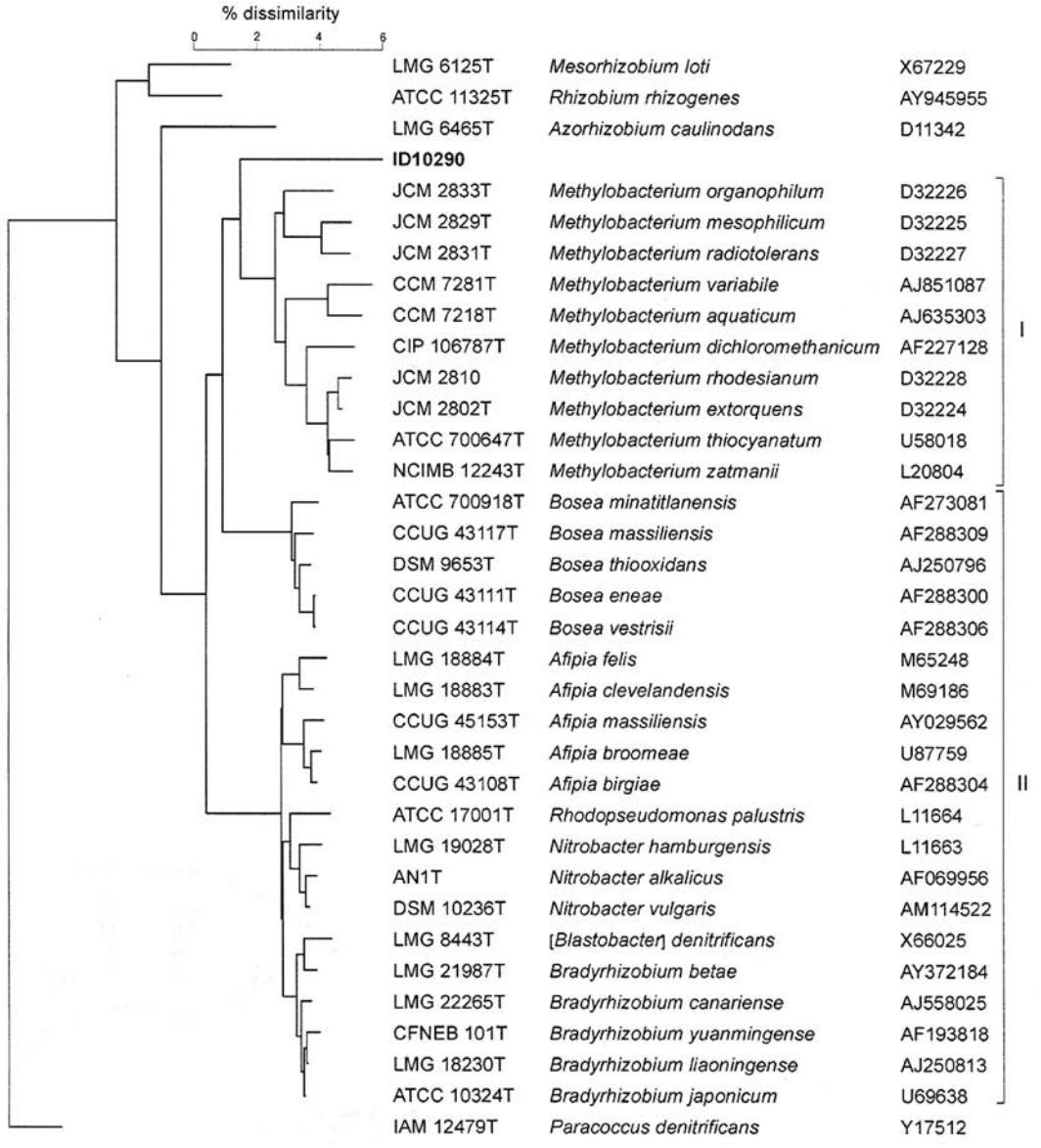

Figure 5. Phylogenic tree constructed using the neighbor-joining method using partial 16S rDNA sequence analysis (ID10290 = bacteria to identify).

Table 1. Ability of Various Microorganisms To Produce MDMP in Synthetic Media

identification	strain origin and identification	medium[a]	production of MDMP[b]
P. perolens	LMG13577/ATCC10757	1	
P. fluorescens	LMG5822/ATCC11251	1	
S. odorifera	LMG7885/ATCC33077	2	0.082
C. crocatus	DSMZ 14606	4	0.088
R. excellensis	NCIMB11808/LMG24089	1	100
P. glabrum	MUCL35034	3	

[a] For composition of medium, see Materials and Methods. [b] Comparative relative production of MDMP analyzed by GC/MS directly in the culture media.

two amino acids because of the difficulty in condensing an α-amino acid with a carbonyl compound. This reaction is, in fact, perfectly normal. Furthermore, it is the root cause of the chemical synthesis of MDMP (*22*): the alanine amide condenses with the methylglyoxal in a basic medium to produce 2-hydroxy-3,5-dimethylpyrazine (2-hydroxy-3,5-DMP) and 2-hydroxy-3,6-dimethylpyrazine (in a proportion of 85:15). The methylation of 2-hydroxy-3,5-DMP directly produces DMDP.

Culturing *R. excellensis* in a minimum mineral medium supplemented with various amino acids shows (**Figure 6**) that L-leucine (**3**) and L-alanine (**4**) plus, to a lesser degree, L-valine (**5**) and phenylalanine (**6**) are mainly responsible for the highest biosynthesis of MDMP (**1**). The nature of the nitrogen source thus has an enormous influence on the formation of MDMP. The presence of an ammonium salt as the sole source of nitrogen is not sufficient to synthesize pyrazine. It seems that only certain amino acids with an apolar chain derived from pyruvate induce the largest quantities of MDMP.

Contamination of Bottled Wine with Cork Stoppers by MDMP. During a tasting of various bottles of a red wine from a Classified Growth of Saint-Emilion (Bordeaux, France) from the 2001 vintage, a large number of corked bottles, with corks from the same batch and from the same producer, had an organoleptic profile very different from the rest of the same wine bottled under

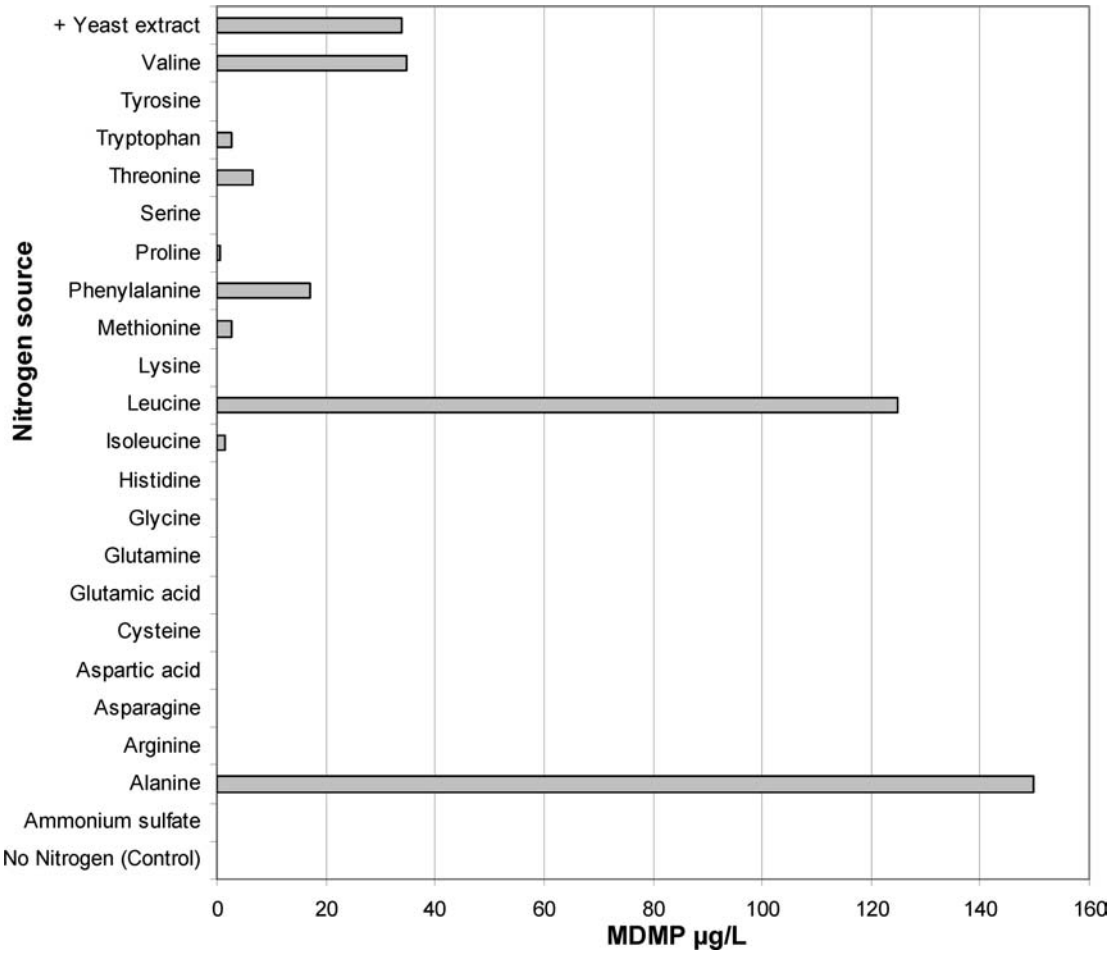

Figure 6. Biosynthesis of MDMP in a minimum medium supplemented with various nitrogen sources (L-amino acid, ammonium sulfate, and yeast extract) by *R. excellensis*.

the same conditions and at the same time, but with corks from three other cork producers. While several bottles had a defect clearly attributable to a "corked flavor" due to a highly particular "moldy" character (average of 1% of the total lot), the person in charge of production was much more worried about the large proportion of bottles (> 30%) that did not have this characteristic flaw but rather a deep and negative change of the original organoleptic profile. In light of the descriptors used (a "corky" and "herbaceous" flaw on the nose, accompanied by a "dry, woody" character on the palate), the fault seemed more likely due to a lack of quality of the wine, rather than to an obvious external contamination due to the cork stoppers.

Wine and corks from several bottles with the problem described above were analyzed, with results presented in **Table 2**. Various compounds such as haloanisoles (*1*−*3*, *23*) thought to confer a "moldy", "corked", and "dusty" character were assayed simultaneously with MDMP in wines and corks after individual maceration for 72 h in a model water and alcohol solution (12% v) to simulate wine. In this instance, 2,4,6-tribromoanisole (TBA) was never detectable (< LOD 0.2 ng/L). The concentration of 2,3,4,6-tetrachloroanisole (TeCA) was consistently identical in all instances, both in wine and in macerated corks. In every case, as confirmed by the presence of pentachloroanisole (PCA) in the same proportions, the contaminant came from an environmental pollution of the wine and cork from the atmosphere of the wine cellar (*24*). Cork stoppers were thus unrelated to the presence of these compounds. Furthermore, the content of these molecules was far beneath detection thresholds and thus was in no way responsible for flaws noted during tastings. Certain wines were found to contain quantities of 2,4,6-trichloroanisole (TCA) that reached or exceeded the detection threshold of this contaminant and thus displayed a "moldy" odor and very typical "corked" characteristics when concentrations reached approximately 2 ng/L (*2*). This compound, whose concentration varied greatly from one bottle to another, seemed evidently to come from the corks that were used (**Figure 7**). In the same way, MDMP measured at levels of 1.4−3.5 ng/L in affected wines was correlated to MDMP in corresponding macerated corks (**Figure 8**). According to Simpson et al. (*4*), as opposed to TCA, which has a strong affinity for cork tissue, it seems much easier for wine to extract MDMP from corks. This property may explain why there is a less obvious correlation between MDMP in wine and the residual quantity detectable *a posteriori* in incriminated corks in certain instances. There is no obvious relationship between the concentration of TCA and MDMP in either wines or corks (**Figure 9**), which inevitably led to positing totally different biochemical origins.

MDMP Content in Different Batches of Natural Cork Stoppers. We analyzed the total MDMP content obtained from several batches of finely ground natural corks (49 × 24 mm, first grade by visual aspect) randomly sampled by extraction with dichloromethane before use. These corks came from five suppliers (all in Portugal) and were proposed to the same wine estate over a period of 36 months (43 lots for a total of just under 500000 corks).

We found that MDMP was present (86% > 2.0 ng/cork) (**Figure 10**) in a large majority of batches (minium = 0, maximum = 29.97, average = 9.28, median = 8.70, standard

Table 2. Wine and Corresponding Cork Analysis of Various Bottles of the Same Wine with More or Less Organoleptic Alteration Detected by Sensorial Analysis for "Musty" and "Corky" Contaminants

	cork, ng/L per cork[a]					wine, ng/L					
bottle	MDMP	TCA	TeCA	PCA	TBA	MDMP	TCA	TeCA	PCA	TBA	taint by tasting
control	nd	nd	1.0	2.5	nd	nd	nd	1.5	1.8	nd	no
1	nd	0.9	1.2	2.5	nd	nd	0.8	1.4	2.0	nd	weak
2	nd	0.8	1.2	2.5	nd	nd	0.6	1.6	2.4	nd	weak
3	traces	0.6	1.2	2.3	nd	1.4	1.4	1.5	2.8	nd	yes
4	nd	0.8	1.0	2.6	nd	nd	1.6	1.5	1.8	nd	yes
5	3.5	0.8	1.0	2.5	nd	2.9	1.1	1.6	2.0	nd	strong
6	traces	0.8	1.1	2.0	nd	3.2	2.9	1.6	2.5	nd	strong
7	5.1	1.0	1.0	2.5	nd	3.5	0.8	1.2	1.8	nd	strong
8	nd	1.7	1.1	2.7	nd	nd	1.8	1.5	1.8	nd	musty (TCA)
	Characteristics of the Analytical Method in ng/L[b]										
LOD	0.7	0.3	0.1		0.2						
LOQ	1.8	0.5	0.7		0.7						
RSD%	2.6	2.0	6.5		1.8						

[a] Releasable contaminants from the maceration of individual cork in a model hydroalcoholic solution. [b] From ref *16*. Key: LOD, limit of detection; LOQ, limit of quantification; RSD%, relative standard deviation.

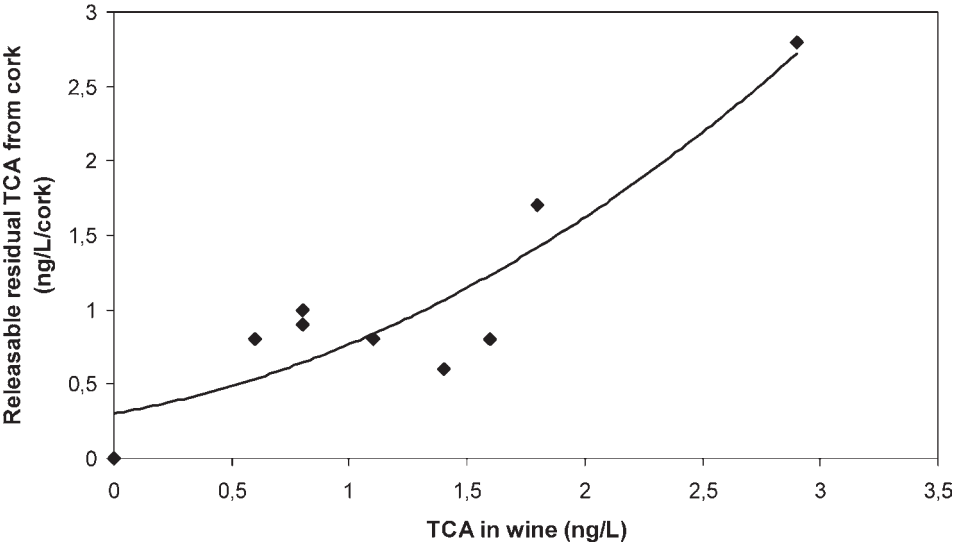

Figure 7. Correlation between residual releasable TCA analyzed in the cork and released TCA in bottles of a same wine with a "corky" and "green nuts" character detected by sensorial analysis.

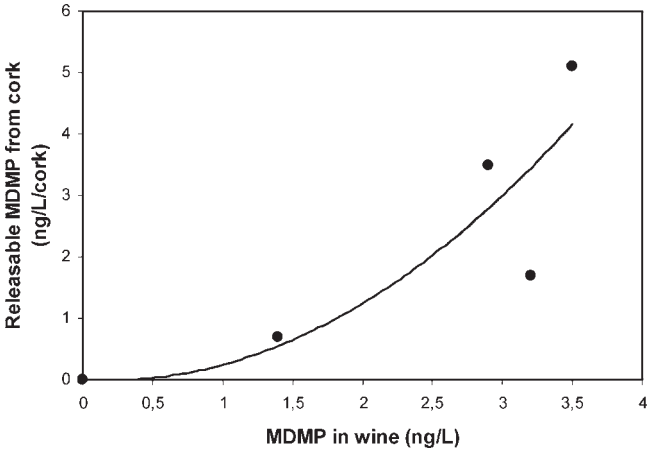

Figure 8. Correlation between residual releasable MDMP analyzed in the cork and released MDMP in bottles of a same wine with a "corky" and "green nuts" character detected by sensorial analysis.

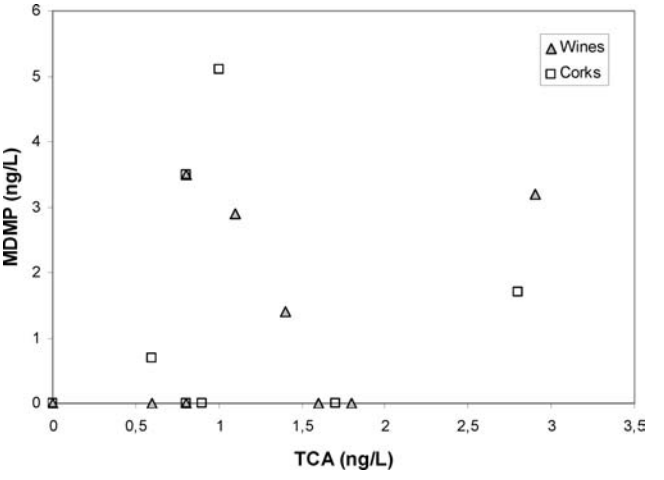

Figure 9. Correlation between TCA and MDMP in bottled wine and their corresponding cork stoppers.

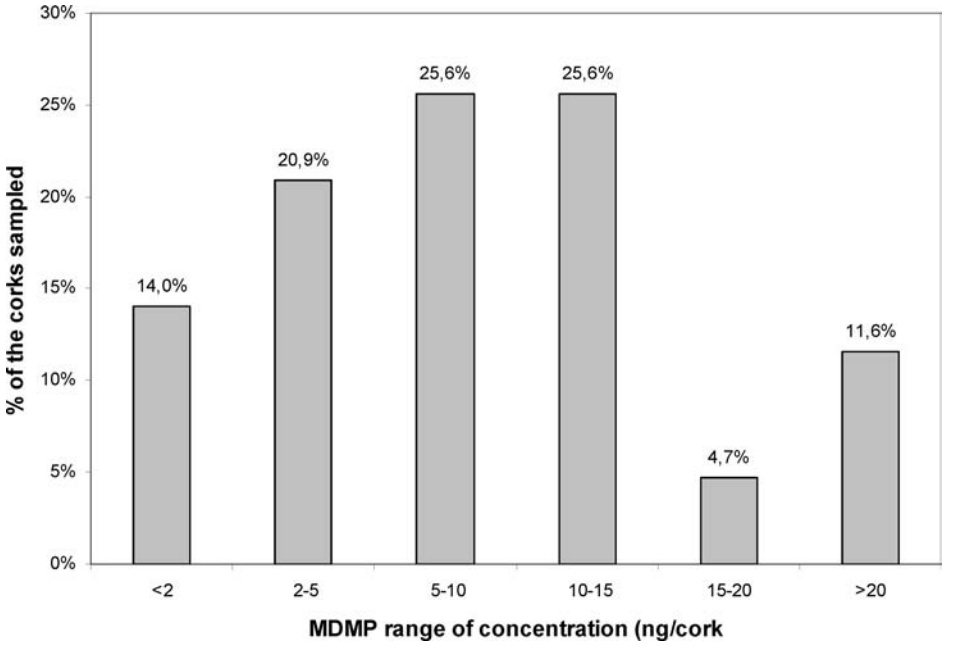

Figure 10. Repartition of the MDMP content in various lots ($n = 43$) of new cork stoppers offered to the same winery during a period of 36 months (LOQ = 2.0 ng/cork) and controlled before their use.

deviation = 7.34 ng/cork). At the present time, we do not know the relationship between measurable MDMP in an organic extract of ground cork and the amount likely to be actually extracted in bottles of corked wine. We believe that stoppers with less than 5.0 ng/cork (corresponding to 34.9% of batches) represent little or no risk of contamination, that batches with 5.1−15.0 ng/cork of MDMP concerning 51.2% of batches undoubtedly represent a more serious risk for bottled wine, whereas strongly contaminated lots (> 15.1 ng/cork) corresponding to 16.3% of the commercial batches analyzed in this particular case obviously represent an extreme risk.

These same batches of corks were systematically monitored using the extractable TCA assay protocol (50 corks/1000 mL of model solution) by HSSPME/GC/MS (*25*, *26*). All of the batches were accepted for use because they contained less than 2.4 ng/L extractable TCA (results not presented), a content presenting a statistical risk of 3%, and the maximum level judged to be acceptable (*27*). With regard to MDMP, even if the proportion of corks individually contaminated within batches was not known, over three-quarters (77.5%) of these batches could be considered to present a risk. This proportion is much greater than the worst statistics for TCA contamination, up until now considered the most crucial for corks.

Identification of MDMP in Wines in Contact with Oak but Not with Cork. Red wine from a same lot produced in the Languedoc region of France from Syrah and Grenache grapes (30:70) was divided into three separate stainless steel vats of varying capacities. Two out of three vats contained slightly toasted oak chips (3 g/L) removed from their original packaging just before utilization. After 4 weeks, the winemaker who regularly tasted all the vats noticed a change in the aroma and taste of the wines in contact with oak chips, whereas the wine that was not in contact with oak was judged not to have any off aromas or flavors whatsoever.

The assay of contaminants in the wines by HSSPME/GC/MS (**Table 3**) showed that both vats of wine of doubtful quality were massively contaminated with MDMP, as much as five to seven times the detection threshold. The wine in vat no. 2 containing the most MDMP (15.3 ± 0.40 ng/L) also contained TCA (1.6 ± 0.03 ng/L), an amount approaching the detection threshold. On the other hand, the same wine that was not aged with wood chips had no MDMP or TCA in detectable concentrations. As no residue of the wood chips involved in the experiment was available, we were unable to analyze the oak concerned. However, seeing as the conditions were extremely straightforward, the results showed that sources other than cork stoppers can contaminate wines with MDMP and in particular oak wood.

Table 3. Evidence of MDMP (**1**) and Haloanisole Contamination in the Same Wine Aged in Different Stainless Steel Tanks with or without Oak Wood Chips during Their Aging

	ng/L				
	TCA	TeCA	PCA	TBA	MDMP
control wine	nd	5.2	11.8	traces	nd
tank 1 + chips	traces	5.2	11.5	traces	11.0
tank 2 + chips	1.3	5.6	12.8	traces	15.3

The fact that loads of scrap wood used to make industrial wood chips are often stored on the ground (*28*) may explain their contamination by the telluric bacteria identified in this study and thus the formation of MDMP before toasting.

Toasting oak is a very common step in barrel making and the preparation of alternative products made with oak fragments. The thermal treatment of oak takes place at 180−220 °C and exposes the wood to radiative or convective heating for a duration of approximately 15−60 min (*29*). We have shown that heating quickly eliminates MDMP (**Figure 11**). Thus, the thermal treatment of oak under conditions usually found in cooperages and in the production of alternative forms of oak aging should be sufficient to guarantee the elimination of this source of pollution. However, oak that is toasted only lightly, or not at all (which is frequently the case with industrial alternatives to traditional barrel aging), always runs the risk of containing residual amounts of MDMP likely to affect the aromatic quality of wines. Heating to a temperature of 105 °C for 10 min only reduced the MDMP content of the oak originally contaminated with 27.4 ± 0.67 ng/g by about 50% (12.6 ± 2.4 ng/g) but toasting at 220 °C during the same time reduce by 93% the original MDMP content.

In conclusion, our study identified a new species of proteobacteria of the *Rhizobium* genus able to form large quantities of MDMP, a

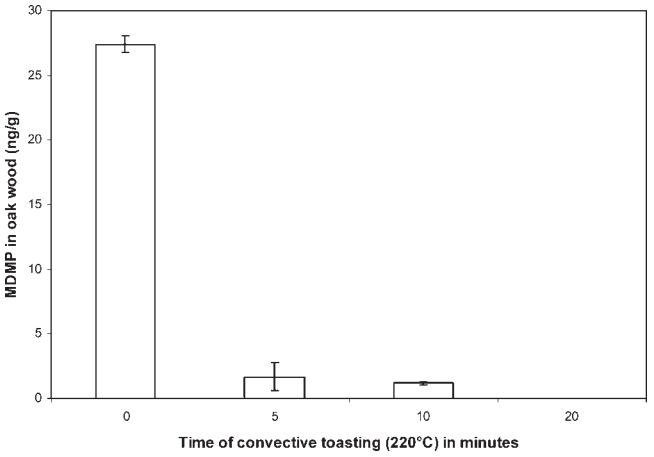

Figure 11. Thermal degradation of MDMP during the convective toasting at 220 °C of oak wood chips.

volatile and strong-smelling substance. This compound is detectable in cork stoppers and in wines with a "corky" and "dusty" olfactory character that is fairly different from the typical "fungal" or "moldy" odor of TCA. In light of various previously published studies and the results of our own study, it appears extremely probable that *R. excellensis* is the microorganism primarily responsible for the presence of this molecule in cork. However, other observations show that cork is not the only vector for polluting wine. Oak can also be contaminated and thus affect wines with which it comes into contact. Nevertheless, in light of MDMP's sensitivity to temperature, oak should not represent a major risk of pollution if sufficiently toasted. It would be interesting to monitor a larger number of samples of wood chips in order to become better informed of the extent to which MDMP is present in this type of vector. As for cork stoppers, in light of the physicochemical characteristics of this molecule, it is likely that decontamination techniques that have proved effective to reduce the concentration of TCA (treatment with water vapor, a combination of vapor and ethanol, and extraction with supercritical CO_2) are also perfectly suited to eliminating MDMP. On the other hand, due to the frequent presence of MDMP in natural cork stoppers in quantities greater than 10 ng/cork (> 40%), this type of stopper seems to possess an extremely worrying potential for polluting wine, which implies the need for systematic monitoring before use, in much the same way as has been done with regard to TCA these past few years. At the present time, we do not know at exactly what stage the contamination of wine by MDMP occurs. The origin of the microorganism in question makes it seem likely that the raw material (sheets of cork, scrap wood) stored on, or near, the soil are easily contaminated. Further investigations are necessary in order to confirm the presence of *R. excellensis* in cork and oak and to define the conditions under this substance appears, accumulates or disappears during processing and storage.

NOTE ADDED AFTER ASAP PUBLICATION

This paper published ASAP November 8, 2010 with errors in the text, and in Table 2 footnote b. The correct version published November 11, 2010.

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Received for review July 23, 2010. Revised manuscript received October 18, 2010. Accepted October 18, 2010.