Evidence for differences between *B. bruxellensis* strains originating from an enological environment

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Abstract: The aim of this paper is to study and compare the physiological diversity of different strains of a wine spoilage yeast species: *Brettanomyces bruxellensis*. The minimum inhibitory concentrations of several drugs on different *B. bruxellensis* strains were scored on solid nutrient media. This revealed variations in resistance among the *B. bruxellensis* strains. Their capacity to develop in different wine and must environments: pH, ethanol, and SO_2 concentrations, were evaluated by measuring the direct incubation survival rate. The results, compared with those obtained for other wine yeast species, confirmed the remarkable resistance of *B. bruxellensis* strains to various conditions which inhibit the growth of other species. Nevertheless some differences were observed among the *B. bruxellensis* strains, thus confirming their physiological diversity. A comparison of their volatile phenol production revealed intraspecific heterogeneity among *B. bruxellensis* strains. *B. bruxellensis* is one of the microbial species most resistant to environmental constraints in wine. It is the best adapted to growing in wine and spoiling it by volatile phenol production. However, different *B. bruxellensis* strains exhibit varying characteristics, particularly their capacity to produce volatile phenols. This implies that certain strains are more prejudicial than others. Further studies are required to determine the molecular causes of this intraspecific diversity.

Keywords: *Brettanomyces bruxellensis*, strain diversity, physiology, volatile phenols

Introduction

Among microbial deteriorations of wine, the production of volatile phenols, 4-ethylphenol and 4-ethylguaiacol, by *Brettanomyces bruxellensis* yeast is certainly the most dreaded by winemakers and the most criticized by consumers. A great deal of research has focused on this subject.[1] Previous work investigated the origins of *B. bruxellensis*, the conditions it required to develop in wine,[2,3] and its interactions with other microorganisms.[4] Several techniques for eliminating these microorganisms were also studied.[5] Fast, effective detection methods were also developed.[6]

Microbial identification had always been restricted to the species level, until a recently-reported macrorestriction analysis of the chromosomal DNA by pulsed-field gel electrophoresis (REA-PFGE) method made it possible to differentiate among *B. bruxellensis* strains.[7] Distinguishing among strains is particularly useful as it makes it possible to clarify how contamination occurs and study intraspecific diversity.[8] Studies of differences between *Brettanomyces* isolates were essential[9,10] to explain the differences in spoilage due to volatile phenol synthesis observed during production.

The goal of this work is to characterize the physiology of the *B. bruxellensis* strains isolated at different stages, from grapes on the vine to bottled wine. These *B. bruxellensis* strains were also compared with other yeast genera. Firstly, we measured the resistance of each strain to some inhibitory compounds, and then we tested their capacity to establish colonies in grape must and wine, at various levels of pH, alcohol, and SO_2. Finally, volatile phenol production in grape must was evaluated in the laboratory.

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Materials and methods

Microorganisms

B. bruxellensis strains used in this study are listed in Table 1. They were isolated from a Bordeaux domain at different stages in the winemaking process, from grapes through to bottled wine. Wines samples were used to inoculate solid nutritive medium specific to the non-*Saccharomyces* yeast species. This medium is composed of glucose (20 g/L), yeast extract (10 g/L), bactotryptone (10 g/L), agar (20 g/L), biphenyl (150 mg/L), cloramphenicol (100 mg/L), cycloheximide (500 mg/L), pH = 5.0 with H_3P0_4 and aerobic incubation at 25 °C for 10 days. After colonies were tested by the species-specific polymerase chain reaction (PCR) species developed by Ibeas[11] in order to identify *B. bruxellensis* isolates at the species level. After positive colonies of *B. bruxellensis* were compared from point of the strain typing by REA-PFGE developed by Miot-Sertier.[7] This method is the most relevant method to type strains of *B. bruxellensis* isolated throughout the winemaking process[1,7,12] by providing a specific PFGE pattern for each strain. For each different pattern obtained by the REA-PFGE method during our monitoring a strain were conserved at –80 °C in the IOEB (Institute of Oenology of Bordeaux) collection. The list and the nomenclature of these strains are provided in Table 1.

Microorganism cultures

Starter cultures were prepared in a grape-juice medium consisting of 200 mL/L commercial grape juice, 40 mL/L ethanol, 1.5 g/L malt extract, 1.5 g/L yeast extract, 0.5 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4$. The pH was adjusted to 5.0 using KOH 5N. Growth was monitored by epifluorescence, using the protocol developed by Millet and Lonvaud-Funel.[13] For each experiment, microbial cells were collected at the end of the exponential growth phase.

Table 1 List of strains used in this study

Species	Designation	Origin
Brettanomyces bruxellensis	*IOEBL0411	Fermenting must during alcoholic fermentation
	IOEBL0407	Wine during aging
	IOEBL0447	Fermenting must during malolactic fermentation
	IOEBL0462	Wine during aging
	IOEBL0468	Grapes
	IOEBL0469	Grapes
	IOEBL0506	Bottled wine
	IOEBL0522	Bottled wine
	CLIB300	Belgian beer
Candida cantarellii	IOEBL0416	Fermenting must
Metschnikowia fructicola	IOEBL0416	Must after pressing
Piichia anomala	IOEBL0534	Fermenting must
Rhodotorual mucilaginosa	CLIB370	Grapes
Saccharomyces cerevisiae	Levain 522 Davis	Industrial strain

Notes: *IOEBL, Yeast collection of the Institute of Enology of Bordeaux, Talence, France; CLIB, collection de levures d'intérêt biotechnologique, INRA, Thiverval Grignon, France.

Measurement of minimum inhibitory concentrations

Drug sensitivity was assayed by measuring the minimum inhibitory concentration (MIC) in a medium similar to that used for the starter cultures, with 20 g/L of agar, containing different drug concentrations between 10 g/L and 10 mg/L (10, 5, 2.5, 1.25, 0.6, 0.4, 0.25, 0.2, 0.15, 0.100, 0.050, 0.025, 0.010 g/L). The initial drug solutions were prepared in ethanol (70% v/v). 10 µL viable yeasts were plated on the Petri dishes using the Steer's equipment, at an initial concentration of 10^6 cells/mL. In all cases, drug resistance was scored by the presence or absence of observation colonies on the agar surface after ten days' incubation at 25 °C. A growth positive control was prepared without any drugs for each strain. Triplicate assays were carried out at each concentration.

The drug tested were Congo red (Sigma-Aldrich, St. Louis, MO), Calcofluor white (Sigma-Aldrich), and vanillin (Sigma-Aldrich). Vanillin has a phenolic function that interacts with enzymes involved in essential redox reactions.[14] Its amphilic properties disturb the phospholipid organization in the cytoplasm membrane.[15] Calcofluor white binds microfibrils of cellulose or chitin, thus disrupting their assembly and leading to cell lysis.[16] This inhibitory activity is similar to that of Congo red, which also affects the cell-wall organization.[17]

Measuring the yeasts' development capacity

The strains' capacity to establish themselves at varying levels of pH, ethanol, and free SO_2, was evaluated by the relationship, τ48, between the population counted two hours after direct inoculation of cells pre-cultured and collected at the end of the exponential phase into must or wine previously sterilized by filtration (0.22 µm). The concentration was adjusted to 10^3 cells/mL in the medium inoculated. The population was counted after forty-eight hours of incubation at 25 °C.

Two pH values were tested, 3 and 3.6, in grape must (glucose + fructose = 200 g/L, total phenolic index = 35, L-malic-acid = 4.2 g/L). Ethanol was tested at 12.5% v/v and 15% v/v in homogenized wine (pH = 3.6, total phenolic index = 76, free SO_2 = 4 mg/L, total SO_2 = 22 mg/L). The two

SO_2 levels tested were 10 mg/L and 30 mg/L free SO_2 in wine collected at the end of malolactic fermentation (MLF) (pH = 3.6, ethanol = 12.5% v/v, total phenolic index = 78). All these media were sterilized by filtration (0.22 μm).

Measuring volatile phenol production

Volatile phenol production was evaluated in grape-juice cultures (pH = 3.6, glucose + fructose = 200 g/L, L-malic acid = 4.2 g/L, total phenol index = 35). Microbial cells collected at the end of exponential growth phase were inoculated at an initial concentration of 10^3 CFU/mL. Alcoholic fermentation (AF) was monitored by measuring glucose and fructose, using an enzymatic kit (Boehringer Mannheim GmbH, Mannheim, Germany). In each assay, volatile phenols were measured when AF was completed. Each assay in triplicate was compared with negative control without microbial cells. Volatile phenols were measured by dichloromethane extraction from 50 mL grape juice, followed by gas chromatography.[18] The temperature was 25 °C.

Results

Minimum inhibitory concentrations

Table 2 lists the MIC of the species and strains tested. Three types of resistance were distinguished. Firstly, some species were highly sensitive to the drugs used: *C. cantarelli* and *M. fructicola*. *S. cerevisiae* exhibited intermediate resistance and two species were highly resistant: *B. bruxellensis* and *P. anomala*. There was considerable intraspecific diversity within the *B. bruxellensis* species in terms of resistance to Calcofluor white and vanillin, whereas the MICs for the other two drugs were the same for each strain. The same strain (IOEBL 0447) was the most resistant to Calcofluor white and vanillin, but it was difficult to identify a correlation between these two resistances, as the IEOBL 0468 strain was one of the most resistant to vanillin and one of the most sensitive to Calcofluor white, and inversely for IOEBL 0522.

Capacity to develop following inoculation

B. bruxellensis had a greater capacity than other species to resist direct inoculation in wine (Table 3) and was less affected by variations in pH, alcohol content, and SO_2. Unsurprisingly, *S. cerevisiae* was the species best suited to developing in grape juice. Its development rate was three times higher at pH = 3.6 than at pH = 3, whereas this value did not change significantly for *B. bruxellensis* strains. After AF, the survival rate of *S. cerevisiae* decreased by 100-fold at an ethanol content of 12.5% v/v, and 1000-fold at 15% v/v, while it remained relatively stable for *B. bruxellensis* strains. Unlike the other species tested, the latter exhibited strong resistance to SO_2. In two cases, the cell-count decreased by 10-fold (*R. mucilaginosa*) or 100-fold (*M. fructicola*) after forty-eight hours of incubation in wine containing only 10 mg/L free SO_2.

The key observation concerned the significant differences between *B. bruxellensis* strains. The CLIB300 strain was the least and IOEBL 0447 the most resistant to direct incubation under all conditions. The latter was more resistant to inoculation in must than in post-fermentation wines, irrespective of pH. This was not the case for the IOEBL 0462 strain, which developed twice as well at pH = 3.6 than pH = 3.0.

Table 2 Comparison of minimum inhibitory concentrations for different drugs on solid medium

		Calcofluor white (g/L)	Congo red (g/L)	Vanillin (g/L)
B. bruxellensis	CLIB300	0.6	2.5	1.25
	IOEBL0468	0.3	2.5	5
	IOEBL0469	0.3	2.5	1.25
	IOEBL0447	1.25	2.5	5
	IOEBL0407	0.3	2.5	1.25
	IOEBL0462	1.25	2.5	2.5
	IOEBL0453	0.3	2.5	1.25
	IOEBL0506	0.3	2.5	2.5
	IOEBL0522	1.25	2.5	1.25
S. cerevisiae	522 Davis	0.3	1.25	1.25
Metschnikowia fructicola	IOEBL0530	0.3	0.3	0.3
Rhodotorula mucilaginosa	CLIB370	0.6	2.5	2.5
Candida cantarelli	IOEBL0416	0.15	0.3	0.6
Pichia anomala	IOEBL0534	0.6	2.5	2.5

Table 3 Comparison of the capacity to develop following inoculation

		Must pH = 3	Must pH = 3.6	Wine after AF and before MLF pH = 3.6 ethanol content = 12.5% v/v	Wine after AF and before MLF pH = 3.6 ethanol content = 15% v/v	Post-fermentation wine pH = 3.6 ethanol content = 13% v/v, free SO_2 = 10 mg/L H_2SO_4	Post-fermentation wine pH = 3.6 ethanol content = 13% v/v, free SO_2 = 30 mg/L H_2SO_4
B. bruxellensis	CLIB300	1.6 ± 0.7	5.8 ± 2.3	1.5 ± 0.5	0.7 ± 0.1	1.8 ± 0.4	0.6 ± 0.2
	IOEBL0468	4.2 ± 0.3	4.4 ± 0.7	1.9 ± 1.0	1.5 ± 0.2	0.8 ± 0.1	0.7 ± 0.3
	IOEBL0469	5.3 ± 2.6	9.5 ± 1.6	6.5 ± 3.9	1.6 ± 0.9	1.7 ± 0.4	0.9 ± 0.1
	IOEBL0447	24.1 ± 2.9	27.1 ± 1.7	4.6 ± 2.6	5.4 ± 0.9	9.8 ± 1.4	3.4 ± 0.3
	IOEBL0407	12.1 ± 1.9	13.0 ± 1.1	1.2 ± 0.4	1.4 ± 0.4	1.3 ± 0.4	0.3 ± 0.2
	IOEBL0462	11.0 ± 1.2	21.1 ± 6.8	2.7 ± 1.0	0.8 ± 0.1	4.5 ± 0.8	2.4 ± 0.4
	IOEBL0453	15.4 ± 1.2	8.6 ± 2.6	1.4 ± 0.5	1.3 ± 0.1	0.9 ± 0.1	0.5 ± 0.3
	IOEBL0506	1.2 ± 0.3	1.30 ± 0.03	2.0 ± 0.8	1.6 ± 0.2	2.4 ± 1.1	0.6 ± 0.2
	IOEBL0522	1.2 ± 0.5	1.0 ± 0.1	1.7 ± 1.4	1.6 ± 0.2	4.7 ± 2.3	5.1 ± 0.2
S. cerevisiae	522 Davis	32.7 ± 4.7	93.9 ± 2.5	0.2 ± 0.05	0.06 ± 0.02	0.15 ± 0.06	0.08 ± 0.02
Metschnikowia fructicola	IOEBL0530	1.0 ± 0.7	27.0 ± 1.8	0.7 ± 0.2	0.06 ± 0.04	0.04 ± 0.03	0.02 ± 0.01
Rhodotorula mucilaginosa	CLIB370	0.1 ± 0.06	2.0 ± 1.4	0.07 ± 0.03	0.05 ± 0.03	0.15 ± 0.05	0.02 ± 0.01
Candida cantarelli	IOEB0L416	2.8 ± 1.0	2.0 ± 0.8	1.5 ± 0.5	0.03 ± 0.01	0.09 ± 0.05	0.04 ± 0.01
Pichia anomala	IOEBL0534	10.2 ± 0.8	19.6 ± 0.2	2.9 ± 0.8	1.3 ± 0.2	1.5 ± 0.3	0.07 ± 0.02

Abbreviations: AF, alcoholic fermentation; MLF, malolactic fermentation.

Volatile phenol production

All the *B. bruxellensis* strains produced volatile phenols, especially 4-ethylphenol and 4-ethylguaiacol. However, amounts of volatile phenols produced varied by over three-fold according to the strain: 330 µg/L 4-ethylphenol for the IOEBL 0469 strain and 1200 µg/L for IOEBL 0462). Moreover, the ratio of 4-ethylphenol and 4-ethylguaiacol produced varied from one strain to another. Under the test conditions, it was nearly two for most strains. However, certain strains, such as IOEB L0447, produced equal amounts of 4-ethylphenol and 4-ethylguaiacol.

The other yeast species tested also produced volatile phenols, but only compounds with vinyl functions: 4-vinylphenol and 4-vinylguaiacol. Among these species, *S. cerevisiae* and *C. cantarelli* produced the largest amounts. Finally, while the *B. bruxellensis* strains produced more 4-ethyphenol than 4-ethylguaiacol, the other yeast species produced more 4-vinylguaiacol than 4-vinylphenol, except *P. anomola*, which also produced little 4-ethylphenol.

Discussion

The inhibitory concentrations for the drugs tested were obviously too high to be used to prevent *B. bruxellensis* development in wine but the findings revealed physiological differences among *B. bruxellensis* strains in terms of sensitivity or resistance to toxic constraints. Even if *B. bruxellensis* strains were more resistant than the other species to the drugs tested, the MIC measurements revealed significant differences in responses to Calcofluor white and vanillin. These compounds act on cell-wall and membrane organisation, resulting in different resistance to various stressors, notably alcohol, one of the most important in wine, and others, due to cross-tolerance phenomena.[19] The high resistance of *B. bruxellensis* strains to toxic pressure on a laboratory scale was not sufficient to determine their capacity to resist in wine, but revealed that they were able to adapt their metabolism to constraining environmental conditions.

That was confirmed by their ability to grow after direct incubation in wine. The most resistant strain to Calcofluor white and vanillin had also tolerated incubation in wine with a higher ethanol level. In general, *B. bruxellensis* strains were more resistant to ethanol than the other species, while *S. cerevisiae* performed better at high sugar concentrations. This explains the prevalence of *S. cerevisiae* strains during the first phases of AF, when the medium has a high sugar content and osmotic pressure.[20] *S. cerevisiae* had a higher growth rate under these conditions than the other genera. In contrast, *B. bruxellensis* prospers at the end of sluggish fermentations, when the ethanol content reaches high values.[4,21,22] Moreover *B. bruxellensis* is not very demanding from a nutritional point of view.[23] Certain strains, notably those which originated from fermented wines, established themselves more easily in must than others, and were more resistant to high ethanol and SO_2 levels. These findings, however, did not establish a direct correlation between the stage where the strains were isolated and their intrinsic capacity to develop, but simply indicated

Table 4 Volatile phenol production

		4-ethylphenol (µg/L)	4-ethylguaiacol (µg/L)	4-vinylphenol (µg/L)	4-vinylguaiacol (µg/L)
B. bruxellensis	CLIB300	605 ± 60	390 ± 40	8 ± 2	22 ± 2
	IOEBL0468	775 ± 35	345 ± 65	0	0
	IOEBL0469	330 ± 20	180 ± 22	0	6 ± 2
	IOEBL0447	950 ± 20	540 ± 40	12 ± 3	20 ± 2
	IOEBL0407	780 ± 70	760 ± 50	16 ± 2	22 ± 6
	IOEBL0462	1200 ± 90	690 ± 40	4 ± 2	26 ± 2
	IOEBL0453	770 ± 30	380 ± 10	4 ± 2	2 ± 2
	IOEBL0506	850 ± 68	520 ± 8	0	0
	IOEBL0522	1060 ± 110	800 ± 20	0	0
S. cerevisiae	522 Davis	0	0	60 ± 6	264 ± 12
Metschnikowia fructicola	IOEBL0530	0	0	26 ± 4	120 ± 8
Rhodotorula mucilaginosa	CLIB370	0	0	14 ± 3	40 ± 14
Candida cantarelli	IOEBL0416	10 ± 3	0	100 ± 4	530 ± 20
Pichia anomala	IOEBL0451	54 ± 4	0	226 ± 12	47 ± 3
Control: sterile must		**0**	**0**	**12 ± 4**	**22 ± 2**

that they were preferentially selected during fermentation. Some strains were clearly more sensitive than others to variations in pH, ethanol content, and SO_2, resulting in the preferential selection of some strains and the elimination of others, resulting in the decrease in intraspecific heterogeneity during the winemaking process observed by Miot-Sertier.[7]

Volatile phenol production also varied according to the strain. Some strains are likely to be more prejudicial than others with regard to the problem of volatile phenols. Further work in molecular characterization, focused on identifying enzymes, genes, and the regulation patterns involved, is required to elucidate the volatile phenol production mechanism. Comparisons with other species that produce only vinyl derivatives will be made.

The *B. bruxellensis* strains collected in vineyards[24] differed from one estate to another,[25] so the physiological differences identified in this research may explain why certain cellars are more exposed to this problem than others and why nonobservance of hygiene recommendations is not the only relevant parameter. The prevalence and degree of spoilage may vary according to the ability of the strains present to develop under winemaking conditions and their capacity to produce volatile phenols.

Disclosure

The authors report no conflicts of interest in this work.

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