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Ustilago maydis killer toxin as a new tool for the biocontrol of the wine spoilage yeast *Brettanomyces bruxellensis*

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ABSTRACT

Brettanomyces bruxellensis is one of the most damaging species for wine quality, and tools for controlling its growth are limited. In this study, thirty-nine strains belonging to *Saccharomyces cerevisiae* and *B. bruxellensis* have been isolated from wineries, identified and then tested against a panel of thirty-nine killer yeasts. Here, for the first time, the killer activity of *Ustilago maydis* is proven to be effective against *B. bruxellensis*. Mixed cultures in winemaking conditions show that *U. maydis* CYC 1410 has the ability to inhibit *B. bruxellensis*, while *S. cerevisiae* is fully resistant to its killer activity, indicating that it could be used in wine fermentation to avoid the development of *B. bruxellensis* without undesirable effects on the fermentative yeast. The characterization of the dsRNAs isolated and purified from *U. maydis* CYC 1410 indicated that this strain produces a KP6-related toxin. Killer toxin extracts were active against *B. bruxellensis* at pH values between 3.0 and 4.5 and temperatures comprised between 15 °C and 25 °C, confirming their biocontrol activity in winemaking and wine aging conditions. Furthermore, small amounts (100 AU/ml) of killer toxin extracts from *U. maydis* significantly reduced the amount of 4-ethylphenol produced by *B. bruxellensis*, indicating that in addition to the growth inhibition observed for high killer toxin concentrations (ranging from 400 to 2000 AU/ml), small amounts of the toxin are able to reduce the production of volatile phenols responsible for the aroma defects in wines caused by *B. bruxellensis*.

1. Introduction

The transformation of grape into wine by alcoholic fermentation is the result of the sequential development and metabolic activity of various species and strains of yeasts (Fleet and Heard, 1993; Sangorrín et al., 2007). Nowadays, the growth of spoilage microorganisms is one of the most serious problems facing the wine industry, causing serious financial loss (Loureiro and Malfeito-Ferreira, 2003; Snowdon et al., 2006). Yeast of the genus *Brettanomyces*, or its teleomorph *Dekkera*, is among the many naturally occurring types of yeast in the winemaking process. *Brettanomyces* yeasts, in particular *Brettanomyces bruxellensis*, are regarded as spoilage microorganisms with the potential to drastically alter the final outcome of red wine under uncontrolled conditions. These yeasts are well known to be involved in the production of volatile phenols in wines that lead to aroma defects usually described as "horse sweat", "leather" and "animal" (Chatonnet et al., 1995, 1997; Heresztyn, 1986; Suárez et al., 2007).

Phenolic acids, also called hydroxycinnamic acids, are present in red grapes (Golderg et al., 1998; Shinohara et al., 2000). The transformation of hydroxycinnamic acids into volatile phenols is known to be predominantly associated with the activity of the genus *Dekkera/Brettanomyces* (Chatonnet et al., 1995; Coulon et al., 2010; Heresztyn, 1986; Oelofse et al., 2009; Romano et al., 2008). Therefore, the monitoring and control of these organisms both in the wine itself and on its contact surfaces is of major importance for wine producers (Chatonnet et al., 1993; Oelofse et al., 2009). The financial loss and reputational damage associated with *Brettanomyces* mean that the wine industry is constantly seeking to optimize current methods and find new ways of monitoring and controlling this problem (Dai et al., 2010; Du Toit et al., 2005; Puértolas et al., 2009; Santos et al., 2009).

Biological control is a non-hazardous alternative to the use of chemical compounds that involves the use of biological processes to reduce crop and environmental damage. In the food and beverage industries, killer yeasts and their toxins have many potential applications and have been proposed to combat contamination by wild yeasts (Ciani and Fatichenti, 2001; Goretti et al., 2009; Jacobs and Van Vuuren, 1991; Liu and Tsao, 2009; Lowes et al., 2000; Palpacelli et al., 1991; Santos and Marquina, 2004). An interesting application of this biological activity in winemaking involves the killer yeasts used to control the proliferation of spoilage microorganisms (Comitini et al., 2004; Comitini and Ciani, 2010; Santos et al., 2009).

The species and strains of the genus *Ustilago* are well-known killer toxin-producers, especially *Ustilago maydis* (Gage et al., 2002; Park et al., 1996a; Tao et al., 1990). *U. maydis* killer toxins are small proteins

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encoded by dsRNA mycoviruses. Three toxins from *U. maydis* are known, KP1, KP4 and KP6, which are encoded on specific medium-size (M) segments of the *U. maydis* viruses P1, P4 and P6, respectively (Tao et al., 1990). Although the molecular nature of the killer aspect of *U. maydis* has been well understood, there have been no attempts to use this feature in food and beverage preservation. This paper addresses the inactivation of *Brettanomyces* populations in wine by killer yeasts. The resistance/sensitivity profiles of several strains of *B. bruxellensis* have been evaluated against a panel of different killer yeasts from collections isolated from wineries. *U. maydis* CYC 1410 was able to inhibit all tested strains of *B. bruxellensis*. Taking into account that three killer toxins from *U. maydis* have been previously identified, here we also show the identification of the killer toxin produced by *U. maydis* CYC 1410, the one responsible for such killer activity. In order to biocontrol *B. bruxellensis* for oenological purposes, mixed cultures of killer/sensitive strains were grown. In short, given the need to develop natural means of beverage and food preservation due to consumer demand, we have sought to investigate the killer aspect of *U. maydis* as a biopreservative for controlling *B. bruxellensis*.

2. Materials and methods

2.1. Microorganisms and media

Thirty-seven yeast strains of *S. cerevisiae* and *B. bruxellensis*, isolated from different Spanish wineries, were tested as sensitive strains against a panel of thirty-nine killer strains (Tables 1 and 2) from the CYC (Complutense Yeast Collection, Complutense University of Madrid, Spain) and the IGC (Portuguese Yeast Culture Collection, New University of Lisbon, Portugal).

Yeast strains were maintained at 20 °C on YMA: 1% glucose, 0.3% yeast extract (Difco), 0.3% malt extract (Difco), 0.5% proteose peptone no. 3 (Difco) and 2% agar. Strains were isolated on YMA-CH-BR (YMA supplemented with 0.1 g/L chloramphenicol and 0.05 g/L rose bengal). Incubation was routinely performed at 30 °C for 48 h. For killer/sensitive cross-reaction trials between yeasts isolated from wineries, killer activity was determined in YMA-MB (Santos et al., 2009) at 20 °C for 72 h.

The growth media for mixed cultures was sterile grape juice obtained from Spanish Tempranillo red grapes (pH 3.5, sugar 220 g/L) without the addition of SO_2. Incubation was carried out at 20 °C with orbital shaking at 150 rev/min. DBDM (*Dekkera*/*Brettanomyces* differential medium) plates incubated for 72 h at 30 °C were used for the distinction and enumeration of *B. bruxellensis* strains after mixed cultures in must (Rodrigues et al., 2001). Additionally, grape must agar (GMA) was developed with sterile must from Tempranillo grapes and 2% agar. Unless specifically indicated otherwise, no additives were added. GMA was used for activity plates in conditions similar to wine fermentation to determine the killer activity at different pH values and temperatures.

The red wine used in this work was Rioja from Alava (Spain), with the following physicochemical specifications: 14.2% ethanol, pH 3.51, free SO_2 31 ppm, total SO_2 63 ppm and a total polyphenolic index of 72.6.

2.2. Isolation and identification

The sensitive strains used in this work were isolated by Agrovin, S.A. from grapes, wines and barrels from diverse regions of Spain. Isolation was made on YMA-CH-BR. Yeast colonies were selected after 48–72 h incubation at 30 °C and then maintained on YMA.

Yeast isolates were characterized at species level according to previously described discriminatory morphological and biochemical tests (Barnett et al., 1990; Kurtzman and Fell, 1998). Additionally, sensitive strains were identified by using PCR-RFLP (restriction fragment length polymorphism). Indigenous yeast identity was performed by analyzing the ITS1-5.8S-ITS2 rDNA region amplified by PCR using primers ITS1 (5′-CCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Esteve-Zarzoso et al., 1999; Guillamón et al., 1998). The thermal cycling parameters were an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 52 °C for 2 min, and extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min. The amplified products (0.5–10 μg of DNA) were

Table 1
Killer yeasts from CYC and IGC used in this study. Sensitive yeasts were from Agrovin.

Killer strains		
Candida boidinii CYC 1024	*Pichia membranifaciens* CYC 1090	*Ustilago maydis* CYC 1611
Candida diddensiae CYC 1098	*Pichia membranifaciens* CYC 1095	*Ustilago maydis* CYC 1613
Candida ethanolica CYC 1097	*Pichia membranifaciens* CYC 1116	*Ustilago maydis* CYC 1647
Candida parapsilosis CYC 1050	*Rhodotorula mucilaginosa* CYC 1092	*Ustilago maydis* CYC 1675
Candida rugosa CYC 1044	*Rhodotorula aurantiaca* CYC 1094	*Ustilago maydis* CYC 1721
Candida valida CYC 1028	*Saccharomyces bayanus* IGC 4465	*Ustilago maydis* CYC 1722
Candida valida CYC 1101	*Saccharomyces cerevisiae* CYC 1102	*Ustilago maydis* CYC 1732
Debaryomyces hansenii CYC 1021	*Saccharomyces cerevisiae* CYC 1115	*Ustilago maydis* CYC 1733
Kluyveromyces lactis IGC 4358	*Saccharomyces cerevisiae* IGC 4456	*Ustilago maydis* CYC 1752
Pichia pseudocaptophila CYC 1029	*Saccharomyces cerevisiae* IGC 4620	*Ustilago maydis* CYC 1753
Pichia anomala CYC 1026	*Saccharomyces exiguus* IGC 4612	*Ustilago maydis* CYC 1771
Pichia membranifaciens CYC 1048	*Ustilago maydis* CYC 1410	*Ustilago maydis* CYC 1773
Pichia membranifaciens CYC 1084	*Ustilago maydis* CYC 1610	*Ustilago maydis* CYC 1800
Sensitive strains		
Saccharomyces cerevisiae Y1	*Saccharomyces cerevisiae* Y9	*Brettanomyces bruxellensis* D017
Saccharomyces cerevisiae Y2	*Saccharomyces cerevisiae* YTTA	*Brettanomyces bruxellensis* D018
Saccharomyces cerevisiae Y3	*Saccharomyces cerevisiae* Y11	*Brettanomyces bruxellensis* D019
Saccharomyces cerevisiae Y ARM	*Saccharomyces cerevisiae* Y522D	*Brettanomyces bruxellensis* D027
Saccharomyces cerevisiae Y5	*Saccharomyces cerevisiae* Y13	*Brettanomyces bruxellensis* D028
Saccharomyces cerevisiae Y6	*Saccharomyces cerevisiae* Y14	*Brettanomyces bruxellensis* D029
Saccharomyces cerevisiae Y RVA	*Saccharomyces cerevisiae* SC1	*Brettanomyces bruxellensis* D031
Saccharomyces cerevisiae Y7	*Brettanomyces bruxellensis* 0263	*Brettanomyces bruxellensis* D032
Saccharomyces cerevisiae Y8	*Brettanomyces bruxellensis* 1D007	*Brettanomyces bruxellensis* D033
Saccharomyces cerevisiae 16	*Brettanomyces bruxellensis* D013	*Brettanomyces bruxellensis* D035
Saccharomyces cerevisiae 17	*Brettanomyces bruxellensis* D014	*Brettanomyces bruxellensis* D036
Saccharomyces cerevisiae 18	*Brettanomyces bruxellensis* D015	*Brettanomyces bruxellensis* D038
Saccharomyces cerevisiae 19		