Journal of Industrial Microbiology (1996) 17, 124–127

Differential killer sensitivity as a tool for fingerprinting wine-yeast strains of *Saccharomyces cerevisiae*

A Vaughan-Martini, G Cardinali and A Martini

Sezione Microbiologia Applicata, Dipartimento di Biologia Vegetale Università di Perugia, Borgo 20 Giugno, 74 I-06121 Perugia, Italy

The extreme variability of the killer phenomenon in nature, expressed differently in different strains of the same yeast species, embodies an exceptional potential for the discrimination of yeasts at the strain level. Killer-sensitive relationships between a killer reference panel of 24 yeasts belonging to 13 species of six genera, and different industrial wine-starters of *Saccharomyces cerevisiae* can be used profitably for a rapid and simple fingerprinting procedure.

Keywords: yeasts; killer toxin; fingerprinting; *Saccharomyces cerevisiae*; selected starters; wine-making

Introduction

The introduction of the concept of microbiological invention in patent protection inevitably brought forth the need for a precise taxonomic characterization of the culture used in the process. Even though the comparison of whole DNA sequences [3] unequivocally and indisputably bears out conspecificity, the proof of ownership of a given microorganism is always intricate since the patentee cannot rely solely upon the name of a species to cover other strains not yet isolated or developed. Nevertheless, it is evident that actual protection in legal disputes can only be enforced if classification discriminates within the species, to the strain level.

Certain yeasts have the ability to secrete low molecular weight glycoproteins (killer toxins) which inhibit the biosynthesis of DNA, RNA, proteins and polysaccharides in sensitive cells of the same species and/or other taxa carrying a specific receptor on the outer cell surface [2,12,17]. Killer activity is common among yeast strains recently isolated from nature as well as those which have been maintained in culture collections [2,6,8,11,20]. Even though the protein moieties of only four toxins have been sequenced [K1, K2 and K3 in *Saccharomyces cerevisiae* Meyen ex Hansen, and pGK1 in *Kluyveromyces lactis* (Dombrowsky) van der Walt], ecological evidence points to the presence of a number of specific toxins, unevenly distributed among a large number of species [19]. In addition, since specificity of action by toxins requires recognition sites on the outer wall of sensitive cells, four or more different receptors must also be distributed among different species and strains. The extreme variability of the killer phenomenon, which has been shown to be expressed differently even in different strains of the same species, may provide an exceptional potential for the discrimination of yeast strains below the species level. This paper reports the results of an investigation of the discriminating performance of a panel of 24 yeast killer cultures (13 species in six genera) on several strains of *S. cerevisiae* isolated from preparations of commercially available selected starters for wine-making.

Correspondence: Dr A Vaughan-Martini, Dipartimento di Biologia Vegetale, Borgo 20 Giugno, 74 I-06121 Perugia, Italy
Received 10 July 1995; accepted 24 July 1996

Materials and methods

Microorganisms

Killer cultures: All yeast strains listed in Table 1 were selected as a result of a series of screening surveys for the presence of killer toxins [8–10] which have been carried out at the DBVPG Industrial Yeasts Collection [5]. These cultures had been isolated over the years from many different natural environments and belong to several ascoporogenous and anascosporogenous genera. To conserve space, each killer culture is identified by using a three-letter abbreviation (one capitalized for the genus and two lower case for the species) followed by a number for the strain. Additional information on the nuclear or plasmid origin of some of the toxins is reported in Table 1. Killer yeast isolates are maintained in the DBVPG collection in lyophilised form and as deep frozen samples at −70°C.

Industrial cultures: Eleven yeast strains were used to test the activity of the killer strains (killer panel) for discrimination at the strain level. These were obtained as pure cultures of several selected starters for wine-making commercialized in Italy, by streaking them on malt-agar. Sensitive cultures are identified by Roman numerals (Table 2). Information on correspondence with respective brand names is available upon request.

Assay of the killing activity

Strains to be tested for sensitivity were grown for 24 h on a malt agar slant; 10^5 cells (determined spectrophotometrically) were then suspended in 1 ml of sterile water. These were mixed thoroughly with 19 ml of molten Bacto Malt Agar (Difco, Detroit, MI, USA) prepared in citrate-phosphate buffer, pH 4.5. The suspension was

Table 1 The cultures used for the killer reference panel

Species	DBVPG[a] Accession No.	Killer designation	Origin	Killer information
Candida berthetii	6203	Cbe 1	gum arabic	
C. freyschussii	6208	Cfr 1	unknown	
C. membranaefaciens	6145	Cme 1	urine	
Debaryomyces sp	3020	Dsp 1	unknown	
Kluyveromyces lactis	6727	Kla 5	creamery	plasmid
K. phaffii	6076	Kpf 1	soil	nuclear
K. wickerhamii	6077	Kwi 1	unknown	nuclear
Pichia anomala	3003	Pan 1	baker's yeast	nuclear
P. anomala	3649	Pan 2	soil	nuclear
P. anomala	3650	Pan 3	soil	nuclear
P. anomala	3863	Pan 4	honey	nuclear
P. anomala	3864	Pan 5	honey	nuclear
P. minuta	3671	Pmi 1	soil	
Saccharomyces cerevisiae	6497	Sce 38	yeast foam	plasmid-K_1, ds RNA, *ca* 4 kbp [19]
S. cerevisiae	6499	Sce 39	brewery	plasmid-K_2, ds RNA, *ca* 4–5 kbp
S. cerevisiae	6567	Sce 40	unknown	Constructed killer strain: $K_1 + K_2$
S. cerevisiae	6744	Sce 41	palm wine	plasmid-K_3, ds RNA, *ca* 4 kbp [19]
Williopsus californica	3657	Wca 1	unknown	nuclear
W. mrakii	6729	Wmr 1	soil	nuclear
W. saturnus	3125	Wsa 1	soil	
W. saturnus	3126	Wsa 2	soil	
W. saturnus	3127	Wsa 3	soil	nuclear
W. saturnus	3128	Wsa 4	soil	
W. saturnus	3129	Wsa 5	soil	
W. saturnus	3130	Wsa 6	soil	

[a]DBVPG = Industrial Yeasts Collection of the Dipartimento di Biologia Vegetale, Università degli Studi, Perugia, Italia.

Table 2 Killer sensitivity of selected wine-starters of *Saccharomyces cerevisiae*

Strain	Killer cultures[a]																								Killer sensitivity formula (KSF)
	C be 1	C fr 1	C me 1	D sp 1	K la 5	K pf 1	K wi 1	P an 1	P an 2	P an 3	P an 4	P an 5	P mi 1	S ce 38	S ce 39	S ce 40	S ce 41	W ca 1	W mr 1	W sa 1	W sa 2	W sa 3	W sa 4	W sa 5	W sa 6	
I													•	•		•			•	•	•	•	•	•	•	Pmi1•Sce38,40•Wmr1•Wsa1,2,3,4,5,6
II													•	•		•	•		•	•	•	•	•		•	Pmi1•Sce38,40,41•Wmr1•Wsa1,2,3,4,6
III	•	•	•	•									•		•	•	•	•	•	•	•	•	•	•	•	Cbe1•Cfr1•Cme1•Dsp1•Pmi1•Sce39,40,41•Wmr1•Wca1•Wsa1,2,3,4,5,6
IV													•	•		•				•	•	•	•	•	•	Pmi1•Sce38,40•Wsa1,2,3,4,5,6
V				•	•	•		•					•	•		•		•	•	•	•	•	•	•	•	Dsp1•Kla5•Kpf1•Pan1•Pmi1•Sce38,40•Wca1•Wmr1•Wsa1,2,3,4,5,6
VI				•	•	•	•	•	•	•	•	•	•	•		•	•		•	•	•	•	•	•	•	Dsp1•Kla5•Kpf1•Kwi1•Pan1,2,3,4,5•Pmi1•Sce38,40,41•Wmr1•Wsa1,2,3,4,5,6
VII													•	•		•						•	•		•	Pmi1,•Sce38,40•Wsa3,4,6
VIII				•		•	•	•			•		•		•	•	•		•	•	•	•	•	•	•	Dsp1•Kpf1•Kwi1•Pan1,4•Pmi1•Sce39,40,41•Wmr1•Wsa1,2,3,4,5,6
IX				•		•	•						•		•	•	•	•	•			•		•	•	Dsp1•Kpf1•Kwi1•Pmi1•Sce39,40,41•Wca1•Wmr1•Wsa3,5,6
X							•		•	•	•				•	•	•					•				Kwi1•Pan2,3,4•Sce39,40,41•Wsa3
XI														•		•						•	•			Sce38,40•Wsa3,4

[a]Explanation of killer strain designations is in Table 1.
• = Sensitive to killer toxin.

poured into a Petri dish (100 × 15 mm) and the agar was allowed to solidify. Each plate was inoculated successively with six different potential 'killer' strains (as separate streaks approximately 10 mm in length) and incubated at 20°C for 72 h. A positive 'killer' reaction was recorded in those cases where an evident and clear zone of inhibition (≥ 5 mm) surrounded a streak on the plate. Assays were performed in duplicate and repeated twice.

Results

Differential response by different *S. cerevisiae* wine-starters to the lethal action of selected killer yeasts is reported in Table 2. Results are displayed as a matrix of presence or absence of killing activity. A condensed representation is also provided in the form of a 'killer sensitivity formula' (KSF), listing in alphabetical order the acronyms of all the yeasts of the killer panel capable of inactivating the sensitive culture.

Each commercial yeast culture exhibited a sensitive phenotype, with inactivation profiles (KSF) ranging from a minimum of three to a maximum of 19 specific killer factors. Individual susceptibility patterns (KSF) were consistently observed in all tests carried out on the eleven commercial wine-making starters, with no two cultures exhibiting the same profile.

Discussion

Even though the occurrence of killer factors has been demonstrated in several yeast genera [2,6,8,11,20], the mode of action and molecular biology of this activity have been studied extensively only in the killer system of the species *S. cerevisiae* [18]. Likewise, applications of the killer phenomenon to genetics and industrial fermentations have been confined mostly to strains of that species [16].

In spite of this limitation, recent studies demonstrated that the killer effect in yeasts is a highly polymorphic character [19]. Hypervariability is regularly expressed among different strains of the same species, as the outcome of the distribution of at least 24 killer-related factors (at least 12 different known toxins and 12 corresponding receptors).

The first attempts of a taxonomic application of killer relationships for the separation of authenticated species of the genus *Kluyveromyces* and of species within the *sensu stricto* group of the genus *Saccharomyces* (certified by *in vitro* nuclear DNA reassociation experiments) [13,14], demonstrated that the sensitivity of yeasts to a defined range of specific toxins is a strain- rather than species-related property [1,15] This conclusion also created the premise for an application of the killer-sensitive relationship as an epidemiological marker for the differentiation of pathogenic yeasts [7].

The above interpretation of the 'killer' phenomenon based on strain-to-strain specificity suggested a preliminary study for the exploitation of the 'killer-sensitive relationship' as a fingerprinting tool for the discrimination of yeasts below the species level [4]. In that study a numerically and taxonomically limited panel of 10 killer strains, belonging to only one species (*S. cerevisiae*), allowed an acceptable fingerprinting of 14 selected starters for wine-making. Immediate application of the method was impaired, however, by the potential instability of expression of the killer phenomenon in that species, since it is characterized by a killer system controlled by extrachromosomal determinants [18] which can be lost during successive vegetative reproduction. Only after other genera were investigated for the presence of lethal factors did the discovery of toxins controlled at the nuclear level make possible the construction of a more genetically stable panel of killer yeasts. The DBVPG collection presently holds about 100 killer cultures which have been well identified to the species level. These strains are maintained under conditions minimizing modifications of the killer genotype. Many of the non-*S. cerevisiae* isolates have already been checked for a nuclear origin of their toxin(s). This together with other salient information on the killer yeasts selected for the present reference panel is listed in Table 1.

The application of individual fingerprinting of industrial strains within the species *S. cerevisiae*, carried out by using a killer panel of 24 strains belonging to 13 different species of six genera, confirmed the highly polymorphic expression of the killing activity. As a consequence, the discriminating potential of this method for fingerprinting at the strain level can be very useful for a simple and rapid characterization of yeast strains of industrial interest.

Acknowledgements

The authors thank Dr Iroshi Fukuhara, Institut Curie—Section de Biologie, Orsay, France for kindly investigating the genomic origin of some of the killer strains. The valuable technical assistance of Stefania Barcaccia and Fausto Maccarelli is also recognized.

This study was supported by a grant from the Italian National Council for Research, Special project RAISA, Sub-project 4 (Manuscript No. 2350).

References

1 Cardinali G. 1993. Tassonomia molecolare dei lieviti del gruppo sensu stricto del genere *Saccharomyces* Meyen ex Reess. Doctoral Thesis, University of Perugia, Italy.
2 Golubev WI and VM Blagodatskaya. 1994. Intra- and intergeneric killing patterns of *Pichia punctispora* (Melard 1910) Dekker 1931 mycotoxins. Mikrobiologiya 63: 637–642.
3 Kurtzman CP and HJ Phaff. 1987. Molecular taxonomy. In: The Yeasts: I. Biology of Yeasts (Rose AH and JS Harrison, eds), pp 63–94, Academic Press, London.
4 Martini A. 1983. Principi di classificazione dei lieviti e possibilità di tipizzare i ceppi selezionati. Atti Accad Ital Vite Vino 35: 215–223.
5 Martini A, G Rosini and A Vaughan-Martini. 1987. Industrial Yeasts Collection DBVPG. Ann Fac Agr Univ Perugia 41: 941–997.
6 Philliskirk G and TW Young. 1975. The occurrence of killer character in yeasts of various genera. Antonie van Leeuwenhoek 41: 147–151.
7 Polonelli L, C Archibusacci, M Sestito and G Morace. 1983. Killer system: a simple method for differentiating *Candida albicans* strains. J Clin Microbiol 17: 774–780.
8 Rosini G. 1983. The occurrence of killer characters in yeasts. Can J Microbiol 29: 1462–1464.
9 Rosini G. 1985. Interaction between killer strains of *Hansenula anomala* var *anomala* and *Saccharomyces cerevisiae* yeast species. Can J Microbiol 31: 300–302.
10 Rosini G and M Cantini. 1987. Killer character in *Kluyveromyces* yeasts: activity on *Kloeckera apiculata*. FEMS Microbiol Lett 44: 81–84.
11 Stumm C, JM Hermans, EJ Middlebeek, AF Croes and GJML de Vries. 1977. Killer-sensitive relationships in yeasts from natural habitats. Antonie van Leeuwenhoek 43: 125–128.
12 Tipper DJ and KA Bostian. 1984. Double-stranded ribonucleic acid killer systems in yeasts. Microbiol Rev 48: 125–156.
13 Vaughan-Martini A and A Martini. 1987. Taxonomic revision of the yeast genus *Kluyveromyces* by nuclear deoxyribonucleic acid reassociation. Int J Syst Bacteriol 37: 380–385.
14 Vaughan-Martini A and A Martini. 1987. Three newly delimited species of *Saccharomyces sensu stricto*. Antonie van Leewenhoek 53: 77–84.

15 Vaughan-Martini A and G Rosini. 1987. Killer relationships within the yeast genus *Kluyveromyces*. Mycologia 81: 317–321.
16 Vondrejs V. 1987. A killer system in yeasts: applications to genetics and industry. Microbiol Sci 4: 313–316.
17 Wickner RB. 1986. Double-stranded RNA replication in yeasts: the killer system. Ann Rev Biochem 55: 373–395.
18 Woods DR and EA Bevan. 1968. Studies on the nature of the killer factor produced by *Saccharomyces cerevisiae*. J Gen Microbiol 51: 115–126.
19 Young TW. 1987. Killer yeasts. In: The Yeasts. Vol 2 (Rose AH and JS Harrison, eds), pp 131–164, Academic Press, London.
20 Young TW and M Yagiu. 1978. A comparison of the killer character in different yeasts and its classification. Antonie van Leeuwenhoek 44: 59–77.