Characterization of Killer Yeast Isolates from Chenin blanc Grapes and Grape Skins*

C.J. Jacobs[1], I. Fourie[1] and H.J.J. van Vuuren[2]

1) Stellenbosch Farmers' Winery, P.O. Box 46, 7600 Stellenbosch, Republic of South Africa
2) Department of Microbiology, University of Stellenbosch, 7600 Stellenbosch, Republic of South Africa

Submitted for publication: December 1990
Accepted for publication: May 1991
Key words: Killer yeast, yeasts, grapes, wine

Wild-type killer yeast strains isolated from six South African wineries were identified using classical taxonomic methods. They were further characterized according to their cross-reactions with reference killer yeasts (K_1-K_{11}) and by electrophoresis of their double-stranded RNA molecules. All isolates belonged to the K_2 phenotype and were identified as strains of *Saccharomyces cerevisiae* and *Saccharomyces bayanus*. The killer strains differed substantially in their ability to kill a sensitive wine yeast (Geisenheim GS-1). This phenomenon may be attributed to strain differences among the killer yeasts as was shown by electrophoresis of total soluble cell proteins and gas chromatographic analysis of cellular fatty acids.

Killer yeasts were discovered in laboratory strains of *Saccharomyces cerevisiae* by Bevan & Makower (1963). They also classified yeast strains into one of three phenotypes: killer (K), sensitive (S) and neutral (N). Killer yeasts secrete proteinaceous killer toxins which are lethal to susceptible or sensitive strains of the same species, but are themselves immune to their own toxins. Killer interactions are generally restricted to strains of species within a genus, but interactions between species of different genera have been reported (Bussey, 1974; Mitchell & Bevan, 1983; Tipper & Bostian, 1984).

Many surveys have been conducted since 1963 to determine how widespread killer yeasts are in the environment. They have been found in numerous culture collections and include yeast genera such as *Saccharomyces, Candida, Pichia, Debaryomyces, Hansenula, Kluyveromyces, Torulopsis* and *Cryptococcus* (Phillipskirk & Young, 1975; Stumm *et al.*, 1977; Rosini, 1983). Killer strains of *Sacch. cerevisiae* have also been isolated from natural habitats such as grapes and grape skins (Jacobs, Fourie & Van Vuuren, 1988), wine (Naumov & Naumova, 1973; Naumov *et al.*, 1973; Barre, 1984; Van Vuuren & Wingfield, 1986; Heard & Fleet, 1987), beer (Maule & Thomas, 1973; Rogers & Bevan, 1978) and saké fermentations (Imamura, Kawamoto & Tokaoka, 1974).

The killer phenomenon in *Sacch. cerevisiae* is associated with cytoplasmically inherited virus-like particles (VLPs) or mycoviruses containing the M (medium) and L (large) double-stranded RNA (dsRNA) genomes (Herring & Bevan, 1974). The M genome codes for the toxin and immunity to this polypeptide (Mitchell, Herring & Bevan, 1976), while the L-dsRNA encodes the subunits of the protein coat of the VLPs (Tipper & Bostian, 1984).

Killer yeasts can be classified into at least 11 groups (K_1-K_{11}) according to the size differences of their M-dsRNA molecules and the properties of their respective toxins, i.e. the spectrum of activity against sensitive strains, or by assay of the cross-reactivity (interaction) of killer yeast. The classification of killer yeasts is based on these properties. Five killer types have been reported in *Sacch. cerevisiae* (Naumov & Naumova, 1973; Rogers & Bevan, 1978; Hara, Iimura & Otsuka, 1980; Extremera, Martin & Montoya, 1982; Pfeiffer & Radler, 1982), namely K_1, K_2, K_3, KT28 and K_3GR1. However, Wingfield *et al.* (1990) have recently shown that the M-dsRNA of K_2 and K_3 strains share an extensive homology. They found that the K_2 and K_3 killer types belong to the same class and suggested that the K_3 killer type should be included within the K_2 killer class. The optimum pH for production and stability of toxins from group K_1 ranges between 4,6 and 4,8 (Woods & Bevan, 1968) and is therefore not important in winemaking. However, the optimum pH for the K_2 toxin varies between pH 2,9 and 4,9 (Shimizu *et al.*, 1985), which means that these yeasts might pose a threat to the wine industry. Despite unfavourable conditions related to high concentrations of sugar, SO_2 and polyphenols, the K_2 toxin has been shown to be active in grape must (Barre, 1980; Tredoux, Tracey & Tromp, 1986; Van Vuuren & Wingfield, 1986).

**Presented in part at the 13th Congress of the SASEV, Cape Town, November 1989.*

Acknowledgements: Professor F. Radler from the Institut für Mikrobiologie und Weinforschung, der Johannes Gutenberg-Universität Mainz, Mainz, Germany, is thanked for determining the killer phenotypes; Professor J.P. van der Walt from the CSIR, Pretoria, South Africa, for identifying the killer yeast isolates; and Mr M. Ernstzen from the Department of Microbiology at the University of Stellenbosch for the isolation and electrophoresis of dsRNA molecules.

The industrial interest in killer yeasts stems from the premise that these yeasts, when present, might eventually dominate a fermentation initially inoculated with a sensitive strain. Such contaminations can result in problems such as lagging or stuck fermentations, high volatile acidity, H_2S production and off-flavours (Rosini, 1985; Van Vuuren & Wingfield, 1986; Heard & Fleet, 1987). Killer strains of *Sacch. cerevisiae* have been found to be dominant at the end of fermentations in some wineries but not in others (Cuinier & Gros, 1983; Barre, 1984; Benda, 1985). There are also conflicting reports on the number of killer yeasts needed for sluggish fermentations to occur (Table 1).

In 1986 Van Vuuren & Wingfield reported a case where a killer yeast contaminant (T 206) was responsible for protracted and stuck wine fermentations in a South African wine cellar. The abundance of wild killer yeasts under winemaking conditions is well documented (Rosini, 1985; Heard & Fleet, 1987). Jacobs, Fourie & Van Vuuren (1988) reported on the isolation of two hundred and thirty killer yeasts from Chenin blanc grapes and grape skins collected from six local wineries. The isolates were divided into nine groups based on their colony morphology and colour on modified Wallerstein laboratory nutrient agar. Preliminary work showed that considerable heterogeneity exists among these groups with respect to their ability to kill different sensitive strains. In this study we report on the identification and further characterization of isolates from each of the nine groups.

MATERIALS AND METHODS

Yeasts strains: Wild-type killer yeast strains (K-25; K-54; K-73; K-83; K-102; K-103; K-106; K-125 and K-174), representing nine different colour and morphological groups, were isolated and classified as described previously (Jacobs, Fourie & Van Vuuren, 1988). A strain of *Sacch. cerevisiae* obtained from Geisenheim (GS-1, Stellenbosch Farmers' Winery) was used as the sensitive strain throughout this study. All strains were kept on YM agar slants (1% glucose, 0,5% Bacto peptone, 0,3% malt extract, 0,3% yeast extract and 1,5% Bacto agar) at 5°C.

Identification of isolates: Yeast strains were identified according to the methods and criteria of Kreger-van Rij (1984).

Determination of killer phenotype: The killer phenotype was determined by using the methylene blue agar (MBA) technique of Somers and Bevan (1969). Plates were seeded with 4 x 10^5 cells/ml of a wild-type killer strain and samples of reference killer yeasts were streaked on top. The plates were then incubated at 25°C for 72h. If the inoculated strain was surrounded by a region of blue coloured cells, or by a clear zone of inhibition bounded by coloured cells, it was designated as a killer strain, and the seeded strain as a sensitive one.

Protein electrophoresis: Cell-free extracts were prepared according to the technique of Van Vuuren & Van der Meer (1987). SDS gel electrophoresis was performed in an SE 600 Vertical Slab Gel Unit (Hoefer Scientific Instruments) using the method described by Weber & Osborn (1969). Protein concentrations were determined and standardised by the Bradford method (Bradford, 1976). All chemicals used for the preparation of gels were from E. Merck (Darmstadt, West Germany).

Cellular fatty acid analysis: Cultivation of yeast strains, fatty acid extraction and preparation of methyl esters were done as described in "Method B" of Augustyn & Kock (1989). Fatty acid extracts were analysed by gas chromatography on a J & WDB wax capillary column (30 m x 0,32 mm I.D., coating 0,15µ) according to the method of Augustyn (1989). The relatedness of any two strains was computed by applying the formula developed by Holman (1978):

$$Rx,y = \left(\frac{Cx}{Cy}\right)_1\left(\frac{Cx + Cy}{200}\right)_1 + \ldots\ldots + \left(\frac{Cx}{Cy}\right)_n\left(\frac{Cx + Cy}{200}\right)_n$$

In this formula R represents the index of relationship, x and y are the two strains being compared, C is the concentration expressed as a relative percentage and 1 through n are the fatty acids used to differentiate between the strains (n = 10 in this instance).

Isolation and electrophoresis of double-stranded RNA: Extraction of double-stranded RNA (dsRNA) from

TABLE 1
Ratio of killer: sensitive strains found to cause sluggish wine fermentations.

Country	Ratio Killer:Sensitive	Reference
France	1:50	Barre, 1984
Australia	1:1	Heard & Fleet, 1987
Japan	100:1	Shimizu *et al.* 1985
Japan	25:1	Seki, Choi & Ryu, 1985
U.S.S.R.	1:20	Tyurina *et al.* 1986
Germany	1:1000	Radler, 1988
South Africa	>1:40	Tredoux, Tracey & Tromp, 1986
South Africa	1:500	Van Vuuren & Wingfield, 1986

virus-like particles and electrophoresis in 1% agarose gels were done according to the methods described by Van Vuuren & Wingfield (1986). Sizing of the dsRNA molecules was done according to Wingfield, van Vuuren & Pretorius (1989).

Determination of toxin activity: The lyophilization method described by Radler & Knoll (1988) was used to concentrate killer toxins. Aliquots (50 µl) of concentrated toxin (10x) were assayed in 5 mm diam. wells of methylene blue plates (pH 4.5) seeded with 1 x 10^5 cells/ml of the sensitive strain GS-1.

RESULTS AND DISCUSSION

Nine killer yeast isolates representing nine different colour and morphological groups were identified using classic taxonomic criteria. Eight strains were identified as *Saccharomyces cerevisiae* and one (K-54) as *Saccharomyces bayanus* (Table 2).

TABLE 2

Assimilation of carbon by killer yeasts.

Strains	Carbon source											Identity
	Gluc.	Malt.	Suc.	Treh.	Raff.	Gal.	Gly.	Lac.	EtOH.	Succ.	Citr.	
K-25	+	+	+	+d	+	+	-	-	+	-	-	*Saccharomyces cerevisiae*
K-54	+	+	+	+d	+	-	-	+d	+	-	-	*Saccharomyces bayanus*
K-73	+	+	+	+	+d	+	-	+	+	-	-	*Saccharomyces cerevisiae*
K-83	+	+	+	+	+	+	-	+	+	+	-	*Saccharomyces cerevisiae*
K-102	+	+	+	+	+	+	-	+	+	-	-	*Saccharomyces cerevisiae*
K-103	+	+	+	+	+	+	-	+	+	-	-	*Saccharomyces cerevisiae*
K-106	+	+	+	+	+d	+	-	+	+	-	-	*Saccharomyces cerevisiae*
K-125	+	+	+	+	+	+	-	+d	+	-	-	*Saccharomyces cerevisiae*
K-174	+	+	+	+	+	+	-	-	+	+	-	*Saccharomyces cerevisiae*
K2 (NCYC 738)	+	+	+	+	+	+	-	-	+	+	-	*Saccharomyces cerevisiae*

(+) Assimilation, (-) No assimilation, (+d) Delayed assimilation; Gluc, Glucose; Malt, Maltose; Suc, Sucrose; Treh, Trehalose; Raff, Raffinose; Gal, Galactose; Gly, Glycerol; Lac, Lactose; EtOH, Ethanol; Succ, Succinate; Citr, Citrate.

TABLE 3

Inhibition of a sensitive yeast by killer yeasts and interactions between killer yeasts. Killer isolates were plated and the killer yeasts (K_1-K_{10}) streaked.

Strains	Zone of inhibition (diam. in mm)*	Interaction		Killer Phenotype
		Zones formed	No zones formed	
K-25	18 mm	K_1,K_4	K_2,K_3,K_5-K_{10}	K_2
K-54	17 mm	K_1,K_4	K_2,K_3,K_5-K_{10}	K_2
K-73	14 mm	K_1,K_4	K_2,K_3,K_5-K_{10}	K_2
K-83	16 mm	K_1,K_4	K_2,K_3,K_5-K_{10}	K_2
K-102	16 mm	K_1,K_4	K_2,K_3,K_5-K_{10}	K_2
K-103	10 mm	K_1,K_4	K_2,K_3,K_5-K_{10}	K_2
K-106	14 mm	K_1,K_4	K_2,K_3,K_5-K_{10}	K_2
K-125	14 mm	K_1,K_4	K_2,K_3,K_5-K_{10}	K_2
K-174	15 mm	K_1,K_4	K_2,K_3,K_5-K_{10}	K_2
K_2(NCYC 738)	16 mm	K_1,K_4	K_2,K_3,K_5-K_{10}	K_2

*MBA medium seeded with Geisenheim (GS-1). K_1 = *Sacch. cerevisiae* D587-2A; K_2 = *Sacch. cerevisiae* NCYC 738; K_3 = *Sacch. capensis* NCYC 761; K_4 = *T. glabrata* NCYC 388; K_5 = *H. subpelliculosa* NCYC 16; K_6 = *K. fragilis* NCYC 587; K_7 = *P. membranaefaciens* NCYC 333; K_8 = *H. anomala* NCYC 435; K_9 = *H. mrakii* NCYC 500;K_{10} = *K. drosophilarum* NCYC 575.

It is evident from the carbon assimilation profiles in Table 2 that differences exist among the eight *Sacch. cerevisiae* strains, and that only one strain (K-174) was phenotypically identical to the K_2 reference strain NCYC 738.

All killer strains under investigation were tested for differences in both their ability to kill other killer strains and in their immunity to other killer toxins. No killer yeast was found to be suicidal under the assay conditions used, and only one distinct pattern of interaction was found (Table 3).

All nine wild-type killer strains formed inhibition zones on the K_1 and K_4 background lawns, indicating that these two strains are sensitive to the killer toxins produced by the yeast isolates. No interaction was detected amongst the wild-type killers and the eight reference killer strains K_2, K_3 and K_5-K_{10}, indicating that the nine strains under investigation belong to either the K_2 or K_3 killer phenotype (Young & Yagiu, 1978). The size of the M-ds RNA genomes isolated from the nine wild-type killer yeasts ranged from 1,3 kb to 1,8 kb (Fig. 1).

The reported sizes of the M_2 (K_2) and M_3(K_3) molecules are 1,5 and 1,3 kb respectively (Young, 1987), although variations ranging from 1,5 to 2,0 kb have been reported for the M_2 genome (Sommer & Wickner, 1984; Wingfield, van Vuuren & Pretorius, 1989). These variations might be attributable to variations in both growth conditions and the size of the AU-rich region known to occur in both the M_1 and M_2 genomes (Hannig, Thiele & Leibowitz, 1984; Sommer & Wickner, 1984). However, Wingfield *et al.* (1990) have shown that the K_2 M-dsRNA and K_3 molecules share an extensive homology. Our nine killer strains were therefore regarded as K_2 killer strains.

The killer isolates were also compared in terms of the amount and/or specific activity of the toxins produced. Clear inhibition zones ranging from 10 to 18 mm in diameter were formed on the seeded MBA medium by the various toxin preparations (Table 3). The largest inhibition zones (17 and 18 mm in diameter) were formed by the toxins derived from strains K-54 and K-25 respectively. The toxin from strain K-103 formed a zone of only 10 mm. The halos surrounding the wells also differed in colour and size. These findings are consistent with those of Shimizu *et al.* (1985), who found that the K_2 toxins produced by the commercially available killer yeast Prise de Mouse, 71B, and Montpellier (V-1116) were much more active than that produced by the wild K_2 killer strain G-9. It appears, therefore, that considerable heterogeneity exists between the various K_2 killer strains in the extent to which they produce killer toxins, and perhaps also in the specific activities of these toxins.

Electrophoresis of total soluble cell proteins as well as gas chromatography of cellular fatty acids was used in order to differentiate between the different killer strains. In 1987 Van Vuuren and Van der Meer reported that different genetic groups of *Sacch. cerevisiae* could be distinguished by using electrophoretic profiles, and that this technique was also suitable for characterizing and fingerprinting individual strains. The protein profiles of the nine killer yeast isolates and the reference K_2 killer strain (NCYC 738) are presented in Figure 2.

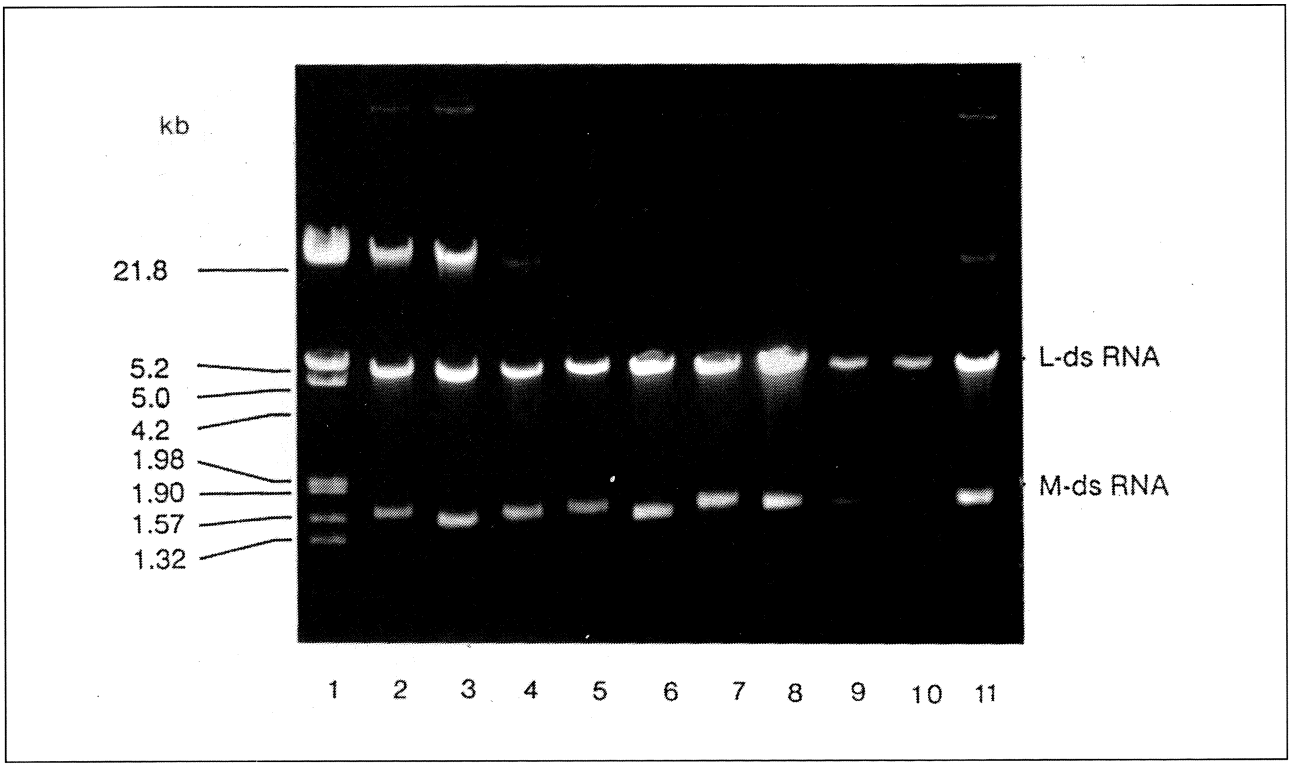

FIGURE 1

Agarose gel electrophoresis of dsRNA isolated from killer yeasts. 1) Phage lambda DNA (cut with *Hin*dIII and *Eco*RI) 2)K-54 3)K-103 4)K-102 5)K-125 6)K-83 7)K-25 8)K-174 9)K-73 10)K-106 11)K_2(NCYC 738). The high molecular weight band in lanes 2 and 3 is chromosomal DNA.

Based on electrophoretic protein patterns, at least four groups of strains were distinguished. *Sacch. cerevisiae* strain K-25, K-174, K-103, K-73, K-106 and K-83 comprised the first group. The second group consisted of *Sacch.cerevisiae* strain K-125 and K-102. Strains within each group showed almost identical protein patterns. The overall protein pattern of *Sacch. cerevisiae* NCYC 738 was similar to those of the other *Sacch. cerevisiae* strains. However, in the lower part of the gel, more peaks were found and variations in the concentration of certain peaks were noted. The overall protein pattern of *Sacch. bayanus* (K-54) was different from those of the *Sacch. cerevisiae* strains.

Another method that can be used to differentiate between yeast species, and between strains within a species, is gas chromatographic analysis of cellular fatty acids. Augustyn (1989) indicated that it was possible to differentiate between 46 strains of *Sacch. cerevisiae* by using the mean relative percentages (MRPs) of 10 fatty acids. In our study the MRPs of 8 of the most important cellular fatty acids, viz. C14:0, C14:1(9), C15:0, C15:1(9), C16:0, C16:1(9), C18:0(9) and C18:1(11), were used to distinguish between the killer yeasts (Table 4).

Holman's index of relationship proved to be a useful tool for indicating the degree of similarity between fatty acid profiles and thus also between strains (Table 5).

Values for R ranged from a minimum of 0,828 [$R_{K\text{-}54}$ with $_{K\text{-}103}$] to a maximum of 0,987 [$R_{K\text{-}83}$ with $_{K\text{-}106}$]. All strains except K-54 had an R value greater than 0,900 with the reference strain NCYC 738. Five strains (K-25, K-73, K-83, K-106 and K-125) had an R value greater than 0,950 and three (K-102, K-103, K-174) an R value greater than 9,000 but less than 0,950.

The killer yeast population on grapes and grape skins under South African conditions consists of K_2 strains of *Sacch. cerevisiae* and *Sacch. bayanus*. Results obtained by protein gel electrophoresis and gas chromatographic analysis of cellular fatty acids confirmed the existence of three groups of strains among the K_2 killer yeast isolates. However, isolates K-103, K-125 and K-174 were not placed in the same group based on results obtained by these two techniques. Furthermore, K_2 killer strains differ in the amount of K_2 toxin produced and/or the activity of their toxins. These differences are now being utilized in mixed culture fermentations in order to obtain a better understanding of the interaction between killer and sensitive yeast strains during wine fermentations.

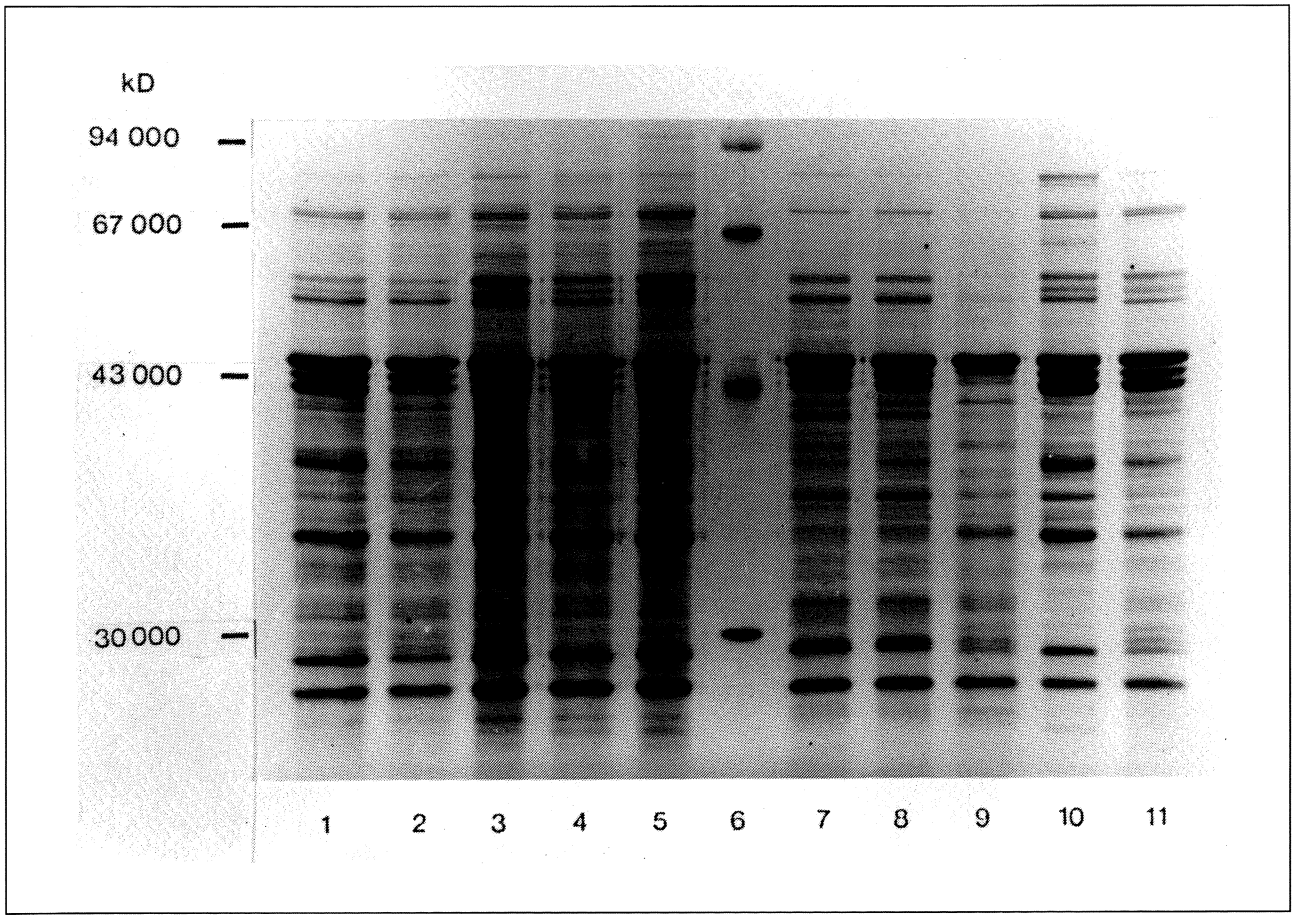

FIGURE 2

Total soluble cell protein patterns of killer yeasts determined by SDS gel electrophoresis. 1) K-174 2) K-103 3) K-73 4) K-106 5) K-83 6) Pharmacia low MW markers 7) K-125 8) K-102 9) K-54 10) K-25 11) K_2 (NCYC 738).

TABLE 4

Mean relative percentage (MRP) of fatty acids from 10 killer yeast strains.

Strains	Killer Yeast Fatty Acid (MRP)							
	C14:0	C14:1(9)	C15:0	C15:1(9)	C16:0	C16:1(9)	C18:0(9)	C18:1(11)
K-25	1,22	0,53	0,16	0,19	9,04	45,47	4,59	37,44
K-54	**1,50**	0,56	**0,35**	**0,24**	**13,81**	**47,23**	3,63	**32,07**
K-73	1,07	0,49	0,14	0,20	8,37	46,26	4,71	37,39
K-83	1,14	0,50	**0,10**	0,13	8,72	46,08	4,55	38,46
K-102	1,11	0,46	0,19	0,20	10,78	**43,57**	**5,89**	37,18
K-103	1,10	**0,58**	0,11	**0,11**	8,37	43,90	**3,62**	**41,57**
K-106	1,01	0,43	0,13	0,13	8,95	45,90	4,77	38,31
K-125	**0,96**	**0,36**	0,19	0,18	10,78	44,48	5,15	37,51
K-174	1,12	0,51	0,16	0,19	**7,15**	46,22	5,06	37,38
K2(NCYC 738)	1,13	0,43	0,13	0,14	9,69	45,07	4,80	38,24
$\bar{x}$	1,14	0,49	0,17	0,17	9,57	45,42	4,68	37,60

Values printed in bold represent the minimum and maximum values for each fatty acid.

TABLE 5

Index of relationship (R), based on fatty acid profiles for all combinations of the killer strains studied.

Strains	K-25	K-54	K-73	K-83	K-102	K-103	K-106	K-125	K-174	K2(NCYC 738)
K-25	1,000	0,870	0,968	0,970	0,939	0,918	0,972	0,955	0,952	0,969
K-54	-	1,000	0,872	0,869	0,860	0,828	0,867	0,870	0,858	0,865
K-73	-	-	1,000	0,973	0,929	0,916	0,971	0,944	0,966	0,956
K-83	-	-	-	1,000	0,926	0,931	0,987	0,943	0,955	0,972
K-102	-	-	-	-	1,000	0,907	0,932	0,973	0,919	0,948
K-103	-	-	-	-	-	1,000	0,926	0,912	0,898	0,927
K-106	-	-	-	-	-	-	1,000	0,952	0,953	0,979
K-125	-	-	-	-	-	-	-	1,000	0,934	0,966
K-174	-	-	-	-	-	-	-	-	1,000	0,941
K2(NCYC 738)	-	-	-	-	-	-	-	-	-	1,000

LITERATURE CITED

AUGUSTYN, O.P.H., 1989. Differentiation between yeast species, and strains within a species, by cellular fatty acid analysis. 2. *Saccharomyces cerevisiae*. *S. Afr. J. Enol. Vitic.* **10,** 8-17.

AUGUSTYN, O.P.H. & KOCK, J.L.F., 1989. Differentiation of yeast species, and strains within a species, by cellular fatty acid analysis. 1. Application of an adapted technique to differentiate between strains of *Saccharomyces cerevisiae*. *J. Microbiol. Methods* **10,** 9-23.

BARRE, P., 1980. Rôle du facteur Killer dans la concurrence entre souches de levures. *Bull. O.I.V.* **53,** 560-567.

BARRE, P., 1984. Le mécanisme Killer dans la concurrence entre souches de levures évaluation et prise en compte. *Bull. O.I.V.* **57,** 635-643.

BENDA, I., 1985. Hefen in der Kellerwirtschaft - Untersuchungen über sogenannte Killerhefen bei der Mostgärung. *Dtsch. Weinbau* **40,** 1166-1171.

BEVAN, E.A. & MAKOWER, M., 1963. The physiological basis of the killer character in yeast. In: GEERTS, S.J. (ed.). Genetics today, XIth Int. Congr. Genet., vol. 1. Pergamon Press, Oxford. pp. 202-203.

BRADFORD, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248-254.

BUSSEY, H., 1974. Yeast killer factor-induced turbidity changes in cells and sphaeroplasts of a sensitive strain. *J. Gen. Microbiol.* **82,** 171-179.

CUINIER, M.C. & GROS, C., 1983. Enquete sur la repartition de levures "killer" en France. *Vignes Vins.* **318,** 25-27.

EXTREMERA, A.L., MARTIN, I. & MONTOYA, E., 1982. A new killer toxin produced by *Saccharomyces cerevisiae*. *Curr. Genet.* **5,** 17-19.

HANNIG, E.M., THIELE, D.J. & LEIBOWITZ, M.J., 1984. *Saccharomyces cerevisiae* killer transcripts contain template-coded polyadenylate tracts. *Mol. Cell. Biol.* **4,** 101-109.

HARA, S., IIMURA, Y. & OTSUKA, K., 1980. Breeding of useful killer wine yeasts. *Am. J. Enol. Vitic.* **31:** 28-33.

HEARD, G.M. & FLEET, G.H., 1987. Occurrence and growth of killer yeasts during wine fermentations. *Appl. Environ. Microbiol.* **51,** 539-545.

HERRING, A.J. & BEVAN, E.A., 1974. Virus-like particles associated with the double-stranded RNA species found in killer and sensitive strains of the yeast *Saccharomyces cerevisiae*. *J.Gen. Virol.* **22,** 387-394.

HOLMAN, R.T., 1978. Quantitative chemical taxonomy based upon composition of lipids. *Prog. Chem. Fats Lipids* **16,** 9-29.

IMAMURA, T., KAWAMOTO, M. & TAKAOKA, Y., 1974. Characteristics of main mash infected by killer yeasts in saké brewing and the nature of its killer factor. *J. Ferment. Technol.* **52**, 293-299.

JACOBS, C.J., FOURIE, I. & VAN VUUREN, H.J.J., 1988. Occurrence and detection of killer yeasts on *Chenin blanc* grapes and grape skins. *S.Afr. J. Enol. Vitic.* **9,** 28-31.

KREGER-VAN RIJ, N.J.W., 1984. The yeasts, a taxonomic study. 3rd. ed. Elsevier Science Publishers, Amsterdam.

MAULE, A.P., & THOMAS, P.D., 1973. Strains of yeast lethal to brewery yeasts. *J. Inst. Brew.* **79,** 137-141.

MITCHELL, D.J., HERRING, A.J. & BEVAN, E.A., 1976. The genetic control of dsRNA virus-like particles associated with *Saccharomyces cerevisiae* killer yeast. *Heredity* **37,** 129-134.

MITCHELL, D.J. & BEVAN, E.A., 1983. SCV "Killer" viruses in yeast. In: SPENCER, J.F.T., SPENCER D.M. & SMITH, A.R.W. (eds.). Yeast Genetics, Springer-Verlag, Berlin, Heidelberg, New York. pp. 371-411.

NAUMOV, G.I. & NAUMOVA, T.I., 1973. Comparative genetics of yeast. XIII. Comparative study of killer strains of *Saccharomyces* from different collections. *Genetika* **9,** 140-145.

NAUMOV, G.I., TYURINA, L.V., BUR'YAN, N.I. & NAUMOVA, T.I., 1973. Wine-making an ecological niche of type K_2 killer *Saccharomyces*. *Biol. Nauki* **16,** 103-107.

PFEIFFER, P. & RADLER, F., 1982. Purification and characterization of extracellular and intracellular killer toxins of *Saccharomyces cerevisiae* strain 28. *J. Gen. Microbiol.* **128,** 2699-2706.

PHILLISKIRK, G. & YOUNG, T.W., 1975. The occurrence of killer character in yeasts of various genera. *Antonie van Leeuwenhoek* **41,** 147-151.

RADLER, F., 1988. Effect of yeast strains on fermentation. In: SMART, R.E., THORNTON, R.J., RODRIQUEZ, S.B. & YOUNG, J.E. (eds.). Proc. Second International Cool Climate Vitic. and Enol. Symposium. Auckland, New Zealand. pp. 298-303.

RADLER, F. & KNOLL, C., 1988. Die Bildung von Killertoxin und die Beeinflussing der Gärung durch Apiculatus-hefen. *Vitis* **27,** 111-132.

ROGERS, D. & BEVAN, E.A., 1978. Group classification of killer yeasts based on cross-reactions between strains of different species and origin. *J. Gen. Microbiol.* **105,** 199-202.

ROSINI, G., 1983. The occurrence of killer characters in yeast. *Can. J. Microbiol.* **29,** 1462-1464.

ROSINI, G., 1985. Effect d'une levure "killer" de *Saccharomyces cerevisiae* sur une souche de levure sensible de la méme espéce non productrice de H_2S et sélectionée pour la vinification dans un milieu de culture mixte. *Bull. O.I.V.* **649,** 214-217.

SEKI, T., CHOI, E.H. & RYU, D., 1985. Construction of a killer wine yeast strain. *Appl. Environ. Microbiol.* **49,** 1211-1213.

SHIMIZU, K., ADACHI, T., KITANO, K., SHIMAZAKI, T., TOTSUKA, A., HARA, S. &DITTRICH, H.H., 1985. Killer properties of wine yeasts and characterization of killer wine yeasts. *J. Ferment. Technol.* **63,** 421-429.

SOMERS, J.M. & BEVAN, E.A., 1969. The inheritance of the killer character in yeast. *Genet. Res.* **13,** 71-83.

SOMMER, S.S. & WICKNER, R.B., 1984. Double-stranded RNA that encode killer toxins in *Saccharomyces cerevisiae*. Unstable size of M double-stranded RNA and inhibition of M_2 replication by M_1. *Moll. Cell Biol.* **4,** 1747-1753.

STUMM, C., HERMANS, J.M.H., MIDDELBEEK, E.J., CROES, A.F. & DE VRIES, G.J.M.L., 1977. Killer-sensitive relationships in yeast from natural habitats. *Antonie van Leeuwenhoek* **43,** 125-128.

TIPPER, D.J. & BOSTIAN, K.A., 1984. Double-stranded ribonucleic acid killer systems in yeasts. *Microbiol. Rev.* **48,** 125-156.

TREDOUX, H.G., TRACEY, R.P. & TROMP, A., 1986. Killer factor in wine yeasts and its effect on fermentation. *S. Afr. J. Enol. Vitic.* **7,** 105-112.

TYURINA, L.V., BUR'YAN, N.I., SKORIKOVA, T.K. & POKROVSKAYA, S.S., 1986. Incidence of *Saccharomyces* yeasts of the killer phenotype in various wine-growing regions and the thermostability of the killer factor. *Mikrobiologiya* **55,** 511-514.

VAN VUUREN, H.J.J. & WINGFIELD, B.D., 1986. Killer-yeasts - Cause of stuck fermentations in a wine cellar. *S. Afr. J. Enol. Vitic.* **7,** 113-118.

VAN VUUREN, H.J.J. & VAN DER MEER, L.J., 1987. Fingerprinting of yeasts by protein electrophoresis. *Am. J. Enol. Vitic.* **38,** 49-53.

WEBER, K. & OSBORN, M., 1969. The reliability of molecular weight determinations by dodecyl sulfate polyacrylamide gel electrophoresis. *J. Biol, Chem.* **224,** 4406-4412.

WINGFIELD, B.D., VAN VUUREN, H.J.J. & PRETORIUS, I.S., 1989. Size differentiation of M_2 genomes among K_2 killer yeasts. *Mycol. Res.* **92,** 364-367.

WINGFIELD, B.D., VAN DER MEER, L.J., PRETORIUS I.S. & VAN VUUREN, H.J.J., 1990. K_3 Killer yeast is a mutant K_2 killer yeast. *Mycol. Res.* **94,** 901-906.

WOODS, D.R. & BEVAN, E.A., 1968. Studies on the nature of the killer factor produced by *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **51,** 115-126.

YOUNG, T.W. & YAGIU, M., 1978. A comparison of the killer character in different yeasts and its classification. *Antonie van Leeuwenhoek* **44,** 59-77.

YOUNG, T.W., 1987. Killer yeasts. In: ROSE, A.H. & HARRISON, J.S., (eds.). The yeasts, vol. 2. Academic Press, London & New York. pp. 131-164.