Group Classification of Killer Yeasts Based on Cross-reactions between Strains of Different Species and Origin

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(Received 14 November 1977)

Strains of killer yeasts of various genera from a variety of sources were tested for their ability to kill each other, two neutral strains and four classes of mutants isolated as being resistant to killer toxin from a stock killer strain $\kappa 12-1$. The strains tested fell into four groups designated TOX1 to TOX4 according to their killing/immunity reaction with one another and their ability to kill the resistant mutants.

INTRODUCTION

Killer yeast strains were first reported by Bevan & Makower (1963) who demonstrated the three phenotypes – killer, neutral and sensitive – with respect to the killer character. Killer strains produce an extracellular toxin which kills sensitive strains (Woods & Bevan, 1968); neutral strains neither produce toxin nor are they killed by it. Most investigations of the killer character have used laboratory stock strains whose origins are uncertain but may have common parentage. The toxin production and immunity of laboratory stock strains are correlated with the presence of extrachromosomal elements which have been identified as double-stranded RNA viruses (Bevan, Herring & Mitchell, 1973; Vodkin & Fink, 1973; Herring & Bevan, 1974). Since the original discovery, killer yeast strains have been found in *Saccharomyces cerevisiae* strains isolated outside the laboratory and in other yeast species (Maule & Thomas, 1973; Naumova & Naumov, 1973; Imamura, Kawamoto & Takaoka, 1974; Philliskirk & Young, 1975; Bussey & Skipper, 1975). Some of these strains have been shown to differ in their pH optimum for killing and, in one case, in their immunity to the toxin produced by the laboratory killer strains (Bussey & Skipper, 1975). Mutants resistant to killing by a laboratory stock killer strain ($\kappa 12-1$) have also been isolated (Al-Aidroos & Bussey, unpublished). In an analogous situation, mutants resistant to colicins in *Escherichia coli* were found to be resistant to a limited number of colicinogenic strains and have been used to characterize them (Davies & Reeves, 1975*a, b*). Experiments reported here were performed to determine the relationship between killing ability and immunity of as many of these strains as were available.

METHODS

Strains and growth media. Details of the strains used in this investigation are shown in Table 1. Strains $\gamma 1$, $\gamma 2$, $x 1$ and $x 2$, obtained from A. P. Maule (Watney-Mann, Mortlake, London), had been isolated as contaminants of a two-stage continuous beer fermenter on separate occasions. It is possible that they are all derived from a common contaminant strain although this would seem unlikely as they differ in ability to flocculate (A. P. Maule, personal communication). Initially, stable single cell isolates were obtained from each strain.

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Table 1. *Yeast strains tested and their pH optima for killing*

Strain	Species	Killer phenotype*	pH optimum for killing	Reference
K4	<i>Saccharomyces cerevisiae</i>	K	4.7	Woods & Bevan (1968)
A364A	<i>S. cerevisiae</i>	K	4.7	Fink & Styles (1972), Wickner (1974)
NCYC 232	<i>S. cerevisiae</i>	K	4.7	Philliskirk & Young (1975)
Y1	<i>S. cerevisiae</i>	K	4.2	Maule & Thomas (1973)
Y2	<i>S. cerevisiae</i>	K	4.2	Maule & Thomas (1973)
X1	<i>S. cerevisiae</i>	K	4.2	Maule & Thomas (1973)
X2	<i>S. cerevisiae</i>	K	4.2	Maule & Thomas (1973)
M437	<i>S. cerevisiae</i>	K	4.2	Naumova & Naumov (1973)
ATCC 15126	<i>Torulopsis glabrata</i>	K	4.0	Bussey & Skipper (1975)
NCYC 575	<i>Saccharomyces drosophilae</i>	K	4.1	Philliskirk & Young (1975)
61	<i>Candida albicans</i>	K	4.7	Mitchell (1974)
68	<i>C. albicans</i>	K	4.7	Mitchell (1974)
N1	<i>S. cerevisiae</i>	N	—	Somers & Bevan (1969)
NCYC 482	<i>S. cerevisiae</i>	N	—	Maule & Thomas (1973)

* K, Killer; N, neutral.

Complete liquid medium contained 1 % (w/v) yeast extract (Difco), 1 % (w/v) Bacto-peptone (Difco) and 2 % (w/v) glucose. Methylene blue agar medium contained, in addition, 1.5 % (w/v) agar and 0.003 % (w/v) methylene blue, and was buffered to the required pH with 0.05 M-citrate/phosphate (CP) buffer. The agar was autoclaved separately from the other constituents of the medium to prevent hydrolysis.

Determination of the killer phenotype. The killer phenotype was determined using the methylene blue agar technique (Somers & Bevan, 1969). The sensitive phenotype was determined only as a background lawn to avoid the problem of blue mutants which are indistinguishable from sensitive strains when streaked on to a killer background (Mitchell & Bevan, 1973).

Determination of the resistant phenotype. The phenotype of the resistant mutants with respect to the various killer strains was determined using crude cell-free toxin preparations in the Well test (Woods & Bevan, 1968). Cells were grown to late-exponential phase (5×10^7 to 7×10^7 cells ml⁻¹) at 23 °C in liquid complete medium buffered to the required pH with CP buffer. The cells were removed by pressure filtration through glass-fibre filters (Whatman GF/A) and cellulose acetate filters (Millipore, 0.45 µm pore size) and concentrated 20-fold by ultrafiltration through an Amicon PM10 membrane in an Amicon model 52 cell [Jencons (Scientific) Ltd, Hemel Hempstead, Hertfordshire] at 4 °C. The resistant strains under investigation were seeded at 5×10^4 cells ml⁻¹ in complete medium with 1 % agar and buffered to the pH optimum of the killer toxin under investigation with CP buffer.

RESULTS AND DISCUSSION

All the strains under investigation were tested for differences in both their killing and immunity cross-reactions. These differences reflect differences in both the killer toxins produced and the immunity system. The information so gained permits the grouping of strains giving the same pattern of cross-reactions.

Initial experiments were performed using the methylene blue agar technique to determine the ability of various strains to kill seven stock sensitive strains on media buffered at various pH values from 4.0 to 5.0. All the strains killed all seven sensitive strains but only over a narrow pH range. All killer strains were tested for their ability to kill each other over a range of pH values from 4.0 to 5.0 to determine the killer phenotype with respect to each other and the effect of pH on the immune reaction. Strain N1, which is neutral to the laboratory killer strains (Somers & Bevan, 1969), and strain NCYC 482, which is neutral to the brewery strains, were also included in these tests. The results are given in Table 2.

None of the strains tested which were resistant to a toxin at its pH optimum were sensitive to the toxin outside the narrow pH band for optimum stability. The immunity

Table 2. *The killing/immunity reactions of killer and neutral yeast strains*

Strains were tested for sensitivity to toxin by spreading them as a background lawn on methylene blue agar and streaking the killer strain on to the lawn.

Killer streak	Background lawn													
	K4	A364A	NCYC 232	61	68	Y1	Y2	X1	X2	M437	ATCC 15126	NCYC 575	N1	NCYC 482
K4	—	—	—	—	—	X	X	X	X	X	X	X	—	X
A364A	—	—	—	—	—	X	X	X	X	X	X	X	—	X
NCYC 232	—	—	—	—	—	X	X	X	X	X	X	X	—	X
61	—	—	—	—	—	X	X	X	X	X	X	X	—	X
68	—	—	—	—	—	X	X	X	X	X	X	X	—	X
Y1	X	X	X	X	X	—	—	—	—	—	X	X	X	—
Y2	X	X	X	X	X	—	—	—	—	—	X	X	X	—
X1	X	X	X	X	X	—	—	—	—	—	X	X	X	—
X2	X	X	X	X	X	—	—	—	—	—	X	X	X	—
M437	X	X	X	X	X	—	—	—	—	—	X	X	X	—
ATCC 15126	X	X	X	X	X	X	X	X	X	X	—	X	X	X
NCYC 575	X	X	X	X	X	X	X	X	X	X	X	—	X	X

x, Killing of background lawn; —, no killing of background lawn.

Table 3. *The immunity of resistant mutants to toxins produced by various killer yeast strains*

The resistant mutants were seeded at 5×10^4 cells ml⁻¹ and tested for their resistance to crude cell-free toxin solution using the Well test.

Strain from which toxin was isolated	Resistant mutant (seeded strain)						
	s14.67 Kre ₁	s14.96 Kre ₁	s30.15 Kre ₁	s14.14 Kre ₂	s14.44 Kre ₃	s14.26 Kre ₃	MB6 Kre ₄
K4	—	—	—	—	—	—	—
A364A	—	—	—	—	—	—	—
NCYC 232	—	—	—	—	—	—	—
61	—	—	—	—	—	—	—
68	—	—	—	—	—	—	—
Y1	—	—	—	X	X	X	X
M437	—	—	—	X	X	X	X
ATCC 15126	—	—	—	X	X	X	—
NCYC 575	—	—	—	X	—	—	—

—, Mutant resistant to toxin; x, mutant sensitive to toxin.

of the strains did not therefore appear to be dependent upon the pH of the medium although the toxins themselves may be rapidly inactivated outside a narrow pH band (Woods & Bevan, 1968; Bussey, 1972; Philliskirk & Young, 1975).

Crude cell-free toxin solutions from the various killer strains were tested against mutants isolated as resistant to killer toxin from laboratory strains using the Well test. A zone diameter consistent with less than 5% of the killing found with a stock sensitive strain was considered a resistant phenotype. The results are given in Table 3.

The strains investigated fell into four groups with respect to their killing and immunity cross-reactions (Table 2). The reactions of the mutants resistant to the toxins showed the same pattern (Table 3). In view of these results, and those of experiments reported elsewhere which showed physicochemical differences between the toxins produced by different killer strains (Bussey & Skipper, 1975; Philliskirk & Young, 1975; Rogers, 1976), one can conclude that the difference in cross-reaction patterns of the strains in each group is due to the production of different killer toxins and immunity systems. Thus it is suggested that the strains are classified in the following manner: (i) TOX1 strains – the laboratory

stock strains, *Candida albicans* strains 61 and 68, strain NCYC 232 and the neutral strain N1; (ii) TOX2 strains – the brewery strains Y1, Y2, X1 and X2, the Russian wine fermentation strain M437 and the neutral strain NCYC 482; (iii) TOX3 strains – *Torulopsis glabrata* strain ATCC 15126; (iv) TOX4 strains – *Saccharomyces drosophilarum* strain NCYC 575. The resistant mutants show different patterns of resistance to strains within these four groups: Kre₁ mutants are resistant to toxins from strains of all four groups; Kre₂ mutants are resistant to toxins from TOX1 strains but are sensitive to all other toxins; Kre₃ mutants are resistant to toxin from TOX1 and TOX4 strains but are sensitive to toxin from strains in groups TOX2 and TOX3; and Kre₄ mutants are resistant to all toxins except those produced by TOX2 strains.

A complete analysis of all known killer strains would no doubt reveal further groups in addition to the four described. Similarly, a search for killer and neutral yeast strains amongst stock cultures may reveal further groups.

The results of the experiments with the resistant mutants suggest each of the toxins has both unique and common sites of attachment or attack on sensitive cells. The killer system in yeast shows many similarities to the colicinogenic system of *Escherichia coli*. The results reported here add further to these similarities, showing different cross-reacting groups as in *E. coli* (Fredericq, 1956; Fields & Luria, 1969*a, b*) and resistant mutants (Davies & Reeves, 1975*a, b*).

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