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# Phenotypic Expression of *Kluyveromyces lactis* Killer Toxin Against *Saccharomyces* spp.

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**The secretion of killer toxins by some strains of yeasts is a phenomenon of significant industrial importance. The activity of a recently discovered *Kluyveromyces lactis* killer strain against a sensitive *Saccharomyces cerevisiae* strain was determined on peptone-yeast extract-nutrient agar plates containing as the carbon source glucose, fructose, galactose, maltose, or glycerol at pH 4.5 or 6.5. Enhanced activity (50 to 90% increase) was found at pH 6.5, particularly on the plates containing galactose, maltose, or glycerol, although production of the toxin in liquid medium was not significantly different with either glucose or galactose as the carbon source. Results indicated that the action of the *K. lactis* toxin was not mediated by catabolite repression in the sensitive strain. Sensitivities of different haploid and polyploid *Saccharomyces* yeasts to the two different killer yeasts *S. cerevisiae* (RNA-plasmid-coded toxin) and *K. lactis* (DNA-plasmid-coded toxin) were tested. Three industrial polyploid yeasts sensitive to the *S. cerevisiae* killer yeast were resistant to the *K. lactis* killer yeast. The *S. cerevisiae* killer strain itself, however, was sensitive to the *K. lactis* killer yeast.**

The killer system in the yeast *Saccharomyces cerevisiae* has been extensively investigated since it was first described by Makower and Bevan (11). Killer strains of *S. cerevisiae* secrete a protein toxin (molecular weight, 11,470) which is lethal to sensitive nonkiller *Saccharomyces* species. The killer system in *S. cerevisiae* is controlled by two double-stranded RNA plasmids, L (4.5 kilobases) and M (1.5 to 1.8 kilobases) (21, 22). Both L and M plasmids are cytoplasmically inherited and separately encapsulated in viruslike particles. M codes for the toxin determinant and for the determinant for immunity to the toxin (1, 20). L is required for production of the capsid protein (9).

Recently, a killer system was described in two strains of the yeast *Kluyveromyces lactis* (7, 8); here the killer system has been attributed to two linear double-stranded DNA plasmids, pGKl1($k_1$) and pGKl2($k_2$). The toxin gene(s) and the gene(s) for immunity to toxin are encoded by pGKl1 (8.8 kilobases), whereas pGKl2 (13.4 kilobases) is required for the maintenance of pGKl1. No viruslike particles have been described as yet for these extrachromosomal plasmids; however, they have been introduced into *S. cerevisiae* by spheroplast fusion (7) and by transformation (6) and expressed normally. The killer toxin from *K. lactis* has been isolated and characterized as a high-molecular-weight protein consisting of two subunits (16). The mode of action of the toxin is not well understood, but it is known to inhibit adenylate cyclase in *S. cerevisiae* and bring about G1 arrest of cells (15), much like the action of *Saccharomyces* α-factor (17).

Yeast cells producing killer toxin are of enormous industrial significance, not only for use in fermentations requiring strict microbiological control (e.g., brewing), but also as models for studying secretory mechanisms involving extracellular proteins and glycoproteins.

In an attempt to provide optimal conditions for toxin production and to improve the detection system (i.e., plate assays), the effects of pH and carbon source on the sensitivity of *Saccharomyces* strains to *K. lactis* toxin was investigated.

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## MATERIALS AND METHODS

**Yeast strains.** The yeast strains employed, with the Labatt Culture Collection numbers, were: *K. lactis* 1437 (killer, IFO 1267), *S. cerevisiae* 1438 (S6, obtained from H. Bussey), *S. cerevisiae* 1465 (killer, A820, obtained from H. Bussey), and the polyploid strains *S. cerevisiae* 1, *Saccharomyces uvarum* (*carlsbergensis*) 21, *S. uvarum* (*carlsbergensis*) 227, and *Saccharomyces diastaticus* 164 (killer).

**Plate assay for killer activity.** Cells of the strain to be tested for killer activity or for sensitivity to killer toxin were grown for 18 h at 28°C in 5 ml of peptone-yeast extract nutrient medium (PYN) which consisted of 0.35% Bacto-Peptone and 0.3% yeast extract (both from Difco Laboratories, Detroit, Mich.), 0.2% potassium phosphate monobasic, 0.1% ammonium sulfate, 0.1% magnesium sulfate septahydrate, and 2% of the carbon source used. Adjustment of the pH (4.5 or 6.5) was made with 1 N HCl or 1 M NaOH. For the plate assays, 2.0% Bacto-Agar (Difco) and 0.002% methylene blue were added.

Approximately $10^5$ killer-sensitive cells were added to test tubes containing 10 ml of molten-agar medium (held at 50°C in a water bath) and vortexed, and lawns were obtained by pouring the agar onto the appropriate predried plates. The overlays were allowed to solidify. With a steel cylindrical tube (6 mm in diameter), wells were made in the agar, ensuring that the bottom of the wells retained a thin layer of agar. A 20-μl portion of the test strain broth or toxin supernatant was placed in the well, and the plates were incubated in an upright position at 21°C for 2 to 3 days. The plates were then examined under light, and zones of inhibition were measured. Clarity of the zones of inhibition was determined qualitatively. Sensitive cells killed by killer toxin accumulated methylene blue, as depicted by a ring of blue cells around the clear zones of inhibition.

The killer toxin was concentrated from a culture broth of *K. lactis* grown in 1 liter of PYN-galactose medium (pH 6.5). After 24 h of incubation, the cells were removed by centrifugation at 8,000 × *g* for 10 min, and the supernatant was subjected to ultrafiltration in an Amicon (model 402) unit fitted with a Diaflo ultrafilter PM30 (30,000-molecular-weight

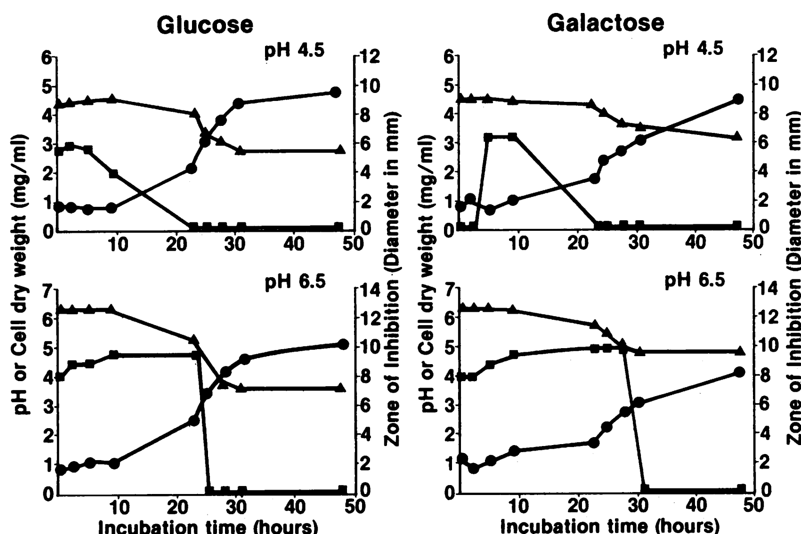


FIG. 1. Growth and toxin production by *K. lactis* 1437 in medium containing glucose or galactose as a carbon source. Symbols: ▲, pH; ●, cell dry weight; ■, zone of inhibition. The medium consisted of Bacto-Peptone (Difco), yeast extract, and 2% (wt/vol) glucose or galactose. Killer toxin supernatant (20 μl) was spotted on a lawn of sensitive cells.

cutoff). Ultrafiltration was performed at 2°C under 60 lb/in$^2$ pressure to obtain a 10-fold concentration of the toxin.

**Growth studies.** *K. lactis* cells pregrown overnight in the appropriate medium were used to inoculate 500-ml Erlenmeyer shake flasks with a 200-ml working volume of the corresponding PYN medium. The flasks were incubated at 21°C and 120 orbits per min in a rotary shaker. Samples (10 ml) were taken periodically and analyzed for pH. The samples were centrifuged at 10,000 × *g* for 10 min, and the supernatant was filter sterilized and analyzed for killer activity as described above, with an *S. cerevisiae* 1438 (sensitive cells) lawn on PYN-maltose plates (pH 4.5). The cell pellet was incubated at 70°C for 48 h to determine the dry weight.

## RESULTS

The inhibitory effect of *K. lactis* broth and supernatant on sensitive *S. cerevisiae* cells was significantly enhanced when plate assays were performed in media at pH 6.5 rather than at pH 4.5 (see Table 1). This would be expected, since the optimum pH for the toxin activity has been reported to be approximately pH 6 (7, 8), although the toxin remains active in a broad pH range of 4 to 8 (16). The individual diameters of the zones of inhibition were dependent on the substrate employed. Thus, with glucose and fructose, the zones of inhibition were consistently smaller than when galactose, maltose, or glycerol was used as the substrate. The degree of clarity of the zones was also enhanced with the latter substrates. Since this effect was observed both with *K. lactis* cells as well as with cell-free toxin, it can be assumed that the effect of the substrate type was primarily on the sensitive *S. cerevisiae* strain. The nonfermentable carbon source glycerol gave the best clearings both with *K. lactis* 1437 cells and cell-free toxin. Similar observations were also made by Sugisaki et al. (16), who found that the activity of the killer toxin was increased 35-fold in the presence of 60% glycerol. It has been established that glycerol also increases the stability of the *Saccharomyces* killer toxin (21).

When *K. lactis* 1437 cells were grown in shake flasks in medium containing glucose or galactose at either pH 4.5 or pH 6.5, the growth of the cells was much faster when glucose rather than galactose was used as the substrate. Growth in glucose corresponded to an early production of killer toxin, as depicted by zones of inhibition in Fig. 1, although the results indicate that toxin production was not directly growth associated. The levels of toxin, however, were much higher at pH 6.5 than at pH 4.5 with either glucose or galactose as the substrate. At pH 6.5, it was also seen that the level of the toxin dropped dramatically after reaching a peak. The reason for this decline, also observed by Sugisaki et al. (16), is not clear. It was not due to a lowering in the pH value, since in PYN-galactose medium the pH value did not drop below 4.9. It is possible that the toxin was degraded by proteases present in the periplasmic space of the cells. The growth studies, in general, indicate that the effect of the carbon source (Table 1) does not seem to be on the production of the toxin, since both glucose- and galactose-containing media gave similar results; nonetheless, the initial pH of the medium does have a significant effect on the production of the toxin. The lack of killer activity during the initial 2 h of incubation in PYN-galactose medium (pH 4.5) is surprising and suggests that at pH 4.5 the uptake of galactose by *K. lactis* cells is slower than the uptake of glucose.

The effect of the *K. lactis* 1437 killer strain on other haploid and polyploid yeasts was investigated and compared with the effects of the *S. cerevisiae* killer strain 1465. Assays were conducted on PYN-maltose plates (pH 4.5), on which the *S. cerevisiae* 1465 killer strain gave the best clearings (C. J. Panchal, unpublished data), and on PYN-galactose plates (pH 6.5), on which the *K. lactis* 1437 killer strain gave large zones of inhibition. Some of the polyploid strains grew poorly on glycerol, and hence PYN-glycerol plates were not used for comparison. Of the polyploid strains tested on PYN-maltose plates (pH 4.5), *S. uvarum* (*carlsbergensis*) 21 and *S. cerevisiae* 1 (both brewing strains) were sensitive to *S. cerevisiae* 1465, but *S. uvarum* (*carlsbergensis*) 227 and *S. diastaticus* 164 were not (Table 2). For *S. diastaticus* 164 this is to be expected, since it is itself a killer strain and thus is immune to the *Saccharomyces* killer toxin. On the same plates, however, both *S. uvarum* (*carlsbergensis*) 21 and *S. cerevisiae* 1 were resistant to *K. lactis* 1437 killer, while *S. uvarum* (*carlsbergensis*) 227 was very sensitive. This could

also be seen on PYN-galactose plates (pH 6.5), although *S. cerevisiae* 1 showed a small degree of sensitivity. *S. diastaticus* 164 was not sensitive to either of the killer strains at the two pH values.

Interestingly, *S. cerevisiae* 1465 killer cells were quite sensitive to *K. lactis* 1437 killer cells, indicating that presence of L and M double-stranded RNA does not provide immunity to the *K. lactis* killer toxin.

## DISCUSSION

The mode of action of the *K. lactis* killer toxin differs from that of the *S. cerevisiae* killer toxin. Whereas the *S. cerevisiae* toxin acts as a protonophore, causing ATP leakage, and as a $K^+$ ionophore (16, 21, 22), the *K. lactis* toxin inhibits adenylate cyclase in yeast cells, causing G1 arrest of sensitive cells (15, 16), much like the action of *Saccharomyces* α-factor pheromone.

The investigations reported here deal with effects of different carbon sources at two pHs on the killing or inhibitory action of *K. lactis* 1437 against sensitive yeast cells. The effects of the sugars glucose and fructose (both implicated in catabolite repression) were quite different from the effects of galactose, maltose, and glycerol (mostly implicated in catabolite derepression [5, 18]). These results could be attributed to the effects of the sugars on cyclic AMP (cAMP) levels in the sensitive cells. Sugisaki et al. (15) reported that the addition of cAMP to sensitive cells of *S. cerevisiae* effectively blocked the inhibitory action of the *K. lactis* killer toxin on the growth and budding of the cells.

cAMP has been reported to be involved in catabolite repression in both bacteria and yeasts (2, 10). Under catabolite repression conditions, when the glucose or fructose concentration is higher than 2%, cAMP levels generally drop, whereas under catabolite derepression conditions (less than 2% glucose and fructose or with galactose, maltose, or glycerol), cAMP levels increase. Thus, cells grown under catabolite repression conditions would be expected to be more susceptible to substances inhibiting adenylate cyclase than would cells grown under catabolite derepression conditions. This has been found to be the case with the action of α-factor pheromone on **a** haploid *S. cerevisiae* cells (14). The extent of G1 arrest of **a** cells was considerably reduced when they were subjected to α-factor treatment in media containing nonfermentable sugars (i.e., derepression). It was postulated (14) that carbon catabolite derepression produced a number of metabolic changes rendering the **a** cells less sensitive to α-factor. One of these changes appeared to be an increased capacity of the **a** cells to inactivate the pheromone, presumably by surface-bound endopeptidases (4), indirectly brought about by increased levels of intracellular cAMP (10, 19). The latter assumption has been challenged by Matsumoto et al. (12, 13), who proclaim that cAMP may not be involved in catabolite repression in *S. cerevisiae*. Eraso and Gancedo (5) recently showed that cAMP-mediated catabolite repression may be strain dependent.

It is thus possible that although the *K. lactis* killer toxin inhibits adenylate cyclase, its action is not affected by catabolite repression in the same manner that the action of α-factor on **a** cells is affected. It is likely that there is more than one mode of action of the toxin, since α-factor only arrests cells in G1 for a short period, except on α-factor-supersensitive strains (3; Panchal, unpublished data), whereas killer toxin inhibits growth of cells for prolonged periods.

The results shown in Table 2 are interesting, since three of the four polyploid yeast strains tested were found to be resistant to the *K. lactis* toxin, although two of them were sensitive to the *S. cerevisiae* killer toxin. This was quite surprising, since the *K. lactis* killer toxin has a broad range of activity against many different yeasts (16). It is possible that the polyploid strains *S. uvarum* (*carlsbergensis*) 21, *S. cerevisiae* 1, and *S. diastaticus* 164 possess some cell wall-membrane surface characteristics (such as lack of toxin-binding sites) that make them resistant to the *K. lactis* 1437 killer toxin.

The results clearly indicate that the killer toxin from *S. cerevisiae* is quite different from that of *K. lactis* with

TABLE 1. Effect of carbon source and pH on the susceptibility of a sensitive *S. cerevisiae* 1438 strain to *K. lactis* 1437 cells and killer toxin

| Substrate (on plates[a]) | Initial pH | Diam (mm) of zone of inhibition with: | |
|---|---|---|---|
| | | 1437 toxin[b] | 1437 cells |
| Glucose | 4.5 | 11.5+[c] | 8+ |
| | 6.5 | 13+ | 10+ |
| Fructose | 4.5 | 11+ | 8+ |
| | 6.5 | 12.5+ | 10+ |
| Galactose | 4.5 | 18+++ | 8+ |
| | 6.5 | 20+++ | 12+ |
| Maltose | 4.5 | 16+++ | 10++ |
| | 6.5 | 19+++ | 12++ |
| Glycerol | 4.5 | 17+++ | 10+++ |
| | 6.5 | 21+++ | 18+++ |

[a] Plates contained Bacto-Peptone (Difco), yeast extract, and 0.002% methylene blue.
[b] Culture supernatants were concentrated 10-fold, and samples (20 μl) of the toxin or culture broth were loaded into agar wells (6 mm in diameter).
[c] +, ++, +++: Relative degree of clarity (in increasing order).

TABLE 2. Effect of carbon source and pH on susceptibility of different yeasts to cells from *S. cerevisiae* 1465 and *K. lactis* 1437 killer strains

| Yeast tested | Diam (mm) of zone of inhibition | | | |
|---|---|---|---|---|
| | PYN[a]-maltose (pH 4.5) | | PYN-galactose (pH 6.5) | |
| | 1465[b] | 1437 | 1465 | 1437 |
| *S. cerevisiae* 1438 (haploid) | 11.5++[c] | 10+ | 0 | 17++ |
| *S. uvarum* (*carlsbergensis*) 21 (polyploid) | 11.5+ | 0 | 0 | 0 |
| *S. uvarum* (*carlsbergensis*) 227 (polyploid) | 0 | 11++ | 0 | 13++ |
| *S. cerevisiae* 1 (polyploid) | 13+ | 0 | 0 | 7+ |
| *S. diastaticus* 164 (polyploid, killer) | 0 | 0 | 0 | 0 |
| *S. cerevisiae* 1465 (haploid, killer) | 0 | 11++ | 0 | 13++ |
| *K. lactis* 1437 (killer) | 0 | 0 | 0 | 0 |

[a] Plates contained Bacto-Peptone (Difco), yeast extract, and 0.002% methylene blue.
[b] Culture broths (20 μl) of the respective strains were loaded into agar wells (6 mm in diameter).
[c] +, ++: Relative degree of clarity (in increasing order).

respect to the phenotypic manifestations on sensitive cells. The presence of the killer phenotype in *S. cerevisiae* 1465 does not make it resistant to the *K. lactis* 1437 killer (Table 2). *Kluyveromyces* yeasts, however, are resistant to the *Saccharomyces* killer toxin (16). In analytical terms, the results reported above indicate that by careful manipulation of medium conditions one can quite readily assay and distinguish between the two killer toxins. Moreover, using this approach one can distinguish, to some degree, between polyploid industrial *S. uvarum* (*carlsbergensis*) strains (e.g., 227 and 21) by determining their sensitivities or resistances to the two different killer toxins, an area of considerable significance in the brewing industry.

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