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Effect of Salt on the Killer Phenotype of Yeasts from Olive Brines

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The killer properties of yeasts isolated from olive brines were examined in the absence and presence of sodium chloride in concentrations of up to 6% (wt/vol). An apparent enhancement of the killing action as the salt concentration increased, as well as changes in the spectra of activity against selected target strains, was observed in a few strains. Culture filtrates from killer strains grown at different NaCl concentrations (0, 3, or 6% [wt/vol]) were tested against sensitive yeasts cultivated under the same conditions. While the sensitivity of the target strain greatly increased in the presence of salt, no significant effect on toxin production was noticed.

Killer-sensitive interactions have been detected in a variety of yeast genera from different sources, though the killer character does not appear to be uniformly distributed (2, 13, 16). Interest in the potential value of the killer property for industrial applications has recently increased (1, 3, 7, 8, 10). However, the ecological role of killer yeasts has been somewhat overlooked, and the conditions governing their behavior in particular habitats are mostly unknown (11, 12). On the other hand, it has been shown that the killing ability may remain unnoticed or be underestimated, depending on the selection of the appropriate sensitive strain and other experimental parameters (6, 14).

In previous work, we found that the majority of yeasts isolated from spontaneously fermenting olive brines possessed the killer character (5) and that strains of *Pichia membranaefaciens*, the dominant species, were particularly active. To assess the importance of the killer phenomenon in this environment, we were interested in determining if the addition of salt, which is common in the production of traditional fermented foods, would affect the expression of the killer phenotype. In preliminary studies, we observed that sodium chloride in concentrations of up to 6% (wt/vol), similar to the salt concentrations in the brines, enhanced the apparent toxicity of a few of the yeasts tested and eventually enlarged their killer spectra. This observation raised the question of whether this higher activity was due to increased production of killer toxin, to increased sensitivity of the strains affected, or to a combination of both. These hypotheses were evaluated in the investigation described in the present article.

Yeast strains (indicated in Table 1) were obtained from the Collection of Yeast Cultures (CYC), Universidad Complutense de Madrid, Madrid, Spain, and from the Portuguese Yeast Culture Collection (IGC), Oeiras, Portugal, and were maintained on agar slants containing 0.5% (wt/vol) yeast extract–1% peptone–2% glucose.

Killer strains were inoculated from a stock culture and grown in 250-ml Erlenmeyer flasks containing 150 ml of YMB (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose) medium, buffered to pH 4.6 with 200 mM phosphate-citrate, in the absence and in the presence of NaCl (3 or 6% [wt/vol]) at 20°C up to the late exponential phase, with slow magnetic stirring. Cells were removed by centrifugation at 3,000 × *g* for 10 min at 4°C, and the supernatant was filtered through a Millipore membrane (0.22-μm pore size). The filtrate was concentrated 10-fold with a Filtron ultrafiltration device with a P10 membrane, working at a nitrogen pressure of 3.1 kg/cm^2. Two methods were used to estimate the killer activity of the concentrated culture filtrate against target strains. In the first method, gradient plates in a method adapted from the Szybalski wedge plate technique (15) were used. Every plate (10 by 10 cm; Sterilin, Hounslow, United Kingdom) consisted of two 15-ml agar layers containing the same basal YMB medium either without NaCl (layer 1) or supplemented with 6% NaCl (layer 2). Once they were autoclaved, the media were maintained at 55°C until they were poured. The plate was slanted before the first layer was poured, allowing the medium to solidify in a wedge shape. The plate was then placed on a flat surface, and the second layer was poured. The plates were left for 24 h to allow the salt concentrations in the layers to equilibrate by diffusion. A suspension of the sensitive strain, directly from the stock solution, in sterile demineralized water was prepared (approximately 10^6 cells/ml) and spread over the surface of the solid medium with sterile cotton buds. Two strips (0.5 by 10 cm) of sterile Whatman no. 1 filter paper were soaked in the concentrated killer culture filtrate and then applied, one on top of the other, onto the surface of the agar seeded with the sensitive strain. The second method was designed to evaluate separately the killing action and the sensitivity of strains grown at different salt concentrations. Aliquots (40 μl) of the concentrated culture filtrate were added to sterile 6-mm-diameter concentration disks (Whatman) previously placed on top of the seeded agar, which was then incubated for 1 week at 20°C. Killer activity was estimated by the width of the growth inhibition zone surrounding the disk.

In the primary screening, the killer activity of culture filtrates from yeasts occurring in olive brines (5) was tested against selected target strains by using gradient plates of sodium chloride in a range of concentrations between 0 and 6% (wt/vol). The results (not shown) varied with both the killer strain and the sensitive strain, but in most cases the apparent toxicity was significantly enhanced as the concentration of salt increased in the medium. An example is shown in Fig. 1. Since the presence of salt in the natural fermentation medium might affect the sensitive yeast as well as the killer yeast, five killer strains (listed in Table 1) representing the various species examined in the preliminary screening were selected to further clarify the effect of salt on the production of killer toxin. The killer yeasts

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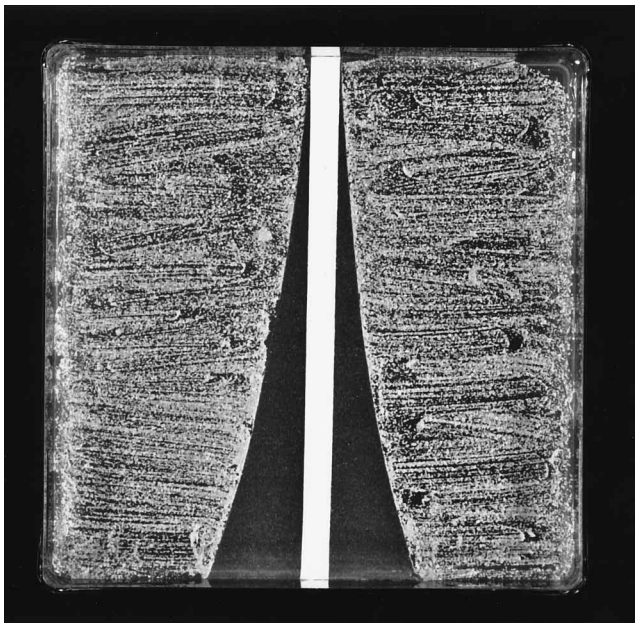

FIG. 1. Growth of the sensitive yeast *C. boidinii* IGC 3430 on a gradient plate of sodium chloride (0% at the top and 6% [wt/vol] at the bottom) incubated for 1 week at 20°C. The filter paper in the middle of the plate contains the concentrated supernatant from a culture of the killer yeast *P. membranaefaciens* CYC 1108.

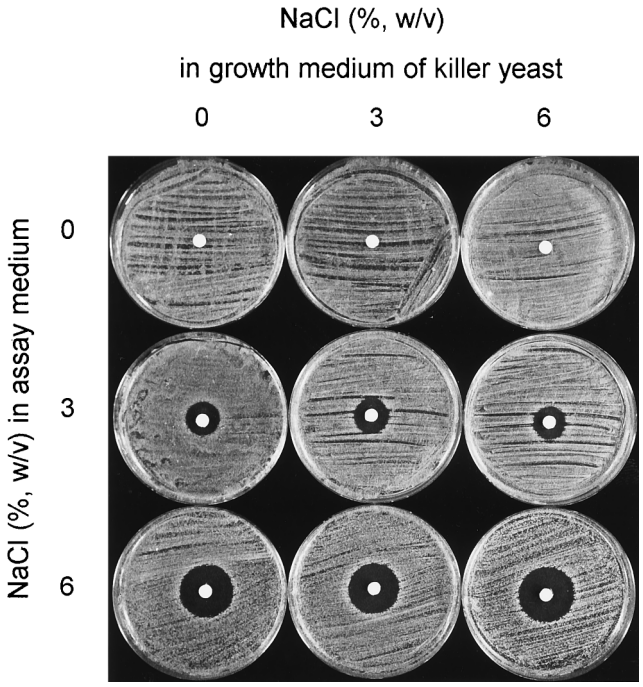

FIG. 2. Inhibitory activity of culture filtrates (in disks) from *P. anomala* CYC 1027, grown in the absence or presence of NaCl, against the sensitive yeast *C. boidinii* IGC 3430 in assay media with different NaCl concentrations.

were grown in liquid medium containing 0, 3, or 6% sodium chloride, and the activity of each culture filtrate thus obtained was tested against three target strains (indicated in Table 1) growing in the assay medium at those different salt concentrations. The results are shown in Table 1. The toxicities of the yeasts did not vary significantly with the concentration of salt used in their cultivation media (Fig. 2). The only exception was *Saccharomyces cerevisiae* CYC 1115, the culture filtrate of which did not display any killer activity when the strain was grown in the presence of either 3 or 6% NaCl. Even when cultivated in the absence of sodium chloride, this strain showed a rather weak activity and was active only against *Kluyveromyces lactis* IGC 4358. Table 1 also shows that the presence of salt in the assay medium may be necessary to reveal the killer phenotype of some yeasts. Salt may enlarge the activity spectra of a killer yeast against the selected target strains, an observation in agreement with the results obtained by Suzuki et al. (14), who reported that halotolerant yeasts were able to kill a larger number of strains in medium containing 4% NaCl than in salt-free medium.

TABLE 1. Effect of salt on the toxicity and sensitivity of selected yeasts

Killer yeast	NaCl concn (% [wt/vol])[a]	Inhibition zone width (mm) for sensitive strain at indicated NaCl concn (% [wt/vol])[b]								
		C. boidinii IGC 3430			*S. exiguus* IGC 4612			*K. lactis* IGC 4358		
		0	3	6	0	3	6	0	3	6
Debaryomyces hansenii CYC 1021	0	—	12	17	—	—	16	—	—	—
	3	—	13	17	—	—	13	—	—	—
	6	—	12	17	—	—	15	—	—	—
Pichia anomala CYC 1027	0	—	14	19	—	—	—	—	9	12
	3	—	14	19	—	—	—	—	9	12
	6	—	14	19	—	—	—	—	9	15
P. membranaefaciens CYC 1106	0	—	17	28	—	15	19	14	20	23
	3	—	19	26	—	14	18	14	20	22
	6	—	20	28	—	14	18	13	20	23
P. membranaefaciens CYC 1108	0	—	20	28	—	13	15	11	20	22
	3	—	20	28	—	13	15	11	20	22
	6	—	15	22	—	12	15	11	20	21
S. cerevisiae CYC 1115	0	—	—	—	—	—	—	9	14	—
	3	—	—	—	—	—	—	—	—	—
	6	—	—	—	—	—	—	—	—	—

[a] Concentration of NaCl in the growth medium of killer yeasts.
[b] Inhibition zone width is calculated as the total diameter of the halo minus the disk diameter. —, no inhibition zone observed. The NaCl concentration for the sensitive yeasts is that of the assay medium.

Toxins produced at any given salt concentration consistently exhibited the highest activity against sensitive strains at the highest concentration of NaCl used in the detection medium (Fig. 2), except for the strain of *S. cerevisiae* mentioned above. These results indicate that the sensitivity of the target strain increases with the concentration of salt in the medium. Since this higher sensitivity could be ascribed to a lower specific growth rate in the presence of salt, the effect of 3 and 6% NaCl on the kinetics of growth of both killer and sensitive yeasts was determined. The effect, as expected, correlated with the halotolerance of the strain (Table 2). Interestingly, and in contrast to the killer strains studied, the target strains did not show a prolongation of the latency phase when transferred from a medium with no salt added to another with 3 or 6% NaCl (not shown). This was particularly surprising in the case of *Candida boidinii* IGC 3430, for which the latter concentration is the maximum allowing sustained growth. The hypothesis of an increased sensitivity of the target strain in the presence of salt being, at least in part, a consequence of a decreased growth rate was reinforced by the observation that the relative enhancement in toxicity follows the same order as the degree of growth inhibition by salt: *C. boidinii* > *K. lactis* > *Saccharo-*

TABLE 2. Effect of sodium chloride on the specific growth rates of killer and sensitive yeasts

Yeast	Maximum tolerated NaCl concn (% [wt/vol])	Specific growth rate (h^{-1}) at indicated NaCl concn (% [wt/vol]) in growth medium		
		0	3	6
Killer				
D. hansenii CYC 1021	15	0.39	0.42	0.40
P. anomala CYC 1027	15	0.36	0.36	0.32
P. membranaefaciens CYC 1106	12	0.35	0.34	0.24
P. membranaefaciens CYC 1108	12	0.33	0.27	0.26
S. cerevisiae CYC 1115	8	0.33	0.27	0.17
Sensitive				
C. boidinii IGC 3430	6	0.28	0.24	0.09
K. lactis IGC 4358	8	0.41	0.34	0.22
S. exiguus IGC 4612	10	0.47	0.34	0.27

[a] The specific growth rates were determined upon growth in flasks containing YMB medium (see text), buffered to pH 4.6 with 200 mM phosphate-citrate, at 28°C with mechanical shaking. Inocula were prepared in the same medium without NaCl. The values shown were obtained in one of two independent experiments.

myces exiguus. The reasons for such an increased vulnerability of the sensitive cell to the toxin's action remain to be elucidated. It is well known that the composition of the yeast plasma membrane and, therefore, its permeability depend on the specific growth rate (9). It has also been reported that killer toxins induce the formation of ion-permeable channels in lipid bilayer membranes (4). The disruption of the ionic equilibrium across the plasma membrane may well lead to an increased mortality of the intoxicated cells in the presence of NaCl. These questions cannot be answered with the available data and will have to be addressed by further study.

Despite the eventual effect of salt on the kinetics of growth of killer strains (Table 2), the amounts of toxin found in the supernatants at the end of the exponential phase were remarkably similar (Fig. 2). Among the killer yeasts analyzed in the present study, *P. membranaefaciens* was the most toxic species, and the two tested strains (CYC 1106 and CYC 1108) behaved alike. The purification and characterization of the toxin(s) produced by *P. membranaefaciens* CYC 1106 are under way.

The incorporation of sodium chloride in the medium used in killer assays may result in the detection of more killer strains among the original yeast isolates from fermenting olive brines. We may also infer, from the results of this study, that salt can be expected to confer an additional selective advantage on killer strains in competition with sensitive cells, eventually influencing the development of spontaneous fermentations and the organoleptic characteristics of the final product.

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