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Yeasts Killer/Sensitivity Phenotypes and Halotolerance

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Summary

Yeast killer phenotype is not uniformly distributed within certain genera or even within some species. Salt has been described as enhancing killer action, especially in strains that have been isolated from salt environments. The aim of this work was to evaluate the extent of the correlation, if any, between killer/sensitivity and salt-stress tolerance phenotypes. A set of 58 different yeast strains (46 species) was studied. As it has been previously described, tolerance to salt stress can be classified in four major classes of tolerance: 1, 2, 3 and 4 M NaCl. Investigation of killer and sensitivity phenotypes was carried out in the absence and in the presence of NaCl from 0 to 3.5 M, in 0.5 M increments. On the basis of this study, two different groups of yeasts were established. One group was mainly composed of the more halotolerant killer yeasts, which also displayed an increase of killer *spectrum* in the presence of salt in the assay. The other group included the less halotolerant strains, whose killer *spectrum* was less significant and either did not vary consistently with salt stress in the assay or decreased in its presence. Although killer activity was found in yeasts belonging to the various classes of salt-stress tolerance, the percentage of strains showing this capacity increased significantly for the classes of higher halotolerance, while the percentage fraction of sensitive strains remained approximately constant. This suggests a phenotypic relationship between high halotolerance and killer capacity manifestation in the presence of high salt concentrations, which is not a consequence of an increase in sensitivity to salt stress of the target strains.

Key words: yeasts, halotolerance, killer activity

Introduction

Yeast killer phenotype appears to be widely distributed within different genera and species (*1–3*). In halotolerant yeast strains, especially salt-food isolates (*4,5*), killer activity has been associated with the enhancement of cell death by killer toxin in the presence of salt stress (*6,7*). It has also been reported that some killer toxins induce the formation of ion-permeable channels (*8,9*). The disruption of ionic equilibrium across the plasma membrane has been suggested as a functional damage of the sensitive cell which might increase the mortality in the presence of salt (*7*). However, a broader diversity of modes of action of the killer toxins has been reported (*10,11*), which cannot be directly related with an effect of salt stress over the action of killer toxins. So far, the toxin produced by the halotolerant yeast *Pichia farinosa* was the only one whose killer activity manifestation was associated with salt stress (*6*). It was described as a chromosome-encoded protein, named SMK (**s**alt-**m**ediated-**k**iller) toxin, which presents a maximum killer activity at 2 M NaCl due to a salt-mediated post-translational mechanism of toxin secretion control (*12,13*).

The objective of this work was to evaluate the possible correlation between killer/sensitivity phenotypes and salt-stress tolerance. For this purpose a set of 58 different yeast strains, corresponding to 46 different spe-

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cies, using the same criteria for its selection and grouping as Lages and co-workers did (*14*), was chosen. In accordance, these strains have been studied in what concerns salt-stress tolerance and classified, as before (*14*), in four major classes of resistance to NaCl.

Material and Methods

Yeast strains and growth conditions

Yeast strains were obtained from the yeast culture collections CBS (Holland) and PYCC (Portuguese Yeast Culture Collection, New University of Lisbon, Portugal – Ref. IGC). Strains were maintained at 4 °C in Bacto YM Agar (YM, Difco) and at –70 °C in glycerol 30 % (w/v).

NaCl maximum tolerance experiments

Some of the strains used in this study were already tested for their growth ability in the presence of different NaCl concentrations, in 1 M increments and classified accordingly (*14*). Strains with unknown salt-stress tolerance, were tested using the same procedure and were included in the four major classes of salt-stress tolerance previously defined (Table 1).

Determination of killer/sensitivity phenotypes in the absence/presence of NaCl

Killer/sensitivity phenotypes were investigated by a cross-reaction procedure (*1*) in plates with YM buffered to pH=4.0 with 0.1 M citrate buffer and containing 0.015 g/L methylene blue (YM-MB). Yeasts to be tested for sensitivity were spread as a lawn on the surface of 3 plates. Subsequently, the surface of the plates was streaked with 10 different yeasts. Inocula cultures used both for lawn and streak were cultivated for 48 h in YM. The plates were incubated at 20 °C for 48 h and then left for 5 days at room temperature. *Kluyveromyces lactis* killer strain CBS 2359 was used as control. The killer effect was considered positive when a clear zone of inhibition of growth and/or a region of bluish-coloured cells surrounded the streaked yeast culture. Results presented were obtained from at least 3 independent experiments (9 plates).

The assays were repeated, in the same culture conditions, in the presence of salt, using YM-MB with NaCl from 0.5 to 3.5 M, in 0.5 M increments. Sensitivity was assayed up to a limit of 0.5 M below the maximum salt concentration allowing growth. Killer activity was determined up to the limit of halotolerance of each strain.

Abbreviations: K, S and N were used for killer, sensitive and neutral strains/phenotypes, respectively (*15*).

Results

According to their salt-stress tolerance, the strains used in this study were grouped in four major classes (Table 1), growth being detected up to 1, 2, 3 and 4 M NaCl, as in Lages and co-workers (*14*). These strains were investigated as to killer and sensitivity phenotypes in cross-reaction assays in the absence and in the presence of salt stress. From a total of 58 different strains, 21

Table 1. List of strains used in the survey, according to their NaCl-tolerance class (*14*); the strains indicated with (*) were classified in this work

1 M NaCl	
Dekkera anomala	IGC 5153
Dekkera bruxellensis	IGC 4179
Lipomyces kononenkoae	IGC 4051
Pichia jadinii	IGC 2541
Pichia stipitis	IGC 4374
Rhodotorula minuta	IGC 4761*
Rhodotorula mucilaginosa	IGC 4617*
Schizosaccharomyces pombe	IGC 2769
Zygosaccharomyces bailii	IGC 4806
Zygosaccharomyces florentinus	IGC 4169
2 M NaCl	
Candida tropicalis	IGC 3097
Debaryomyces castellii	IGC 2839
Fellomyces penicillatus	CBS 5492
Hanseniaspora osmophila	CBS 313
Issatchenkia orientalis	IGC 3806
Kluyveromyces lactis	CBS 2359*
" "	CBS 2360*
Kluyveromyces marxianus	IGC 3014*
" "	IGC 3886
Octosporomyces octosporus	IGC 4180
Pichia angusta	IGC 4129
Pichia anomala	IGC 4121
" "	IGC 4380*
Pichia guilliermondii	IGC 2730*
Pichia haplophila	IGC 2818
Pichia membranaefaciens	IGC 4619*
Rhodotorula glutinis	IGC 4615*
Rhodotorula mucilaginosa	IGC 4791*
" "	IGC 5166*
Saccharomyces cerevisiae	IGC 3507
" "	IGC 4072
" "	IGC 4455*
" "	IGC 4620*
Saccharomyces exiguus	IGC 2543
Schwanniomyces occidentalis	IGC 2829
Zygosaccharomyces bailii	IGC 5167*
Zygosaccharomyces rouxii	IGC 4194
3 M NaCl	
Candida famata	IGC 3056
Candida magnoliae	IGC 2903
Candida parapsilosis	IGC 2545*
Candida silvicultrix	CBS 6269
Citeromyces matritensis	CBS 4462*
" "	IGC 4116
Debaryomyces hansenii	IGC 2968
Pichia etchellsii	IGC 3811
Pichia farinosa	IGC 2459
Pichia membranaefaciens	IGC 3796
Stephanoascus ciferrii	IGC 4164
Torulaspora delbrueckii	IGC 2477*
" "	IGC 2916*
Wingea robertsiae	IGC 3804
4 M NaCl	
Candida cacaoi	IGC 3422
Candida halonitratophila	CBS 5240
Candida halophila	CBS 4019
Candida nodaensis	IGC 3198
Candida versatilis	CBS 1752
Pichia sorbitophila	CBS 7064
Sterigmatomyces halophilus	IGC 4178

Table 2. Strains which presented K and/or S phenotypes, independently of the results obtained in the presence of different salt concentrations; total strains assayed for killer activity: 58 (100 %); total strains assayed for sensitivity: 57 (100 %). *S. pombe*, *H. osmophila* and *C. matritensis* (IGC 4116) were neutral strains

NaCl-tolerance class	1 M	2 M	3 M	4 M
Killer strains 36.2 %	*P. jadinii* *Z. florentinus*	*F. penicillatus* *K. lactis* CBS 2359 *P. anomala* (both strains) *P. haplophila* *R. glutinis* *R. mucilaginosa* (both strains) *Sacch. cerevisiae* IGC 4455 " " IGC 4620	*C. famata* *D. hansenii* *P. farinosa* *P. membranaefaciens* IGC 3796 *T. delbrueckii* IGC 2477	*C. cacaoi* *C. nodaensis* *C. versatilis* *P. sorbitophila*
Sensitive strains 89.5 %	*D. anomala* *D. bruxellensis* *L. kononenkoae* *P. jadinii* *P. stipitis* *R. minuta* *R. mucilaginosa* IGC 4617 *Z. bailii* IGC 4806 *Z. florentinus*	*C. tropicalis* *D. castellii* *I. orientalis* *K. lactis (both strains)* *K. marxianus (both strains)* *O. octosporus* *P. angusta* *P. anomala (both strains)* *P. guilliermondii* *P. membranaefaciens* IGC 4619 *R. glutinis* *R. mucilaginosa* IGC 4791 *R. mucilaginosa* IGC 5166 *Sacch. cerevisiae* (four strains) *Sacch. exiguus* *Sacch. occidentalis* *Z. bailii* IGC 5167 *Z. rouxii*	*C. famata* *C. magnoliae* *C. parapsilosis* *C. silvicultrix* *C. matritensis* CBS 4462 *D. hansenii* *P. etchellsii* *P. farinosa* *P. membranaefaciens* IGC 3796 *S. ciferrii* *T. delbrueckii* (both strains) *W. robertsiae*	*C. cacaoi* *C. halonitratophila* *C. halophila* *P. sorbitophila* *S. halophilus*

Note: *F. penicillatus* was not tested as sensitive strain

showed killer activity (36 %), while 51 (90 %) were sensitive to killer action of at least one strain (Table 2), independently if the results were obtained in the absence or in the presence of salt. Strains presenting either killer or sensitivity phenotypes belong to all classes of salt--stress tolerance. However, while the percentage of killer strains in each halotolerance class increased for the ones of higher salt-stress, the percentage of sensitive strains remained approximately constant (Fig. 1). We chose the most active killer strains to present their *spectra* of action according to the NaCl-tolerance class of the correspondent sensitive strains (Table 3).

The cross-reaction assays were performed in the absence and in the presence of NaCl up to 3.5 M, in 0.5 M increments. Nevertheless, the combinations studied in the presence of salt were limited by the intrinsic halotolerance of each strain (Table 1). In Table 4 we present an example of the salt concentrations used to assay killer capacity of *C. nodaensis*, a strain belonging to the 4 M NaCl-tolerance class. As it can be observed in this table, the assays were performed up to 500 mM below the salt concentration limiting growth of all the target strains. Thus, a reduction in the number of possible combinations was inevitable for higher salt concentrations (Table 5). Despite this reduction, the percentage of combinations in which killer activity was observed remained approximately constant over the whole range of salt molarity, up to 2.5 M NaCl, suggesting an increase in killer activity in the presence of salt. Nevertheless, the total percentage of both killer and sensitivity phenotypes observed decreased for higher salt concentrations, culminating in the total absence of killer activity manifestation in the presence of 3 and 3.5 M NaCl (Tables 4 and 5).

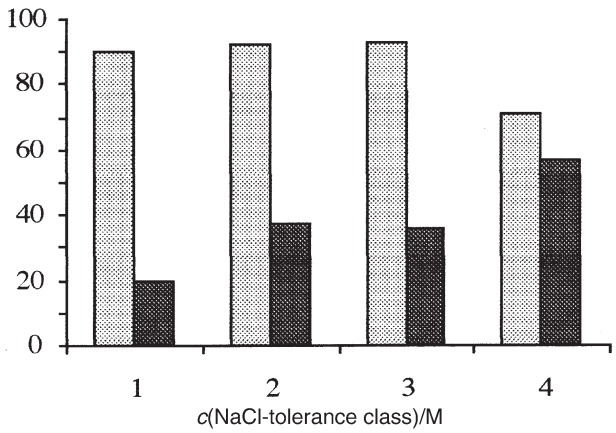

Fig. 1. Percentage of K and S strains from each NaCl-tolerance class; the percentage of K strains increased with halotolerance; S ■ and K ■ strains fraction/%

The expression of killer phenotype significantly differed at different salt concentrations (Table 6). Killer yeasts whose *spectrum* did not exceed one strain were not considered. These strains, showing occasional killer

Table 3. Killer *spectra* of the most active K strains, according to the salt-tolerance class to which S strains belong

K strains		S strains				
NaCl-tolerance class		1 M	2 M	3 M		4 M
P. jadinii	1 M	*D. anomala* *D. bruxellensis* *L. kononenkoae* *R. minuta* *R. mucilaginosa* IGC 4617 *Z. bailii* IGC 4806 *Z. florentinus*	*P. angusta* *P. membranaefaciens* IGC 4619 *R. mucilaginosa* IGC 4791 *Sacch. cerevisiae* (4 strains) *Sacch. exiguus* *Z. bailii* IGC 5167	*P. etchellsii* *P. membranaefaciens* IGC 3796 *T. delbrueckii* (both strains)		*C. halophila* *S. halophilus*
K. lactis CBS 2359	2 M	*L. kononenkoae* *P. jadinii* *R.mucilaginosa* IGC 4617	*O. octosporus* *P. angusta* *P. guilliermondii* *Z. rouxii*	*P. membranaefaciens* IGC 4619 *R. mucilaginosa* IGC 4791 *Sacch. exiguus* *Sacch. occidentalis*	*C. famata* *C. silvicutrix* *D. hansenii*	*C. matritensis* CBS 4462 *P. membranaefaciens* IGC 3796 *T. delbrueckii* IGC 2477	*C. halonitratophila* *C. halophila* *S. halophilus*
P. anomala IGC 4121	2 M	*D. anomala* *L. kononenkoae* *P. jadinii* *P. stipitis* *Z. florentinus*	*C. tropicalis* *D. castellii* *I. orientalis* *K. lactis* (both strains) *P. angusta* *P. guilliermondii*	*K. marxianus* (both strains) *P. anomala* IGC 4380 *P. membranaefaciens* IGC 4619 *Sacch. cerevisiae* (4 strains) *Sacch. occidentalis*	*C. famata* *C. magnoliae* *C. parapsilosis* *C. silvicultrix* *P. etchellsii* *P. farinosa*	*C. matritensis* CBS 4462 *P. membranaefaciens* IGC 3796 *S. ciferrii* *T. delbrueckii* (both strains) *W. robertsiae*	*C. halonitratophila* *P. sorbitophila*
C. famata	3 M		*C. tropicalis* *K. lactis* (both strains) *P. guilliermondii*	*P. anomala* (both strains) *K. marxianus* IGC 3014	*C. magnoliae* *C. parapsilosis* *C. silvicultrix*	*C. matritensis* CBS 4462 *P. farinosa* *T. delbrueckii* (both strains)	*P. sorbitophila*
D. hansenii	3 M	*L. kononenkoae*	*C. tropicalis* *D. castellii* *I. orientalis* *P. angusta*	*K. marxianus* IGC 3014 *P. guilliermondii* *P. anomala* (both strains) *Sacch. exiguus*	*C. magnoliae* *C. parapsilosis* *C. silvicultrix* *P. farinosa*	*C. matritensis* CBS 4462 *T. delbrueckii* (both strains)	*C. cacaoi* *P. sorbitophila*
P. farinosa	3 M	*P. jadinii* *R. mucilaginosa* IGC 4617	*C. tropicalis* *D. castellii* *K. lactis* (both strains) *P. guilliermondii*	*P. membranaefaciens* IGC 4619 *R. glutinis* *R. mucilaginosa* IGC 5166 *Sacch. exiguus*	*T. delbrueckii* (both strains)		
C. nodaensis	4 M	*L. kononenkoae*	*C. tropicalis* *D. castellii* *I. orientalis* *P. angusta*	*K. marxianus* IGC 3014 *P. anomala* (both strains) *P. guilliermondii* *Sacch. exiguus*	*C. magnoliae* *C. parapsilosis* *C. silvicultrix* *P. farinosa*	*C. matritensis* CBS 4462 *T. delbrueckii* (both strains)	*C. cacaoi* *P. sorbitophila*
P. sorbitophila	4 M	*R. mucilaginosa* IGC 4617	*D. castellii* *K. lactis* (both strains) *P. guilliermondii*	*R. mucilaginosa* IGC 5166 *R. glutinis* *Sacch. exiguus*	*C. magnoliae* *T. delbrueckii* IGC 2477		*S. halophilus*

Table 4. *Candida nodaensis* killer phenotype variation with salt concentration in the assay

NaCl-tolerance class	S strains	*c*(NaCl in the assay)/ M	0	0.5	1	1.5	2	2.5	3	3.5
1 M	*Lipomyces kononenkoae*		+	+	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
2 M	*Candida tropicalis*		-	+	+	+	n.a.	n.a.	n.a.	n.a.
	Debaryomyces castellii		+	+	+	+	n.a.	n.a.	n.a.	n.a.
	Issatchenkia orientalis		-	+	-	-	n.a.	n.a.	n.a.	n.a.
	Kluyveromyces lactis CBS 2359		-	-	-	+	n.a.	n.a.	n.a.	n.a.
	Pichia anomala IGC 4121		-	+	+	+	n.a.	n.a.	n.a.	n.a.
	Pichia anomala IGC 4380		+	+	+	+	n.a.	n.a.	n.a.	n.a.
	Pichia guilliermondii		+	+	+	+	n.a.	n.a.	n.a.	n.a.
	Saccharomyces exiguus		-	-	+	-	n.a.	n.a.	n.a.	n.a.
3 M	*Candida magnoliae*		-	-	+	+	+	+	n.a.	n.a.
	Candida parapsilosis		+	+	+	+	+	+	n.a.	n.a.
	Candida silvicultrix		+	+	+	+	+	-	n.a.	n.a.
	Citeromyces matritensis CBS 4462		+	+	+	+	+	-	n.a.	n.a.
	Pichia farinosa		-	+	+	+	+	+	n.a.	n.a.
	Torulaspora delbrueckii IGC 2477		-	-	-	+	+	-	n.a.	n.a.
	Torulaspora delbrueckii IGC 2916		-	+	+	+	-	-	n.a.	n.a.
4 M	*Candida cacaoi*		-	-	+	+	+	+	-	-
	Pichia sorbitophila		-	+	+	+	+	+	-	-

n.a. not assayed
- no killer activity detected
+ killer activity detected

phenotype, are spread through all the classes of salt--stress tolerance, but with higher incidence in the ones of lower halotolerance. From strains in Table 6, some presented killer activity over a broad range of target strains, which increased when the assays were performed in the presence of salt. This enhancement of killer *spectrum* in the presence of salt attained a maximum percentage value at an intermediate salt concentration, varying from 0.5 to 2 M NaCl, above which a steep decrease in killer phenotype manifestation was observed. This variation under salt stress was observed in yeasts from all the classes of salt-stress tolerance, with a higher incidence in the more halotolerant strains. On the other hand, as it can also be seen in Table 6, other yeasts, like *P. jadinii* and *P. membranaefaciens* IGC 3796, decreased their *spectra* of action in the presence of salt, killing a smaller diversity of target strains.

The percentages in Table 6, in a few cases, did not match the variation observed in the number of sensitive strains killed by a given species at each salt concentration assayed, as illustrated in Fig. 2 with the results from *C. versatilis*. The reason for this is that, as it can be seen in Fig. 2 (*Inbox*) as well as in Table 5, the number of strains assayed as sensitive, decreased with the increase of salt molarity in the assay. In the same Figure, *C. nodaensis* illustrates an example in which proportionality between the percentage and the correspondent number of killed strains was maintained.

When analysing the results of the expression of the sensitivity phenotype in the presence of increasing salt in the assay medium, a pattern of variation similar to the one described for killer phenotype was observed. Some strains, like for example *C. tropicalis*, were more sensitive at higher salt concentrations. This yeast was killed by 3.4 % of the total of the strains assayed as killer in the absence of salt, and by 5.2, 8.8 and 10.4 % in the presence of, 0.5, 1 and 1.5 M NaCl, respectively. In some other cases, like for example *Sacch. cerevisiae* IGC

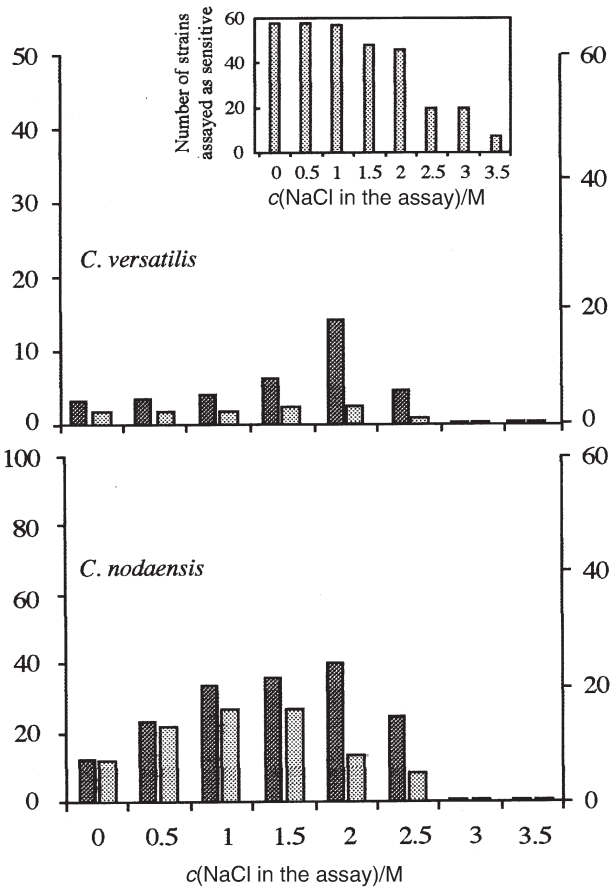

Fig. 2. Comparison of the number and percentage of S strains with the action of *C. versatilis* and *C. nodaensis* obtained at each salt concentration assayed; *inbox*: total number of strains assayed as sensitive at each salt molarity
Fraction of S strains (■)/% and number of S strains (■)

Table 5. Variation of killer phenotype and total percentage of K and S strains at each salt molarity assayed; combinations were considered positive whenever killer phenotype (weak or strong) was observed. The combinations do not necessarily correspond to the same strains for assays performed at different salt molarity

c(NaCl in the assay)/ M	0	0.5	1	1.5	2	2.5	3	3.5	Total
Number of strains assayed (KxS)	58 x 57	58 x 56	57 x 47	48 x 45	46 x 20	20 x 20	20 x 7	7 x 5	58 x 57
Number of combinations	3306	3248	2679	2160	920	400	140	35	12 888
Fraction of positive combinations/%	3.1	3.7	3.6	3.8	3.9	4.0	0	0	3.5
Fraction of K phenotypes/%	31	28	18	19	11	20	0	0	36
Fraction of S phenotypes/%	72	77	64	49	55	35	0	0	90

Table 6. Killer phenotype variation with salt concentration in the assay; results are expressed as the percentage of killed strains, from the total assayed as sensitive, at each salt molarity. Only K strains which killed more than one strain were considered

			S strains / %							
NaCl- tolerance class	*K strains*	*c*(NaCl in the assay)/M	0	0.5	1	1.5	2	2.5	3	3.5
		Number of strains tested for S phenotype (100 %)	57	56	47	45	20	20	7	5
1 M	*P. jadinii*		38.6	12.5	0	n.a.	n.a.	n.a.	n.a.	n.a.
	Z. florentinus		5.3	7.1	6.4	n.a.	n.a.	n.a.	n.a.	n.a.
2 M	*K. lactis* CBS 2359		26.3	32.1	4.3	0	0	n.a.	n.a.	n.a.
	P. anomala IGC 4121		35.1	48.2	46.8	44.4	50.0	n.a.	n.a.	n.a.
	Sacch. cerevisiae IGC 4620		8.8	8.9	10.6	4.4	0	n.a.	n.a.	n.a.
3 M	*C. famata*		0	1.8	21.3	28.9	30.0	15.0	0	n.a.
	D. hansenii		14.0	23.2	34.0	35.6	40.0	35.0	0	n.a.
	P. farinosa		10.5	21.4	21.3	13.3	5.0	0	0	n.a.
	P. membranaefaciens IGC 3796		8.8	3.6	0	0	0	n.a.	n.a.	n.a.
4 M	*C. nodaensis*		12.3	23.2	34.0	35.6	40.0	25.0	0	0
	C. versatilis		3.5	3.6	4.3	6.7	15.0	5.0	0	0
	P. sorbitophila		5.3	17.9	17.0	11.1	0	0	0	0

n.a. = not assayed

4455 or *Sacch. halophilus*, sensitivity decreased with salt in the assay. These strains were killed, respectively, by 5.2 and 6.9 % of the total strains assayed in the absence of salt, by 1.7 and 3.4 % of the strains assayed in the presence of 0.5 M, and by none at 1 M NaCl. Yet, most strains, regardless of the stress-tolerance class to which they belong, either maintained the sensitivity or varied it without a defined tendency, according to salt in the assay (not shown). The exceptions were *Sacch. pombe*, *H. osmophila* and *C. matritensis* (IGC 4116) which did not exhibit killer activity and were immune to the action of all strains.

Discussion

The yeast strains used in this work, classified in four classes of salt-tolerance, included species from very different genera. They were selected, as in an earlier study (*14*), to represent different degrees of resistance of yeasts to salt stress, some of them being well-known osmotolerant food contamination and spoilage yeasts like, for example, *Z. bailii*, *P. membranaefaciens* or *Z. rouxii*. However, as discussed elsewhere (*14,16*), the degree of halotolerance of one strain is not always proportional to its degree of osmotolerance and thus, the salt-stress tolerance classes presented do not necessarily translate osmotic stress resistance of these very same strains. Some of the more osmo and/or halotolerant yeasts we identified as killer strains had already been reported as such: *Sacch. cerevisiae* (*9*), *P. etchellsii*, *D. hansenii* (*17*), *P. anomala* (*2*) and *P. farinosa* (*4*).

Killer activity was found in yeasts from the various classes of salt-stress tolerance, but the percentage of strains presenting this ability increased for the classes of higher halotolerance. Not all of these strains are common contamination or spoilage yeasts. The reason why the ecological advantages or the evolutionary restraint, which may have favoured the maintenance of killer phenotype for the more salt-tolerant ones is not clear. We cannot ignore the possibility that other strains from the same species may behave differently, in particular environment or food isolates, since we used mainly laboratory strains. Furthermore, results could also be different because assays for toxin production and immunity response are highly dependent on the choice of sensitive strains and appropriate conditions for toxin activity (*17*). However, the dimension of this survey, comprising almost 13 000 combinations at different salt concentrations, puts emphasis on the tendency observed between the two phenotypes: halotolerance and killer activity.

An enhancement in killer *spectrum* in the presence of salt was observed in most of the strains presenting a

significant killer activity. Similar results have been reported for *P. membranaefaciens* (*7*), *D. hansenii* and *P. farinosa* (*4*), but these studies seldom expanded salt stress to very high concentrations. *P. farinosa* has been, up to this moment, the only yeast reported to present a maximum of killing activity in the presence of 2 M NaCl (*4,6*). Instead, and in agreement with the strain dependence of killer phenotype discussed above, the type-strain of *P. farinosa* used in this screening presented its maximum killer activity at 1 M NaCl. All the strains included in Table 5 behaved similarly, and presented their maximum of activity between 0.5 and 2 M NaCl. Above that salt concentration an abrupt decrease in *spectrum* was observed. For example, as it can be seen in Table 4, the strains of 4 M salt-stress tolerance level, which were killed in the presence of 2.5 M were not killed in the presence of 3 or 3.5 M NaCl. This suggested that salt was not enhancing killer phenotype expression, since above a variable threshold more salt was not equivalent to higher sensitivity.

Like suggested by Starmer and co-workers (*3*), sensitivity was more widespread than killer activity and few species were immune to all toxins. Sensitivity was not exclusive for the less salt-resistant strains, being approximately equally distributed among all classes of stress-tolerance (Fig. 1). However, this decreased as a whole when the expression of the killer phenotype was assayed in the presence of increasing salt concentrations (Table 5).

The type of results obtained in the presence of salt, in our opinion, are not consistent with the mode of action described for *P. kluyvery* (*8*) or *Sacch. cerevisiae* K1 (*9*) toxins, *i.e.* with the production of already mentioned ion-permeable channels. The reason for this is that there is no reasonable evidence to assume that, above 2 M NaCl, the depletion of internal K^+, as well as the acidification of intracellular pH that such channels occur, should be different than at lower salt concentrations. Instead, one could speculate about a possible reduction of plasma membrane permeability to the toxic agent responsible for growth impediment in cells growing under severe salt stress.

Thus, at the moment, no explanation can be foreseen for the steep decrease observed in killer *spectrum* at the higher salt concentrations. Since the halotolerant killer yeasts described here behaved in a fashion similar to *P. farinosa*, presenting a phenotypic pattern identical to the one of SMK toxin, the possibility arises that these yeasts share a common mode of action with *P. farinosa*. This warrants further attention in the near future.

Conclusions

Although killer activity was found in yeasts from the various classes of salt-stress tolerance, the percentage of strains showing this ability increased for the classes of higher halotolerance. On the other hand, the percentage of sensitive strains remained approximately constant over all the tolerance classes. Moreover, the more halotolerant yeasts displayed a broader killer *spectrum* in the presence of intermediate salt concentrations. Considering that sensitivity, in general, decreased along with the increase in salt-stress, the killer *spectrum* increment might possibly not be a consequence of salt-stress growth inhibition. From the results presented here, a phenotypic relationship between high halotolerance and killer capacity in the presence of high salt concentrations is suggested.

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Osjetljivost/ubilačka svojstva fenotipova i otpornost prema soli

Sažetak

Fenotip ubilačkog kvasca nije jednoliko rasprostranjen unutar određenih vrsta ili čak unutar nekoga soja. Poznato je da sol pojačava ubilačko djelovanje, osobito onih sojeva izoliranih iz slanog okoliša. U ovom se radu nastojao procijeniti stupanj korelacije (ako postoji) između osjetljivosti ubilačkih svojstava te otpornosti prema soli pojednih fenotipova. Kao što je prije opisano, proučena je osjetljivost prema soli 58 različitih sojeva kvasca (46 vrsta). Kvasci su prema osjetljivosti razvrstani u četiri glavne skupine, a rast je utvrđen pri 1, 2, 3 i 4 M NaCl. Ispitivanje ubilačkih svojstava i osjetljivosti fenotipova provedeno je u odsutnosti i prisutnosti NaCl, u rasponu od 0 do 3,5 M, s porastom od 0,5 M. Utvrđene su dvije različite skupine kvasaca. Jedna je uglavnom bila sastavljena od ubilačkih kvasaca jače otpornih na sol, koji su pokazivali povećane ubilačke sposobnosti u prisutnosti soli. Druga je pak obuhvaćala sojeve manje otporne na sol, čija su ubilačka svojstva bila manje izražena, a nisu se bitno mijenjala u prisutnosti soli ili su se čak smanjivala. Određujući otpornost na sol uočeno je ubilačko svojstvo kvasaca koji pripadaju raznim razredima. Postotak sojeva koji su imali to svojstvo bitno je porastao kod onih s većom otpornosti prema soli, dok je postotak osjetljivih sojeva ostao približno konstantan. To upućuje na to da postoji fenotipski odnos između velike otpornosti prema soli i pojave ubilačkih svojstava uz veliki udjel soli, što nije posljedica povećane osjetljivosti sojeva prema stresu u prisutnosti soli.