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Genetic Characterisation of dsRNA Plasmids in Italian *Saccharomyces cerevisiae* Wine Yeasts

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Summary

Yeasts are responsible for the production of fermented foods and, in particular, Saccharomyces cerevisiae is considered as the principal wine yeast. The existence of dsRNA plasmids in the cytoplasm of yeast has been correlated with the production of a killer toxin lethal to other strains of the same species, making this character of great interest in enology. In the present study, we have analysed a total of 56 strains of Sacch. cerevisiae from two different geographical areas of Italy: 27 of them came from the Basilicata region (South) while the remaining 29 strains were from Northern regions. These strains were assayed for the presence of dsRNA plasmids in their cytoplasm. The same strains were also tested for the production of the killer toxin. On the basis of length of dsRNAs, most strains were classified as K_2-K_3. The K_2-$K_{"3"}$ type was much less common and K_1 killer was absent.

These results were corroborated by plate assay and have been explained as a consequence of the pH of the fermenting musts. The existence of neutral strains that showed immunity to killer toxins has been mainly correlated to variation in length in the M genome of dsRNAs. However, the absence of viral particles of the M genome characterised most sensitive strains, while in some cases small M genomes were present. In those cases in which the M genome was present and the killer activity was lost, insertions/deletions affecting the killer preprotoxin gene has been suggested.

Key words: Saccharomyces cerevisiae, killer toxin, dsRNA plasmids, wine yeasts

Introduction

The killer character in yeasts was first described by Bevan and Makower (*1*) in laboratory strains of *Saccharomyces cerevisiae*. The killing action is due to the production and secretion of toxins by the killer strains (K), which are lethal to sensitive yeasts (S). Killer strains are immune to the toxins they produce and some yeast strains, called neutral (N), can resist the action of these toxins even though they do not secrete them. In the case in which resistance is encoded by nuclear genes, the strain is called resistant (R).

The killer phenomenon, has been previously described in smut fungi, slime moulds and bacteria, other than in yeasts (2). Among yeasts, killer activity has been described at present in about 80 species representing 20 genera among both ascomycetous and basidiomycetous yeasts, resulting in more than 100 different killer types (2).

The genetic determinants of the killer property are not the same in all yeast species, but in most of them they are cytoplasmically inherited. Killer production in *Kluyveromyces lactis*, *Pichia acaciae* or *P. inositovora* is associated with linear double-stranded DNA (dsDNA) plasmids. In *Sacch. cerevisiae*, as well as in *Hanseniaspora uvarum*, *Sporidiobolus johnsonii* and *Cystofilobasidium bis-*

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poridii, the killer property is associated with double-stranded RNA (dsRNA) plasmids (*2,3*). These dsRNA plasmids are encapsulated in virus-like particles that are non-infectious and, subsequently, are transmitted by vegetative cell division or hyphal sexual fusion, both very frequent in nature (*4,5*). Finally, the KHR and KSR systems of *Sacch. cerevisiae* (*6,7*) and some killer proteins of *Candida glabrata* (*2*) are encoded by chromosomal genes. In many other cases, the genetic basis of the killer activity is still unknown.

The two known dsRNA viruses of *Sacch. cerevisiae* are called L-A and L-BC. Each of them is a family of structurally and functionally distinct viruses, even though both have a genome size of 4.6 kb. Many strains containing L-A also carry a satellite dsRNA, called M dsRNA, that codes for the killer toxin, as well as for the immunity to that toxin, while the L genome codes for polymerase and capsid proteins for both genomes (*5*). In *Sacch. cerevisiae*, different M dsRNAs have been described each encoding a different killer protein, namely K_1, K_2, K_3, $K_{-3''}$, and K_{28} (*8*).

The killer protein is produced as a preprotoxin that consists of a signal peptide followed by a peptide (δ component) of unknown function, and then by two toxin subunits, α and β, which are separated by the central glycosylated γ peptide. Once the processed protein is secreted in its functional form, the β component would be mainly responsible for binding to the glucans of the cell wall, conferring each killer protein a specific cell-wall binding site. The α component is involved in the interaction with the toxin cytoplasmic membrane receptor and subsequent ion channel formation or inhibition of DNA synthesis, that constitute the effective toxic action (*9*). The N-terminus of γ and the central domain of α are essential for immunity and, it has been suggested that the preprotoxin may bind to the toxin membrane receptor in such a way as to prevent the active toxin from binding, conferring thus immunity (*4,10*).

The toxin genes corresponding to killer proteins K_1, K_2, and K_{28} have been cloned and sequenced (*11–13*). It has been shown that no amino acidic sequence similarity is found among them, in spite of the significant parallels between the toxins, such as the presence of δ, α, β, and γ components, processing of the toxins and binding to cell wall and to cytoplasmic membrane receptors.

Killer yeasts are of great interest in wine making. On one hand, because they can be used as starter cultures in order to kill certain wild yeasts which can cause problems such as delay of fermentation, and induce fermentation and production of off-flavours. On the other hand, the killer selected strains will have immunity against the killing action of wild yeasts and, subsequently, have a greater chance of dominating the fermentation.

In the present study, we have analysed the occurrence of killer activity in natural strains of *Sacch. cerevisiae* from South and North Italy with the aim of correlating the observed phenotypes with the presence of different viral dsRNA plasmids and estimating the killer incidence in natural strains.

Materials and Methods

Yeast strains

Reference yeast strains. The following strains of *Sacch. cerevisiae* from the DBVPG collection (University of Perugia, Italy) were used: K_1 (# 6497), K_2 (# 6499), K_3 (# 6567) and S (# 6500), being. K_1, K_2 and K_3 killer strains and S a sensitive strain to all three killer toxins.

Oenological yeast strains. 56 natural strains, classified as *Sacch. cerevisiae* according to the methods of Kurtzman and Fell (*14*) were used: 27 of them were isolated from Aglianico of Vulture wine (South), while the remaining 29 strains came from different regions of North Italy.

Killer plate assay

Killer assay was determined using the method described elsewhere (*15*). For the sensitivity test, natural strains were inoculated on Petri dishes and a background lawn culture was obtained, and then the different killer yeast were seeded on it. For testing killer activity, both killer and sensitive strains were inoculated as a lawn culture and the natural strains were seeded on them. Sensitivity to the killer protein or the production of killer toxin was evaluated by the formation of inhibition zones around the seeded colonies on the lawn culture.

Molecular characterisation of dsRNA plasmids

Nucleic acids were extracted from overnight cultures according to the method of Coakley *et al.* (*16*) with some modifications. Yeasts were incubated overnight in 5 mL of YPD (1 % Yeast Extract, 1 % Peptone, 2 % Glucose) at 28 °C. 1.5 mL of this culture was harvested in a microfuge tube at 5 000 rpm for 5 min, and the pellet was resuspended in 200 μL of lysis buffer (1 % SDS, 2 % Triton X-100, 100 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl pH = 8.0). Then, 0.3 g of acid washed glass beads (106 microns and finer) and 200 μL of phenol: chloroform:isoamyl alcohol (25:24:1) were added and the mixture was vortexed for 2 min followed by centrifugation at 12 000 rpm for 5 min. The aqueous phase was transferred to a new tube and stored at 4 °C.

The presence of dsRNA plasmids was evaluated by loading 20 μL of the aqueous phase onto a 1.2 % agarose gel. The electrophoresis was carried out in 0.5X TBE buffer (0.045 M Tris-borate, 0.045 M boric acid, 0.001 M EDTA, pH = 8.0) containing EtBr (10 mg/mL). Phage lambda DNA digested with *Hin*d III and a 100 bp ladder were used as molecular markers. The gels were run at 80 V for 3 hours and visualised under UV transillumination.

Results

Among the 56 natural strains, five different phenotypes have been detected based on the sensitivity and activity test (Table 1): strains with the same phenotype as the reference sensitive strain (I), strains which show the same killer activity as K_2 and K_3 reference strains (II), a killer strain different from any of the three refer-

Table 1. Yeast classification based on killer plate assay. The number of natural strains showing each phenotype are also reported

Phenotype	Sensitivity test (seed)			Activity test (lawn)				Strains from	
	K1	K2	K3	S	K1	K2	K3	North	South
Reference strains:									
S	+	+	+	–	–	–	–		
K1	–	+	+	+	–	+	+		
K2	+	–	–	+	+	–	–		
K3	+	–	–	+	+	–	–		
Natural strains:									
I	+	+	+	–	–	–	–	25	14
II	+	–	–	+	+	–	–	3	7
III	+	+	+	+	+	+	+	–	1
IV	+	–	–	–	–	–	–	–	4
V	–	–	–	–	–	–	–	1	1

Table 2. Yeast classification based on the presence of dsRNA plasmids
The number of natural strains for each genotype group are also reported between parentheses

Genotype	L-A/L-BC dsRNA	Satellite dsRNA	Plate phenotype (Table 1)	
Reference strains:				
S	———	———	S	
K1	4.60 kb	1.90 kb	K1	
K2	4.60 kb	1.70 kb	K2	
K3	4.60 kb	1.50 kb	K3	
Natural strains:			North	South
A	———	———	I (14), V (1)	I (2), V (1)
B	4.60 kb	———	I (10)	I (10)
C	4.60 kb	1.70 kb	II (1)	II (2)
D	4.60 kb	1.62 kb	II (1)	II (1)
E	4.60 kb	1.57 kb	II (1)	II (2)
F	4.60 kb	1.50 kb	———	II (2)
G	4.60 kb	2.10 + 1.55 kb	———	III (1)
H	4.60 kb	1.95 kb	———	IV (2)
I	4.60 kb	1.40 kb	———	IV (2)
J	4.60 kb	1.30 kb	I (1)	I (2)

ence killer strains (III), non-killer strains that can resist the action of K_2 and K_3 toxins (IV), and non-killer strains able to resist the action of all three reference killer toxins (V).

Both reference and natural strains have been genotyped for the presence of dsRNA plasmids (Table 2).

All three reference killer strains showed a 4.6 kb plasmid that corresponded to the L-A viral particle, but they differed in the length of the satellite genome, M dsRNA: K_1, K_2 and K_3 were characterised by the presence of a 1.9, 1.7 and 1.5 kb plasmid, respectively (Fig. 1, lanes 1–3).

Among the natural strains, ten different genotypes have been found (Table 2), based on the absence of viral plasmids (A in Table 2; Fig. 1, lanes 5–6), or the presence of different dsRNA plasmids (B – J in Table 2; Fig. 1, lanes 7–16).

As it can be observed in Table 2, there is a relationship between the absence/presence of different dsRNA plasmids and the phenotype obtained by plate analysis. Thus, in the case of reference strains, the sensitive one

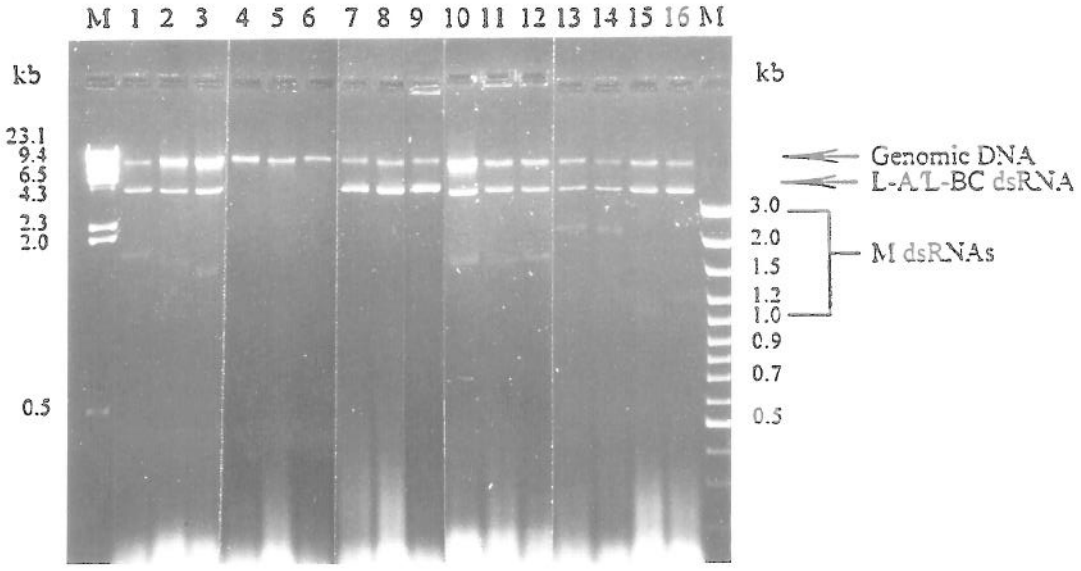

Fig. 1. Agarose gel electrophoresis of nucleic acids extracted from reference and natural yeast strains showing genomic DNA and the presence of viral particles: L-A/L-BC and M dsRNAs. Lane 1, K_1 killer strain (#6497). Lane 2, K_2 killer strain (#6499). Lane 3, K_3 killer strain (#6567). Lane 4, S sensitive strain (#6500). Lanes 5 – 6, natural strain without viral particles (A in Table 2). Lanes 7 – 9, natural strains carrying a 4.6 kb dsRNA (B in Table 2). Lanes 10 – 12, natural K_2-K_3 killer strains (C – F, in Table 2). Lane 13, natural K_2-K_3 neutral strain (H in Table 2). Lane 14, natural K_2-$K_{"3"}$ killer strain (G in Table 2). Lane 15, natural sensitive strain carrying a 1.3 kb dsRNA plasmid (J in Table 2). Lane 16, natural K_2-K_3 neutral strain (I in Table 2). M, molecular mass markers: phage lambda digested with *Hin*d III and 100 bp ladder are shown on the left and the right side, respectively

carries no viral-like particle (Fig. 1, lane 4), while killer strains are characterised by different dsRNA plasmids (Fig. 1, lanes 1–3). It is noteworthy that K_2 and K_3 reference strains present dsRNA plasmids of different length, but both of them show the same phenotype in plate assays. Indeed, it has been shown that K_3 killer yeasts are mutants of K_2 ones, showing both dsRNAs a great sequence similarity tested by hybridisation experiments (*17*). Furthermore, the difference in length of these genomes would not involve the preprotoxin gene and subsequently killer and immunity is maintained in the mutant as in the original genome. Thus, from this point we will refer to these killers as K_2-K_3.

Regarding natural strains, most of them present no viral-like particles (A in Table 2; Fig. 1, lanes 5–6) or only a 4.6 kb dsRNA (B in Table 2; Fig. 1, lanes 7–9) and belong to the group of sensitive strains (I in Table 1). Killer activity and immunity in *Sacch. cerevisiae* have been mainly associated with the presence of satellite dsRNAs (*2,3,18*) thus, those strains lacking this satellite genome could produce killer proteins and/or show immunity only if these characters are encoded by chromosomal genes. Indeed, we observed two strains without viral-like particles (A in Table 2) that are immune to the action of all three reference killer toxins (V in Table 1), suggesting that, in this case, resistance should be encoded by chromosomal gene(s), and thus, the strain may be considered resistant (R) (*19*).

Strains that carry a 1.7 to 1.5 kb dsRNA (C-F in Table 2; Fig. 1, lanes 10–12) are classified as K_2-K_3 killer yeasts (II in Table 1). The existence of length variation in the M dsRNA molecule of K_2-K_3 killer yeast has been described previously (*20,21*), showing that its size can range from 1.3 to 2.0 kb. The fluctuations have been attributed to variations in the AU-rich region at the end of the preprotoxin gene (*20,22*). However, deletions in other regions of the M genome cannot be ruled out.

The natural strain that carries two dsRNA plasmids of 2.1 and 1.5 kb, respectively (G in Table 2; Fig. 1, lane 14) corresponds to a killer strain (III in Table 1) completely different from the three reference killer strains. According to the presence of these two dsRNA plasmids, it could be classified as a K_2-$K_{"3"}$ killer strain (*23*). This would be in agreement with the fact that this strain is able to kill all the reference killer strains, due to the production of the $K_{"3"}$ toxin that is encoded by the 2.1 kb dsRNA. On the other hand, although we should expect that the existence of a 1.5 kb dsRNA should confer immunity to K_2-K_3 killer toxins, we observed that this strain is sensitive to these toxins (III in Table 1). This could be due to the fact that the 1.5 kb dsRNA is present in low quantity (Fig. 1, lane 14) and the amount of produced preprotoxin is not able to prevent the active toxin from binding to the membrane receptor. Another possibility is that the 1.5 kb dsRNA produces no preprotoxin or a mutant one, not able to bind the membrane, and subsequently lacking immunity to K_2-K_3 toxins.

Strains with 1.95 or 1.4 kb dsRNAs (H-I in Table 2) have in common immunity to K_2-K_3 killer toxins (IV in Table 1). A similar strain to the former case (Fig. 1, lane 13) has been described (*24*) and an inverted duplication of a fragment from a K_2-K_3 dsRNA positioned in the middle of the molecule has been demonstrated to be the origin of such length variation. In the other case (Fig. 1, lane 16), a deletion of a part of the K_2-K_3 dsRNA would result in a shorter plasmid. In both cases, the modifica-

tion involves the preprotoxin gene, leading to a non-killer strain, due to the lack of secretion of the toxin or to the production of a non-functional toxin. Anyway, the modification does not affect immunity to K_2-K_3 toxins, suggesting that the central domain of the α component and the N-terminus of the γ component should not be involved (*10*).

Finally, the strain with a 1.3 kb dsRNA plasmid (J in Table 2; Fig. 1, lane 15) corresponds to a sensitive strain (I in Table 1). Most likely this dsRNA is a deletion mutant in which the preprotoxin gene is involved in such a way that both killer activity and immunity have been lost. A deletion mutant of a K_1 killer yeast has been isolated and its M genome of 0.8 kb has been sequenced, showing that the deletion involves the complete β and δ components and the C-terminus of α. In this mutant both killer activity and immunity have been lost (*25*).

Discussion

The analysis of 56 natural wine strains has revealed that there is a correlation between the killer phenotype and the presence of specific dsRNA plasmids. It is striking to note that all killer types detected in the samples here analysed correspond to the K_2-K_3 and $K_{-3''}$ types. Optimum pH for K_1 killer toxin has been described in the range of 4.5–4.9, loosing its activity at pH values lower than 3.5. For K_2-K_3 toxins, optimum pH ranged from 2.9 to 3.7 and that of $K_{-3''}$ from 2.9 to 4.0 (*8,26*). The pH of fermenting musts is about 2.8–3.1 and so, the lower pH for optimal killing activity are more advantageous and this would explain the absence of K_1 killer strains in the samples we have analysed. These results are in agreement with previous surveys (*8,27* and references therein) which show that wild killer strains mainly belong to the K_2-K_3 type and are world wide distributed. $K_{-3''}$ phenotypes are much less common and they have only been found in Spanish (*23*) and Czechoslovakian (*26*) wine yeasts, other than the present study. In contrast with these results, natural killer strains isolated from Japanese vineyards belong to the K_1 type (*8,28*).

The distribution of the killer phenotype is significantly different in the two sampled regions, with a significant prevalence of the killer character in the South (29.6 %) with respect to the North (10.3 %) ($\chi^2_1 = 9.64$, $p < 0.05$), and it is also different from the distribution of the killer types in both samples (Table 2). The occurrence of wild killer yeasts in other countries is very heterogeneous, ranging from 0 to 90 % (*8,27* and references therein). Furthermore, an enormous variability in the incidence of killer yeast has been described in Italy, even in closely geographic areas, resulting in values that ranged from 17–95 % (*29–31*). These differences have been mainly associated with the stage of fermentation, the length of vintage and microecological factors (*27, 31*).

Conclusions

Industrial winemaking in Italy is mainly carried out by using commercially selected wine yeast. However, nowadays, spontaneous fermentation makes up the highest percentage of all fermentation of well-known wines. Nevertheless, a way of improving the fermentation process and maintaining at the same time the specific original properties of each wine requires the use of selected strains to be used as starters cultures. At this point, the presence of the killer character and the immunity to killer toxins are two important criteria that should be taken into account in industrial winemaking. The killer character can represent a tool to select yeast strains with desirable characters to be used as starter cultures, because doing so the inoculated strain have greater chance for dominating the fermentation. Natural yeasts present in the fermenting musts, however, can also exhibit the killer character or present immunity to the killer toxin produced by the inoculated strain, and subsequently could persist along the fermentation.

The analysis of the killer character becomes, therefore, of technological importance, both for selecting suitable natural strains and for knowing how wild killer or immune yeast present in the musts can affect the characteristics of the final product.

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Genetička karakterizacija dsRNA u talijanskim vinskim kvascima *Saccharomyces cerevisiae*

Sažetak

Kvasci sudjeluju u proizvodnji fermentirane hrane, a Saccharomyces cerevisiae je najvažniji vinski kvasac. Postojanje dsRNA plazmida u citoplazmi kvasca uspoređeno je s proizvodnjom ubilačkih toksina letalnih za druge sojeve iste vrste, što je uvelike važno u enologiji. Autori su analizirali ukupno 56 sojeva Sacch. cerevisiae iz dviju različitih geografskih područja Italije: 27 iz područja Basilicata (jug) dok je preostalih 29 sojeva bilo iz sjevernih područja. U sojevima je ispitana prisutnost dsRNA plazmida u njihovoj citoplazmi. Isti su sojevi bili testirani kako bi se utvrdilo proizvode li ubilačke toksine. Na osnovi duljine dsRNA najveći broj sojeva može se klasificirati kao K_2-K_3. Tip K_2-$K_{"3"}$ puno se rjeđe pojavljuje, a nije nađen ubilački toksin K_1. Ti su rezultati potkrijepljeni i rezultatima na pločama, a objašnjeni su posljedicom pH fermentiranih moštova. Postojanje neutralnih sojeva, koji su bili imuni na ubilačke toksine, uglavnom korelira s promjenom duljine dsRNA u M genomu. Međutim, odsutnost virusnih čestica u M genomu značajka je najvećeg broja osjetljivih sojeva, dok su u nekim slučajevima prisutni mali M genomi. Kada je prisutan M genom, a izgubljena ubilačka aktivnost, pretpostavlja se da je došlo do insercije/delecije ubilačkog preprotoksina gena.