SHORT COMMUNICATION

Incidence of symbiotic dsRNA 'killer' viruses in wild and domesticated yeast

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Abstract

Viruses are found in almost all organisms and physical habitats. One interesting example is the yeast viral 'killer system'. The virus provides the host with a toxin directed against strains that do not carry it, while the yeast cell enables its propagation. Although yeast viruses are believed to be common, they have been actually described only for a limited number of yeast isolates. We surveyed 136 *Saccharomyces cerevisiae* and *S. paradoxus* strains of known origin and phylogenetic relatedness. Of these, 14 (*c.* 10%) were infected by killer viruses of one of the three types: K1, K2 or K28. As many as 34 strains (*c.* 25%) were not sensitive to at least one type of the killer toxin. In most cases, resistance did not disappear after attempts to cure the host strains from their viruses, suggesting that it was encoded in the host's genome. In terms of phylogeny, killer strains appear to be more related to each other than to nonkiller ones. No such tendency is observed for the phenotype of toxin resistance. Our results suggest that even if the killer toxins are not always present, they do play significant role in yeast ecology and evolution.

Virus elements that can be found in yeast cells include retroviruses, ssRNA and dsRNA viruses. Most of them are noninfectious and apparently symptomless in their typical hosts; hence, they are often named 'virus-like particles' (VLPs; Ghabrial, 1998). It has been repeatedly reported that their presence determines the production and secretion of low-molecular mass proteins and glycoprotein toxins (Makower & Bevan, 1963). Toxins typically kill sensitive strains of the same and closely related species or genera (Schmitt & Breinig, 2006). The so-called 'killer phenotype' in *Saccharomyces* depends on the presence of dsRNA viruses belonging to the *Totoviridae* family, a class of mycoviruses (Magliani *et al.*, 1997). VLPs consist of two separately encapsulated dsRNA viruses: an LA helper virus and a toxin-coding M-satellite virus. The LA dsRNA component of 4.6 kilobase pair (kb) is an autonomously replicating virus and is responsible for encoding the capsid protein (Gag) and the viral RNA-dependent RNA polymerase (Pol). The M dsRNA subunit of 1.7–2.1 kb is a satellite virus and contains genes for the production of toxins and associated immunity factors. The presence of both subunits together is required for the production of active toxin, which determines the killer phenotype of the host (Marquina *et al.*, 2002). Mutants that have lost the ability to kill but at the same time harbour the resistance to killing are named 'neutral' (Schmitt & Radler, 1990). They produce protein toxins that are inactive due to defective mutations in the toxin gene of the M dsRNA. VLPs tend to be lost at elevated temperature. In this way, normal killer strains can be 'cured' of both toxicity and resistance while neutral strains of resistance only. The action of toxins is mediated by cell surface receptors (Schmitt & Radler, 1990). The toxins kill sensitive yeast either by distorting the cell-membrane pH gradient or by blocking DNA synthesis and thus yeast growth. Based on killing-resistance profiles, three *Saccharomyces* viruses (ScV-M1, ScV-M2 and ScV-M28) have been characterized (Schmitt & Breinig, 2002). Transfer of the virus is strictly vertical (Schmitt *et al.*, 1996). Therefore, killer viruses are inherited either after cell division or through mating with a donor cell, but not by 'horizontal' infection (Wickner, 1974, 1992).

Killer strains are thought to be ubiquitous in both *Saccharomyces* sp. and other yeast species. They have been

incidentally found in cultures derived from the wild (fruits, mushrooms, spontaneous fermentation, soil, decaying plant material), as well as human-made habitats (Starmer *et al.*, 1987; Schmitt & Breinig, 2006; Vadkertiova & Slavikova, 2007). We investigated two collections of *S. cerevisiae* and *S. paradoxus* strains, 136 in total, which were isolated from a variety of habitats including laboratories, soil, wineries, fermentation facilities and human patients. The strains have been sequenced, and therefore, we knew how related they were in terms of phylogenetic distance (Liti *et al.*, 2009; Schacherer *et al.*, 2009). Our goal was to test which of the isolates showed a killer phenotype when confronted with a susceptible laboratory strain (i.e. one known not to harbour a killer virus). We also asked whether the strains that did and did not host viruses differed in their susceptibility to three known yeast killer strains (producing K1, K2 and K28 toxin). In this way, we could estimate how prevalent the viruses are and whether the phenotypes of toxicity and resistance are strictly associated with each other. Moreover, we hoped to see whether the phenotypes of toxicity and resistance show a dependence on the ecological and phylogenetic differentiation of the host strains.

We used standard medium for the propagation of all strains, YPD broth, containing 10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 2% glucose. YPD-MB agar (YPD containing 0.01% methylene blue and 1.5% agar, adjusted with citric-phosphate buffer to pH = 4.6) was used for assaying the killer activity and the presence/absence of resistance. This was carried out by seeding YPD-MB plates with cells of the sensitive M 984 strain and then overlaying a tested strain onto them. A zone of growth inhibition indicated toxin production and thus the presence of active virus. The next step was to classify the discovered killers into one of the three known phenotypes. This was carried out by introducing reference killers – K1, K2 or K28 – hosted by the Y55 and MS300b strains. These were overlaid onto MBA plates seeded with a lawn of every discovered here killer strain. Resistance of a tested strain to a reference killer was confirmed if no signs of clearance through 3 days of incubation were seen. Our survey identified 14 strains infected with viruses (Supporting Information, Table S1). Presence of the viral dsRNA (Castillo *et al.*, 2011), inferred from the observed toxicity, was verified by gel electrophoresis (Supporting Information, Fig. S1). We also confirmed that the host strains can be cured of their killer phenotype by cultivation at increased temperature (37 and 40 °C) (Wickner, 1974).

Figure 1 shows how the killer strains are distributed across different branches in the phylogeny. In the collection consisting of 71 strains (Liti *et al.*, 2009), there were five killers. Of those, one was in *S. cerevisiae* (Fig. 1a) and four in *S. paradoxus* (Fig. 1b). Among the *S. paradoxus* strains, the four killer strains appear to be generally closer to each other than to the remaining, nonkiller, strains. To test whether this could be coincidental, we repeatedly drew at random four strains from an entire tree and calculated a mean phylogenetic distance between them. After 10 000 trials, we found that only in four random sets, the distance was smaller than that actually observed. The type I error as low as $P = 0.0004$ suggests that the killers are indeed phylogenetically grouped. In another collection of *S. cerevisiae* strains, there were five killers located on a common tree (Fig. 1c). An analogous randomization test yielded $P = 0.0016$ and thus again indicated relatedness between the killer strains. In the latter case, however, the killer viruses were of three types: K1, K2 and K28. This precludes common single infection in the past. Rather, some related groups of strains are more likely to acquire, or maintain, viruses than others. Common environment is another potential factor. There were 28 strains isolated from wineries/bakeries (of 60), and they contained as many as four killers (of five) (Fig. 1c). However, the sample of viruses is so small that is does not allow any conclusion about killers being more common in wineries/bakeries (Fisher's exact test, $P = 0.197$). No test is feasible for *S. paradoxus* because all strains of this species were isolated from virtually the same habitat.

Testing the 122 nonkiller strains, we found that 88 of them were sensitive to all toxins, while the remaining 34 were resistant to at least one toxin (Table S2). Among the latter, 13 showed resistance to all killer types, eight to both K1 and K2, and eight to both K2 and K28. Of those resistant to only one toxin, a single strain was resistant to K28 toxin, while four were resistant to the K2 toxin. The discovered phenotype of toxin resistance could have been coded by partly functional killer particles. To test this possibility, all the 34 identified resistant strains were .subject to the standard protocol of virus curing through propagation at elevated temperatures (37 and 40 °C). Only two of the assayed 34 strains lost their resistances and became sensitive to all three killer toxins. In contrast, all the 14 killer strains became sensitive to all three reference killer strains after applying the same procedure of curing. Considering how straightforward and repeatable curing of the 14 discovered killer strains (and the three reference strains) was, we suggest that the failure to cure the 32 resistant (and originally nonkiller) strains indicates a chromosomal basis of this trait. It originated many times independently. This is suggested by randomization tests carried out in a similar, analogous to those described above. The observed distribution of resistance could result from chance with p equal to 0.635, 0.996 and 0.062 for the trees shown in Fig. 1a–c, respectively.

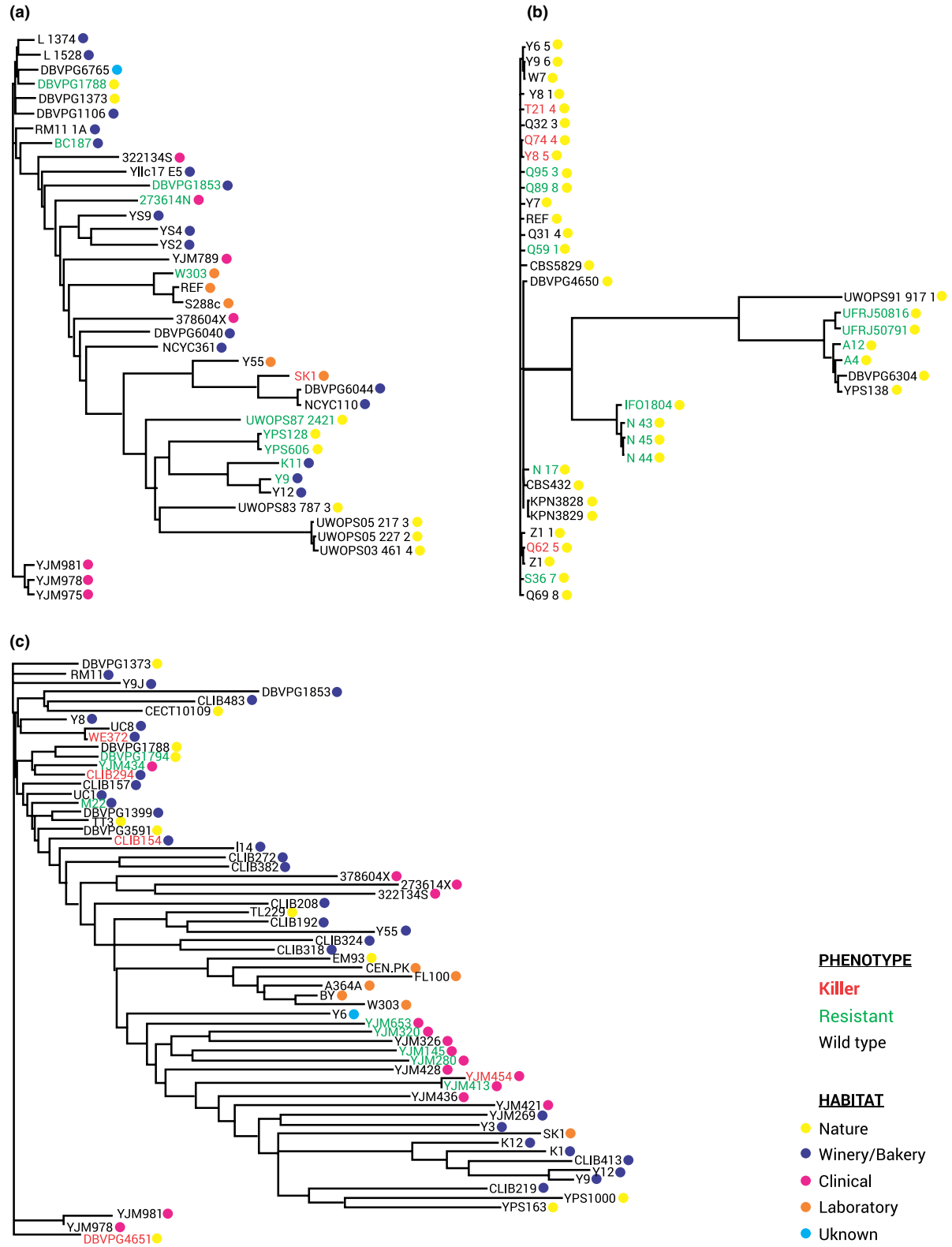

Fig. 1. Phylogenetic relations between killer-producing and killer-resistant yeast isolates. Graphs a and b show, respectively, trees of *Saccharomyces cerevisiae* and *S. paradoxus* strains from the collections of Liti *et al.* (2009). Graph c shows collection of *S. cerevisiae* strains of Schacherer *et al.* (2009). Tables S1 and S2 provide details on the type of toxicity and resistance (K1, K2, K28).

To the best of our knowledge, the present survey of the yeast killer phenotype employed the largest number and the broadest diversity of *Saccharomyces* isolates. It revealed a rather low incidence (10.3%) of the killer phenotype. Loss of killers after isolation is improbable. Any loss of VLPs alters the host gene expression and thus the stability of M dsRNA (McBride *et al.*, 2013). Indeed, the viruses appeared stable in our hands: they were difficult to cure with cycloheximidine and were never lost at the recurring events of freezing and thawing. We got rid of the viruses by applying severe heat stresses, which was probably not experienced by any of these strains after their isolation. We think it is unlikely that the phenotype of being nonkiller but toxin resistant was determined by some viruses overlooked by us. Most of these strains were resistant to more than one toxin, while virus-coded resistance is specific for the partner killer. This can be most likely caused by mutations in the host's genes, perhaps those associated with cell wall components (Page *et al.*, 2003). In addition, our results reveal that resistance is not correlated in any obvious way with habitats from which they were isolated. Neither is genetic relatedness a factor, because the resistant strains lie on branches that were distant from the identified killers. In sum, we found that yeast killer viruses are relatively infrequent, while the resistance to them is rather common. This suggests that wild populations of *Saccharomyces* are confronted with the killer-toxin producing competitors at a rate sufficiently high to promote local origin and maintenance of resistance (Chao & Levin, 1981; Czaran *et al.*, 2002).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. (a) Identification of the killer phenotype. (b) Detection of the viral dsRNA.
Table S1. Killer strains.
Table S2. Toxin resistant strains.