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Geographic Distribution and Genetics of Killer Phenotypes for the Yeast *Pichia kluyveri* across the United States

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Representative strains (n = 61) of the yeast *Pichia kluyveri* from across the United States were studied for their ability to kill 71 other strains (representing 25 species) of yeast. This survey showed killing activity in 69% of the *P. kluyveri* strains tested. More extensive analysis of killer activity of 197 *P. kluyveri* strains against strains of five tester species showed comparable activity (67% of strains tested). This activity was shown to be equally variable within localities, within regions, and across the continent. The genetic basis of the variability was ascertained by tetrad analysis and is most likely due to alleles segregating at three epistatic loci. Evidence for the idea that killer toxins have a role in excluding other yeasts from particular habitats is discussed.

Studies on the distribution of killer toxin-producing yeasts in naturally occurring yeast communities have shown that such yeasts are relatively common, especially in decaying fruit, in which killer toxin-producing strains make up 27% of the yeast community (21). One common fruit yeast, *Pichia kluyveri*, was found to display genetic variability for its ability to kill other yeasts and to be polymorphic for this trait in natural populations. Other observations on the codistribution of *P. kluyveri* and other yeast species suggested an ecological role of this killer species in excluding other yeasts from particular yeast communities. These observations prompted us to investigate the geographic distribution of *P. kluyveri* killer phenotypes, with the goal of understanding why natural populations of this species are polymorphic for their ability to kill other yeasts.

Killer toxin produced by *Saccharomyces cerevisiae* is controlled either by chromosome-dependent genes (7) or by two double-stranded RNA viruslike particles residing in the cytoplasm. The plasmid-encoded system has been reviewed by Bruenn (2), Bussey (3), Wickner (27), and Tipper and Bostian (24). The most studied K_1 killer toxin produced by K_1 killer strains is a small monomeric protein that is heat labile and active only within a pH range of 4.2 to 4.6. The toxin is secreted during exponential growth and kills sensitive cells by coordinated inhibition of macromolecular synthesis and damage to the plasma membrane, after which there is leakage of ATP and potassium ions. Killer toxin K-II of *Hansenula mrakii* also disrupts the permeability of the cell membrane but is stable over a wide range of pH values (4 to 11) and temperatures (1). It is smaller than the K_1 toxin produced by *S. cerevisiae*. Apparently, other *Hansenula* species with Saturn-shaped ascospores also produce toxins against other yeasts (10).

Other yeast species (i.e., *Kluyveromyces lactis*) are known to carry two double-stranded linear-DNA plasmids (8, 9, 25) which produce a high-molecular-weight protein toxin consisting of three subunits (20). This toxin was thought to inhibit the activity of adenylyl cyclase and bring about G_1 arrest in sensitive cells and is thus mechanistically different from the K_1 killer toxin produced by *S. cerevisiae* (23). However, recent evidence indicates that the mode of action is most likely due to permanent arrest of cells in the G_1 phase of the cell cycle and not to a reversible inhibition of adenylyl cyclase (25, 26). The killer toxin from the yeast *P. kluyveri* appears to be a glycoprotein which is stable between pH values 2.5 and 4.7 and at temperatures up to 40°C (13, 14). The mode of action of the *P. kluyveri* toxin is by the formation of ion-permeable channels in phospholipid bilayer membranes, which are similar to bacterial colicins (11).

Classification of killer yeasts on the basis of cross-reactions between strains of different species indicates that several categories of killer and immunity reactions exist in naturally occurring yeasts (3, 4, 12, 16–18, 22, 28). The ascomycetous yeast genera *Debaryomyces*, *Hanseniaspora*, *Hansenula*, *Kluyveromyces*, *Pichia*, and *Saccharomyces* each have one or more species with killer activity. Studies on the properties of their toxins indicate that all killer factors contain a proteinaceous component, in general have low pH optima, and are usually inactivated at elevated temperatures (>35°C), but some may have different genetic determinations (28). Even though killer strains and species are widespread in culture collections, the majority of natural isolates were thought to lack the ability to produce toxins. This apparent low incidence of killer activity in wild yeasts was somewhat misleading because assays for toxin production are highly dependent on the choice of sensitive strains and appropriate conditions for toxin activity (21).

MATERIALS AND METHODS

Strains of *P. kluyveri* used in this study were obtained by shipping styrofoam boxes containing 20 store-bought tomatoes each that had been surface sterilized (by immersion in 70% ethanol for 3 to 5 min) to 15 localities in the United States (Fig. 1). Recipients of these boxes cut a single hole (with a sterile razor blade) in each tomato and placed 10 at one site and the other 10 at a separate site. The sites were backyards, wooded areas, trash heaps, natural areas, etc. The injured fruit attracted insects, especially drosophilids carrying yeasts, and most became infected with insect eggs,

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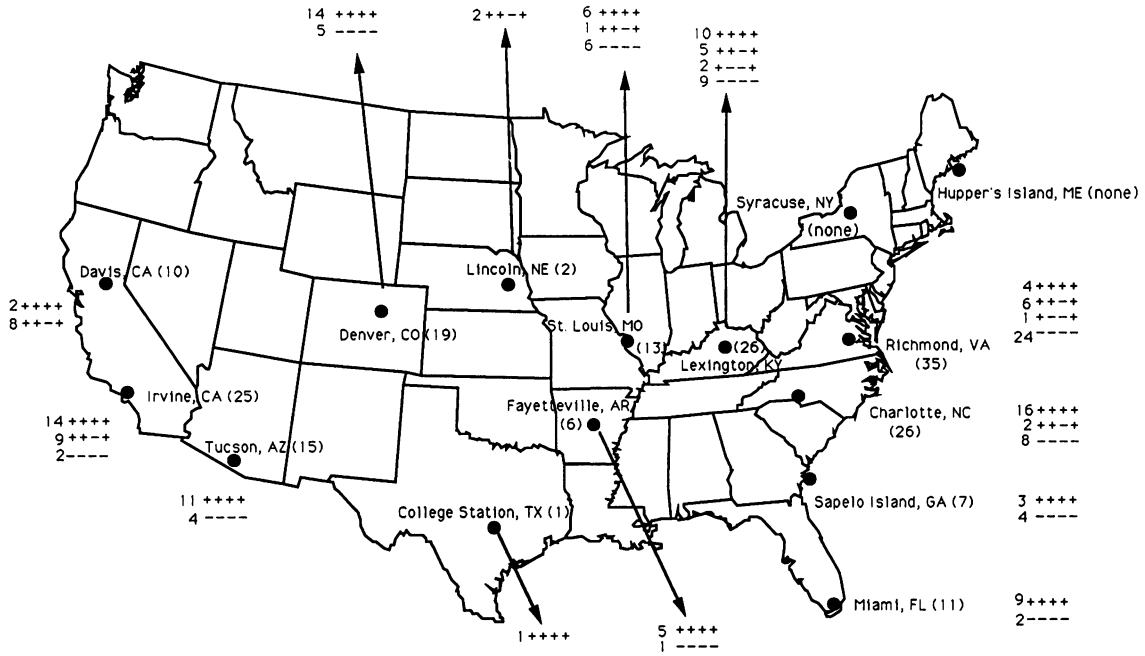

FIG. 1. Geographic distribution of *P. kluyveri* phenotypes across the United States. The number of each killer type is indicated. Killer phenotypes are given in the order Kha, Kcc, Kcm, and Kpa.

larvae, bacteria, filamentous fungi, and yeasts. After 1 week, the rotting tomatoes were packaged in individual sterile bags and shipped to the Syracuse laboratory, where a sample of the rotting material was plated on general (acidified [pH 3.8] yeast extract-malt extract agar) and selective (0.67% yeast nitrogen base [Difco], 0.5% D-glucosamine, 0.1% chloramphenicol, 2% agar) media designed to isolate *P. kluyveri*. Initial isolates were then screened on various media to confirm species identity (15). Strains confirmed to be *Pichia kluyveri* var. *kluyveri* were then tested for their ability to kill 71 strains representing 25 species of yeast (Table 1). These strains were chosen to be representative of cactophilic and tree flux yeast communities because we are particularly interested in how *P. kluyveri* interacts with members of these yeast communities. From these tests, five species of tester yeasts were identified as accounting for most of the variability in killer activity. These species and strains (*Hansenula anomala* 85-256.2, *Cryptococcus cereanus* 83-1112.2, *Candida mucilagina* 84-201.7, *Pichia amethionina* var. *pachycereana* 80-314.1, and *Candida glabrata* Y55) were retested and subsequently used as representatives of killer phenotypes in genetic studies.

Two killer assays were employed. One (the standard assay) was identical to that described by Starmer et al. (21). The second was an assay designed to eliminate false-negative responses due to variable growth rates of tester species. The second method for assaying killer activity was carried out by constructing bilayered plates. The standard killer-strain medium was prepared, autoclaved, and poured into a 9-cm petri dish. After the agar had set, a sterile circular piece of Whatman no. 1 filter paper (trimmed to fit the 9-cm plate) was placed on top of the agar and smoothed flat with a sterile glass rod. A thin layer of sterile hot killer-strain medium was then poured over the filter paper and allowed to cool. Potential killer strains were replicated onto this surface and allowed to grow for 48 h at 25°C, after which the top layer of agar with yeast colonies and filter paper was removed with sterile forceps. Potentially sensitive strains were suspended in water and spread as a lawn (at approximately 10^6 cells per ml) on the exposed agar surface, which contained toxins that diffused through the agar and filter paper. This technique is especially useful when the potentially sensitive strains have extremely different growth rates.

In ascertaining the killer activity of a strain, we adopted a continuous scale of reading the size of both the clear zone of inhibition (i = 0 to 10) and the region of blue cells (k = 0 to 10). We adopted the convention that (i) when readings were $i = 0$ and $k = 0$, the strain was scored as a nonkiller; (ii) when readings were $0 < i \leq 3$ and $0 < k \leq 3$, the strain was scored as a weak killer; and (iii) when readings were $i \geq 4$ or $k \geq 4$, the strain was scored as a killer.

Genetic analysis of natural isolates and laboratory crosses was carried out by dissection of untreated asci with a micromanipulator, as described by Fowell (5). Crosses were made by mixing opposite mating types and dissecting asci containing four ascospores as soon as asci appeared (usually 2 to 4 days). Unordered tetrad analysis was used to ascertain linkage relationships.

RESULTS

Geographic distribution of killer activity. Representative strains of *P. kluyveri* (61 of 197 identified) from sampling localities were tested for their ability to kill 71 strains (representing 25 species) (Table 1) of yeasts typically found in the cactus necrosis-tree flux yeast communities (6). Of the *P. kluyveri* strains, 31% showed no killer activity, while those that did have activity killed, on the average, 12 to 13, or about 20%, of the tester yeasts (Fig. 2). However, at least one representative of 15 species or 60% of the 25 species was killed by one or more *P. kluyveri* strains.

TABLE 1. Strains of yeasts used for screening killer phenotypes

Collection no.	Species[a]	Habitat[b]	Locality[c]	No. of killer-positive *Pichia kluyveri*[d]
84-820.2	*Pichia opuntiae*	*Opuntia stricta*	Trinkey, NSW, Australia	2
84-822.2	*Pichia opuntiae*	*Opuntia stricta*	Trinkey, NSW, Australia	0
84-823.2	*Pichia opuntiae*	*Opuntia stricta*	Trinkey, NSW, Australia	0
84-824.2	*Pichia opuntiae*	*Opuntia stricta*	Trinkey, NSW, Australia	2
83-510.1	*Pichia thermotolerans*	*Carnegiea gigantea*	Ironwood, Tucson, Ariz.	0
83-505.1	*Pichia thermotolerans*	*Carnegiea gigantea*	Ironwood, Tucson, Ariz.	0
82-550A	*Pichia antillensis*	*Cephalocereus royenii*	Fox Bay, Montserrat	0
82-604B	*Pichia antillensis*	*Cephalocereus royenii*	Beef Island, Tortola, Br. V. I.	0
84-821.3	*Candida sonorensis*	*Opuntia stricta*	Trinkey, NSW, Australia	0
84-822.3	*Candida sonorensis*	*Opuntia stricta*	Trinkey, NSW, Australia	0
84-823.4	*Candida sonorensis*	*Opuntia stricta*	Trinkey, NSW, Australia	0
84-824.3	*Candida sonorensis*	*Opuntia stricta*	Trinkey, NSW, Australia	0
84-829.2	*Candida sonorensis*	*Opuntia stricta* fruit	Trinkey, NSW, Australia	0
84-831.2	*Candida sonorensis*	*Opuntia stricta* fruit	Trinkey, NSW, Australia	0
83-522.2	*Candida sonorensis*	*Drosophila nigrospiracula*	Ironwood, Tucson, Ariz.	0
81-672.2	*Candida sonorensis*	*Opuntia lindheimeri*	Ozona, Tex.	0
83-847.2	*Candida sonorensis*	*Opuntia stricta*	Navassa Island	0
84-823.1	*Clavispora opuntiae*	*Opuntia stricta*	Trinkey, NSW, Australia	0
82-445A	*Clavispora opuntiae*	*Opuntia stricta*	Jacmel, Haiti	0
84-822.1	*Kloeckera apiculata*	*Opuntia stricta*	Trinkey, NSW, Australia	0
84-825.1	*Kloeckera apiculata*	*Opuntia stricta* fruit	Trinkey, NSW, Australia	11
84-826.1	*Kloeckera apiculata*	*Opuntia stricta* fruit	Trinkey, NSW, Australia	0
84-830.1	*Kloeckera apiculata*	*Opuntia stricta* fruit	Trinkey, NSW, Australia	0
82-622A	*Kloeckera apiculata*	*Opuntia moniliformes*	Beef Island, Tortola, Br. V. I.	5
84-823.3	*Pichia kluyveri*	*Opuntia stricta*	Trinkey, NSW, Australia	0
84-670.2b	*Pichia kluyveri*	*Opuntia stricta* fruit	O'Hara, NSW, Australia	0
83-466.AM	*Pichia kluyveri*	*Opuntia phaeacantha*	Ironwood, Tucson, Ariz.	0
84-825.2	*Pichia nakasei*	*Opuntia stricta* fruit	Trinkey, NSW, Australia	0
84-826.2	*Pichia nakasei*	*Opuntia stricta* fruit	Trinkey, NSW, Australia	0
84-830.2	*Pichia nakasei*	*Opuntia stricta* fruit	Trinkey, NSW, Australia	0
84-213.2	*Cryptococcus cereanus*	*Carnegiea gigantea*	Ironwood, Tucson, Ariz.	0
84-201.4	*Cryptococcus cereanus*	*Opuntia phaeacantha*	Ironwood, Tucson, Ariz.	0
84-204.2	*Cryptococcus cereanus*	*Opuntia phaeacantha*	Ironwood, Tucson, Ariz.	0
82-608C	*Cryptococcus cereanus*	*Cephalocereus royenii*	Beef Island, Tortola, Br. V. I.	0
83-1112.2	*Cryptococcus cereanus*	*Opuntia stricta*	Grand Cayman Island	28
83-855.2	*Cryptococcus cereanus*	*Opuntia stricta*	Navassa Island	0
83-504.2	*Pichia heedii*	*Drosophila nigrospiracula*	Ironwood, Tucson, Ariz.	40
80-316.8	*Pichia heedii*	*Drosophila mettleri larva*	Ironwood, Tucson, Ariz.	40
83-501.4	*Pichia cactophila*	*Drosophila nigrospiracula*	Ironwood, Tucson, Ariz.	0
83-518.3	*Pichia cactophila*	*Drosophila nigrospiracula*	Ironwood, Tucson, Ariz.	0
85-201.2	*Pichia cactophila*	*Opuntia phaeacantha*	Ironwood, Tucson, Ariz.	0
83-862.1	*Pichia cactophila*	*Opuntia stricta*	Navassa Island	0
82-441A	*Pichia cactophila*	*Opuntia stricta*	Jacmel, Haiti	6
80-314.1	*Pichia amethionina* v.p	Saguaro-soaked soil	Ironwood, Tucson, Ariz.	17
83-514.3	*Pichia amethionina* v.p	*Drosophila nigrospiracula*	Ironwood, Tucson, Ariz.	8
82-554A	*Pichia amethionina* v.f	*Cephalocereus royenii*	Fox Bay, Montserrat	0
84-201.7	*Candida mucilagina*	*Opuntia phaeacantha*	Ironwood, Tucson, Ariz.	32
84-204.5	*Candida mucilagina*	*Opuntia phaeacantha*	Ironwood, Tucson, Ariz.	0
84-237.1	*Hansenula polymorpha*	*Opuntia phaeacantha*	Santa Catalina Mountains, Tucson, Ariz.	40
85-442.1	*Hansenula polymorpha*	*Opuntia phaeacantha*	Ironwood, Tucson, Ariz.	40
85-221.1	*Debaryomyces hansenii*	*Prosopis juliflora*	Happy Valley, Tucson, Ariz.	0
85-240.1	*Debaryomyces hansenii*	*Prosopis juliflora*	Happy Valley, Tucson, Ariz.	0
85-251.2	Unknown #2	*Prosopis juliflora*	Happy Valley, Tucson, Ariz.	0
85-344.1	Unknown #2	*Prosopis juliflora*	Mt. Lemon Hwy., Tucson, Ariz.	0
85-327.1	*Pichia membranaefaciens*	*Prosopis juliflora*	Tanque Verde Wash, Tucson, Ariz.	4
85-343.2	*Pichia membranaefaciens*	*Prosopis juliflora*	Mt. Lemon Hwy., Tucson, Ariz.	0
85-256.1	*Kluyveromyces lactis* v.d	*Quercus emoryii*	Happy Valley, Tucson, Ariz.	40
85-272.1	*Kluyveromyces lactis* v.d	*Quercus emoryii*	Happy Valley, Tucson, Ariz.	40
85-256.2	*Hansenula anomala*	*Quercus emoryii*	Happy Valley, Tucson, Ariz.	40
85-270.1	*Hansenula anomala*	*Quercus emoryii*	Happy Valley, Tucson, Ariz.	40
85-260.1	*Saccharomyces dairensis*	*Quercus emoryii*	Happy Valley, Tucson, Ariz.	39
85-285.1	*Saccharomyces dairensis*	*Quercus emoryii*	Happy Valley, Tucson, Ariz.	29
85-263.1	*Pichia pastoris*	*Quercus emoryii*	Happy Valley, Tucson, Ariz.	24
85-348.1	*Pichia pastoris*	*Quercus emoryii*	Molino Basin, Tucson, Ariz.	0
83-856.1	*Pichia barkeri*	*Opuntia stricta*	Navassa Island	0
83-866.4	*Pichia barkeri*	*Opuntia stricta*	Navassa Island	0
83-1120	*Pichia norvegensis*	*Opuntia stricta*	Grand Cayman Island	1
83-871.4	*Pichia norvegensis*	*Opuntia stricta*	Navassa Island	2
84-826.3	*Candida krusei*	*Opuntia stricta* fruit	Trinkey, NSW, Australia	0
Y55	*Candida glabrata*			43

[a] v.p, var. *pachycereana*; v.f, var. *fermentans*; v.d, var. *drosophilarum*.
[b] Rotting cladodes, stems, or fruits of cactus, slime fluxes of trees or associated adult drosophilids. *Drosophila nigrospiracula* and *Drosophila mettleri* are associated with *Carnegiea gigantea* (saguaro) and saguaro-soaked soil, respectively.
[c] NSW, New South Wales; Br. V. I., British Virgin Islands; Hwy., Highway.
[d] Of 61 strains of *P. kluyveri* tested, number that killed the given organism.

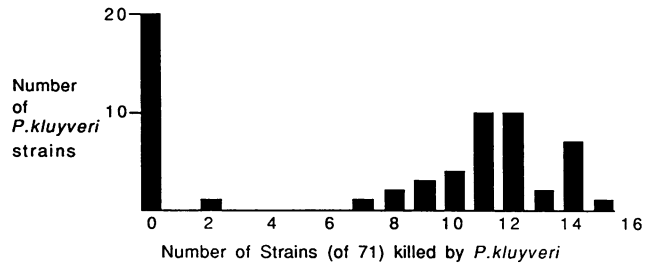

FIG. 2. Distribution of *P. kluyveri* killing activity against 70 tester strains representing 24 species.

Some groups of tester yeasts showed almost identical reactions to the *P. kluyveri* strains. These were *Pichia heedii*, *Hansenula polymorpha*, *Kluyveromyces lactis* var. *drosophilarum*, *H. anomala*, and *Saccharomyces dairensis* (one strain). Ten species were resistant to all of the *P. kluyveri* strains tested.

Of the 197 strains tested for activity against *H. anomala*, *Cryptococcus cereanus*, *Candida mucilagina*, *P. amethionina*, and *Candida glabrata*, 67% showed killing activity. The distribution of killing versus nonkilling was analyzed by grouping the sites within each locality into three regions (western, central, and eastern) (Table 2). A comparison of each region for proportion of strains with killing activity shows a trend of decreasing activity from western to eastern regions (88, 71, and 55%) (Table 2). This trend is also apparent for activity against all of the tester strains except *Candida mucilagina*; activity against this yeast was intermediate in the western, highest in the central, and lowest in the eastern regions (54, 64, and 40%, respectively). However, the trend of decreasing activity from western to eastern regions is not significant, as ascertained by a nested analysis of variance on the arcsine square root of the proportion killed within and between regions ($F = 1.16$; df = 2,10; $P > 0.05$). This indicates that the variability across the United States is equivalent to the variability within regions. Likewise, the variability among localities within regions is not different from the within-locality variability for killing activity ($F = 0.53$; df = 10,10; $P > 0.05$).

Strains from previous collections of *P. kluyveri* associated with decaying cactus in Texas, Mexico, islands in the Caribbean, and localities in Queensland and New South Wales, Australia, were also tested for their ability to kill the tester strains. These strains also show a similar proportion of killer activity (77 to 79%), but isolates from Mexico and the Caribbean have much lower proportional activity against *H. anomala* (33%) (Table 2) and higher proportional activity against *Candida mucilagina* (71%) (Table 2).

We also note that we did not recover any *P. kluyveri* strains from New York or Maine and that the species range may be limited to lower latitudes.

Genetic analysis. Earlier studies on the genetic determination of killer activity showed that at least one nuclear gene was segregating for killer activity against *Candida glabrata* Y55 (21). The variability was found as heterozygotes in naturally occurring isolates. Crosses between spore clones derived from the original isolates also gave supporting evidence for monogenetic control of killer activity against Y55 (21). In this study, we have detected variability for killer activity against different yeast species. Across the United States, the geographic distribution of killer phenotypes indicated that variability for the ability to kill at least three yeast species was considerable within sampling localities (Fig. 1; Table 2). In these assays, we found activity against *H. anomala* and *P. amethionina* to be consistently positive or consistently negative (nonkilling) for both, and in this article, we imply activity against both when referring to activity against *H. anomala*.

Dissection of 10 diploid strains from across all regions showed that segregation for killer activity (against Y55) occurred in one-half of the killer-positive strains. Dissection of nonkiller strains yielded only nonkiller phenotypes. In an attempt to understand the genetic basis of this variability, several crosses were made and resulting spore clones were analyzed for their killing phenotypes.

A cross from a previously isolated spore (84-670.2C) which was not able to kill any of the three tester strains (but was able to kill Y55) was mated to a strain (88-370.2F) which was able to kill all of the tester strains as well as Y55. The progeny from this cross had mainly parental phenotypes, but recombinants did occur. However, most of the recombinants occurred in asci which had fewer than four viable spores, and thus, reciprocal recombinant phenotypes were not recovered from the same ascus. Table 2 gives the distribution of killer types from random spores of this cross. Killer phenotypes are indicated by Kha, Kcc, Kcm, and Kpa, which signify killing of *H. anomala*, *Cryptococcus cereanus*, *Candida mucilagina*, and *P. amethionina*, respectively. The phenotypic distribution (26 Kha^+ Kcc^+ Kcm^+, 12 Kha^+ Kcc^+ Kcm^-, 8 Kha^+ Kcc^{-w} [weak activity], Kcm^-, 15 Kha^- Kcc^- Kcm^-) and a lack of Kha^+ Kcc^- Kcm^+ phenotypes can be explained by a three-locus genetic model. The loci designated *klA*, *klB*, and *klC* are presumed to have a decreasing spectrum of activity against the yeasts tested: klA^+ kills *H. anomala* and has weak or no activity against *Cryptococcus cereanus*; klB^+ kills *H. anomala* and *Cryptococcus cereanus*; klC^+ kills *H. anomala*, *Cryptococcus cereanus*, and *Candida mucilagina*. The parental genotypes were presumed to be klA^- klB^- klC^- (84-670.2C) and klA^+ klB^+ klC^+ (88-370.2F). Table 3 gives the mapping of genotypes to phenotypes for this model. The observed and expected numbers of phenotypes of the cross are given in Table 4. A G-test (distributed as chi-square) yielded a value of 7.08 (df = 3; $P > 0.05$), and thus, the model of three independent genes is acceptable for describing the outcome.

It was probable that spore viability in the progeny was affected by aneuploidy, and thus, further crosses were made from spores derived from this cross. The spores were chosen to carry different marker genes that also segregated in the cross. These markers were (i) the ability to utilize D-glucosamine as the sole source of carbon (gla^+:gla^-), (ii) the ability to use ethyl acetate as a carbon source (eta^+:eta^-), and (iii) the mating type (h^+:h^-). A spore (DX) with the phenotype Gla^+ Eta^- Kha^+ Kcc^w Kcm^- (genotype h^+ gla^+ eta^- klA^+ klB^- klC^-) was mated with a spore (X) of phentoype Gla^- Eta^+ Kha^+ Kcc^+ Kcm^- (genotype h^- gla^- eta^+ klA^+ klB^+ klC^-). Table 5 lists the genotypes for the parental strains used in the cross of DX × X. A total of 45 four-spored asci were dissected. Twenty of those asci yielded four viable spores each. Table 6 gives the tetrad analysis for the ability to kill *Cryptococcus cereanus*, the ability to assimilate D-glucosamine, the ability to use ethyl acetate, and the mating type. It was noted that the sensitivity-resistance response of *Cryptococcus cereanus* was paralleled by reduced (sensitive) or increased (resistance) responses for killing of *H. anomala* and *P. amethionina*. Thus, the epistatic model of gene activity was supported by these observations. The *klB* gene is apparently linked to the *gla*

TABLE 2. Distribution of killer phenotypes for *P. kluyveri* isolates from 23 sites in 12 states in three regions of the United States, Mexico and the Caribbean, and Australia

Isolate source	Site	No. of isolates with Kha Kcc Kcm Kpa phenotype[a]															
		++++	+++−	++−+	++−−	+−++	+−+−	+−−+	+−−−	−+++	−++−	−+−+	−+−−	−−++	−−+−	−−−+	−−−−	Total
Northern California	1	2		8														10
Southern California	1	9		3													2	14
	2	5		6														11
Arizona	1	5															3	8
	2	6															1	7
West region total		27		17													6	50
Colorado	1	14															1	15
	2																4	4
Texas	1	1																1
Nebraska	1			2														2
Missouri	1	6		1													6	13
	2	1																1
Arkansas	1	2																2
	2	3															1	4
Central region total		27		3													12	42
Kentucky	1	4		4				2									2	12
	2	6		1													7	14
Virginia	1	2		4													10	16
	2	2		2				1									14	19
North Carolina	1	9		1													8	18
	2	7		1														8
Georgia	1																4	4
	2	3																3
Florida	1	3															1	4
	2	6															1	7
East region total		42		13				3									47	105
Mexico and Caribbean		3	4	1						9			1		1		5	24
Australia		1	4		2												2	9
84-670.2C × 88-370.2F cross		26		12				8									15	61

[a] Phenotypes are given in the order Kha, Kcc, Kcm, Kpa.

TABLE 3. Genetic model for killer activity

Locus	Allele	Phenotype[a]		
		Kha	Kcc	Kcm
klA	klA^+	+	−(w)	−
	klA^-	−	−	−
klB	klB^+	+	+	−
	klB^-	−	−	−
klC	klC^+	+	+	+
	klC^-	−	−	−

[a] w, weak.

gene by about 20 map units. The other two genes segregating in this cross were independent of one another and of *gla-klB*. However, the linkage relationships among the genes cannot be determined with certainty because of the relatively low number of tetrads available for analysis.

A second cross between strain DX (h^+ gla^+ eta^- klA^+ klB^- klC^-) and a spore strain (CU) with the phenotype Gla^- Eta^+ Kha^+ Kcc^+ Kcm^+ (genotype h^- gla^- eta^+ klA^+ klB^- klC^+ (ascertained from the lack of recombinants between *klA* and *klB* and the lack of nonkiller phenotypes in the progeny) was made (Table 5). A total of 24 asci with four viable spores were obtained. No recombinants with the ability to kill *Cryptococcus cereanus* and *Candida mucilaginа* were obtained (i.e., no Kcc^- Kcm^+ phenotypes), and the epistatic mode of gene activity inferred from results of the original cross (84-670.2C and 88-370.2F) (Table 2) was further supported. Table 6 gives the tetrad analysis for genes segregating in this cross and indicates that all appear to be independent.

A backcross between the spore DX (h^+ klA^+ klB^- klC^-) and the parent 84-670.2C (h^- klA^- klB^- klC^-) yielded 24 viable spores from 40 tetrads (160 spores) dissected. This low spore viability was accompanied by a highly skewed segregation at the mating-type locus (2 h^-:22 h^+) and no tetrads with four viable spores. The killer phenotypes segregated 9:15 (Kha^+:Kha^-); however, two of the Kha^- strains could not kill Y55. These data are difficult to interpret because of the low spore viability and the potential bias caused by linkage to inviability factors. Nevertheless, they do suggest that an epistatic model for killer phenotypes is appropriate.

The alternative hypothesis that the different killer phenotypes are produced by different alleles at the same locus is not acceptable, since this would predict two phenotypes from the original cross (84-670.2C × 88-370.2F) instead of the four observed. A two-locus model with linkage and epistasis can account for the variability for two of the killer characteristics but not the third. We are thus left with a three-locus epistatic model as the best working hypothesis.

It is also apparent from the phenotypic distribution across the continent (Table 2) that other genes are probably present and are responsible for specific phenotypes. Noteworthy are the phenotypes from Mexico that kill *Candida mucilaginа* but no other species of tester yeasts and the strains that kill the other yeasts but do not kill *H. anomala*. However, the strains from Mexico represent *Pichia kluyveri* var. *eremophila* and thus represent a different taxon (15).

TABLE 4. Observed and expected numbers of phenotypes for the cross of 84-670.2C (klA^- klB^- klC^-) and 88-370.2F (klA^+ klB^+ klC^+)

Genotype			Phenotype[a]			No. of spores	
klA	*klB*	*klC*	Kha	Kcc	Kcm	Observed	Expected
+	+	+	+	+	+	26	30.5
−	+	+	+	+	+		
+	−	+	+	+	+		
	−	+	+	+	+		
+	+	−	+	+	−	12	15.3
−	+	−	+	+	−		
+	−	−	+	−(w)[a]	−	8	7.6
−	−	−	−	−	−	15	7.6

[a] w, weak.

TABLE 5. Parental genotypes for genetic analysis

Parental type	Genotype						Phenotype[a]		
	h	*gla*	*eta*	*klA*	*klB*	*klC*	Kha	Kcc	Kcm
DX	+	+	−	+	−	−	+	w	−
X	−	−	+	+	+	−	+	+	−
CU	−	−	+	+	−	+	+	+	+

[a] w, weak.

DISCUSSION

The genetic analysis presented here was done to ascertain the genetic basis of different killer phenotypes within the species and not to determine whether the killer toxin is made by a nuclear gene. All of the crosses were done with strains that could make a toxin against at least one other yeast species (i.e., *Candida glabrata*).

Previous work on the inheritance of killer toxins in *P. kluyveri* var. *kluyveri* indicated that at least one gene is responsible for the ability to kill *Candida glabrata* (21). It is possible that this gene is responsible for toxin production, since strains unable to kill *Candida glabrata* are, in general, unable to kill other yeasts (21). We have attempted to find

TABLE 6. Results of genetic analysis

Cross and genotype	No. of tetrads with segregation pattern[a]			Map distance (cM)[b]
	PD	NPD	TT	
DX × X				
klB-gla	12	2	6	20
klB-eta	2	3	15	53
gla-eta	1	5	14	60
h-gla	6	5	9	48
h-eta	3	2	15	48
h-klB	8	3	9	38
DX × CU				
klA-gla	7	2	15	40
klA-eta	5	7	12	54
gla-eta	5	6	13	52
h-gla	7	2	15	40
h-eta	5	1	18	42
h-klA	3	4	17	52

[a] PD, parental ditype; NPD, nonparental ditype; TT, tetratype.
[b] cM, centimorgans.

both RNA and DNA plasmids by electrophoresis of strains that either do or do not show killer activity (11a, 21). We have attempted to cure killer-positive strains with cycloheximide, acridine orange, ethidium bromide, and elevated temperature. None of these attempts has revealed plasmids or viruslike particles in the strains tested.

The survey across the United States (Fig. 1) and the genetic analysis (Tables 3 and 4) indicate that the variability in killer phenotypes is controlled by several epistatic nuclear genes. The variability in killer activity within localities is approximately equivalent to the variability across the continent. The species is thus polymorphic and not polytypic for killer types. One possibility is that the genes responsible for differences in killing activity are coding for differences in recognition of the killer toxin receptor site of the cell wall of the sensitive species (19) and not for different toxins. Another possibility is that the increased spectrum of killing activity is due to increased production of the toxin and the genes documented here are involved in regulating the quantity and not the quality of the toxin. Our observation that increased killer activity against one species is often paralleled by increases against others supports this possibility.

It is possible that the variability in killer activity is an artifact of some other function of cellular metabolism or is neutral with respect to competitive interaction in the natural community. However, several lines of evidence give support to the idea that the production of killer toxins has a role in excluding other yeasts from particular habitats. (i) Starmer et al. (21) found that yeasts isolated from fruit habitats were more likely to have more killing activity against yeasts of other habitats than against yeasts in their own habitat. The activity (number killed per number tested) of *P. kluyveri* against yeasts in the fruit habitat was 12.5%, while activity against yeasts from other habitats was 64.5%. Results from this study are consistent with this observation; 9% of the yeasts originating from fruit had sensitivity to some of the 71 *P. kluyveri* strains tested, while 42% of the strains from habitats other than fruit were sensitive to the *P. kluyveri* strains tested (Table 1). (ii) The fruit habitat is characterized by relatively low pH values (3.0 to 4.0), which is approximately the range in which *P. kluyveri* toxins are most active. (iii) *P. kluyveri* and *Cryptococcus cereanus* are not independently distributed in decaying *Opuntia* cactus tissue, and when the two species did occur together, the pH of the tissue was higher than 7.5. Further evidence on the role of killer toxins in excluding yeasts from particular habitats will come from field tests.

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