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Genetics

Inheritance of killer phenotypes and double-stranded RNA in *Ustilago maydis*

(virus-like particles/immunity to killer/mycovirus exclusion/polyacrylamide gel electrophoresis)

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ABSTRACT **Three different killer specificities in *U. maydis* are inherited cytoplasmically and transmitted by cell fusion. Each killer generates low frequencies of specifically immune forms in crosses with sensitive strains. The properties of immunity to each killer are also inherited cytoplasmically and transmitted by cell fusion. Killer strains carry virus-like particles about 41 nm in diameter. Each killer possesses distinct double-stranded RNA components that range in molecular weight from 0.46×10^6 to 2.9×10^6. Two components are shared by all three killers. Immune strains possess new forms. Crosses and heterokaryons between different killers revealed unilateral or mutual restrictions that prevent inclusion of two killer specificities in the same cell.**

Interstrain inhibition in *Ustilago maydis* was first reported by Puhalla (1). The inhibition results from the excretion by specific strains, designated P1, of a Pronase-sensitive, thermolabile substance (2) that inhibits the growth of sensitive strains designated P2. The capacity of P1 to inhibit other strains is transmitted as an extranuclear character. Immunity to the inhibitor is also transmitted as a cytoplasmic character (called P3) that can be inherited separately from the inhibitory or killer function. Puhalla concluded that P1 strains carry both killer and immunity determinants. These determinants are transmitted to sensitive P2 strains at heterokaryon formation (3). Resistance to P1 killer is also determined by a chromosomal gene s^+ (1), which we propose be renamed $p1^r$, that is recessive to its allele $p1^s$ (Puhalla, unpublished).

Wood and Bozarth (4) detected virus-like particles (VLPs) in P1 and P3 strains. Sensitive P2 strains examined had no VLPs. The VLPs recovered from a P1 strain contained double-stranded RNA (dsRNA), as do all the known mycoviruses examined to date (5). In the yeast *Saccharomyces cerevisiae* a similar killer phenomenon and related dsRNA are known (6–8).

Puhalla (unpublished) also discovered two other strains of *U. maydis* with inhibitory capacity, called P4 and P6. Although some strains inhibit the growth of sensitive cells whereas others kill sensitive cells, we shall call all such inhibitory strains killers. Killers P4 and P6 can kill P1 strains and strains immune to P1 [we will call such strains P3(1), where P3 denotes cytoplasmically determined immunity and (1) the killer specificity concerned]. All three killers can kill each other as well as sensitive strains (Fig. 1). The nuclear gene $p4^r$ confers resistance to P4 and is independent of $p1^r$. We have observed a third gene $p6^r$ for resistance to P6 that is independent of $p1^r$ and $p4^r$. All three nuclear genes ($p1^r$, $p4^r$, and $p6^r$) that confer resistance to P1, P4, and P6 are recessive to their sensitive alleles. The distinction between cytoplasmic immunity and resistance due to nuclear genes may well prove important when more is known of the mode of action of the killers.

In this paper we examine the correlation between killer phenotypes and the presence of VLPs, the relationships of the killer specificities to their dsRNAs, and the relationships among the three killers in crosses between different killer strains.

MATERIALS AND METHODS

Strains. The strains used are listed in Table 1. P2 strains 5 and 1003 were used in crosses. Strains 18, 15, 1003, and 79 were used as sensitive indicators for identification of killer progeny and killer specificity. Strain 18 is sensitive to all three killer specificities, strain 15 is sensitive to P4 and P6, strain 1003 is sensitive to P1 and P4, and strain 79 is sensitive only to P6.

Crosses and Genetic Analysis. Media, crosses, germination of teliospores, analysis of random progeny and tetrads, and mating compatibility tests were all performed according to the procedures described in Stevens (9).

The effect of cytoplasmic mixing was tested by preparing heterokaryons (3) and by isolating unreduced diploids from germinated teliospores (10). Crosses between killer and sensitive strains in maize plants are successful. In preparing heterokaryons *in vitro* it was necessary either to mate isolates at

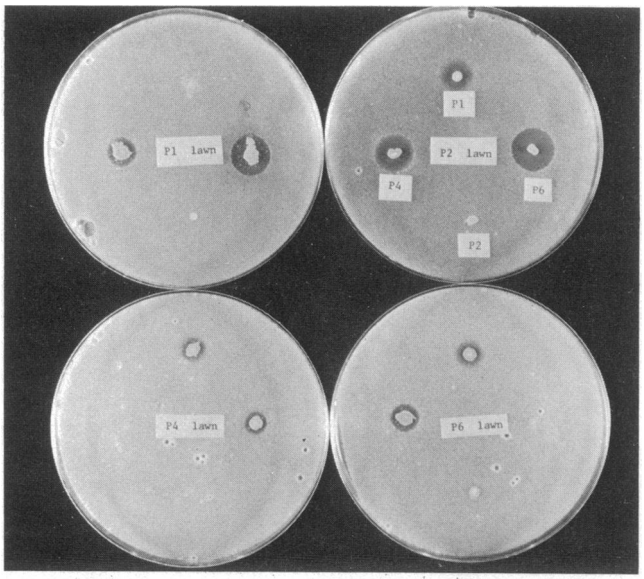

FIG. 1. Upper right: inhibitory effects of P1, P4, and P6 killers on a lawn of sensitive P2. Upper left and lower: sensitivity of each killer to the two others.

Abbreviations: VLPs, virus-like particles; dsRNA, double-stranded RNA; M_r, molecular weight.

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Table 1. Strains used in the study

Strain	Mating type	P state	Origin
5	a1 bC	P2	Conn.
13*	a1 bA	P2	R. Holliday, England
14†	a2 bA	P2	R. Holliday, England
15	a1 bB	P2	R. Holliday, England
18	a1 bG	P2	Conn.
79	a1 bI	P2	Ontario
1003	a2 bI	P2	Conn.
27	a2 bF	P1	Penna.
1268	a2 bD	P1	(3)
77	a1 bM	P4	Ontario
1369	a2 bI	P4	77 × 1003
1380‡	a2 bA	P4	13 × 1369
75	a2 bK	P6	Penna.
1371	a1 bC	P6	75 × 5
1372§	a1 bC or A	P6	14 × 1371
112	a1 bA	P3(1)	(1)
1373	a2 bK	P3(4)	77 × 75
1374	a2 bK	P3(6)	77 × 75

Gene symbols indicate requirements for methionine, adenine, pantothenic acid, reduced nitrogen (*nar-1*), and nicotinic acid.
* *met-15, ade-1, pan-1, nar-1.*
† *met-1, ade-1, nic-3.*
‡ *met-15,* not tested for *nar-1.*
§ *met-1, ade-1, nic-3.*

pH 4 (for P1) or to use a P2 strain carrying a gene for resistance to the killer (for P4 and P6). Progeny were characterized as killers or nonkillers by replication to a lawn of a sensitive strain previously prepared by overlaying complete medium with a cell suspension in 7–8 ml of agar cooled to about 40°. The plates were then incubated at 25° for 24–30 hr. Killers were surrounded by halos of no cell growth, whereas nonkillers had no effect.

Immunity or resistance was determined by streaking an unknown alongside a streak of a killer inoculated 48 hr previously, or by inoculating a suspension of the unknown as a patch on which the killer was spotted. Tests were scored after incubation at 25° for 24–30 hr.

Isolation of Virus Particles. Shake cultures grown at 25° were harvested after 48–64 hr. The cells were washed and suspended in 0.05 M phosphate buffer pH 7.0 and stored at 4°. The cells were disrupted in a Gaulin homogenizer or a Bronwill homogenizer.

The VLPs were precipitated in 1% urea (according to U.S. Patent 3,772,149) as the pH of the buffer was lowered to 5.0–5.3 with 0.01 M HCl at room temperature with continuous stirring for 30 min. The precipitate was resuspended in pH 7.0 buffer and centrifuged (about 1600 × *g*, 10 min). The supernatant was loaded on a 10–40% linear sucrose gradient, and the gradient was centrifuged for 5 hr at 63,000 × *g* in a Beckman model L ultracentrifuge at 4°. Absorbance of the fractions at 260 nm was determined. Samples from those fractions showing maximal absorbance were negatively stained with uranyl acetate and examined in a Zeiss (model Em9S) electron microscope.

Extraction and Electrophoresis of dsRNA. dsRNA was extracted from 2 to 4-g (wet weight) of cells grown in liquid culture for 48–65 hr at 25°. The extraction was performed according to the procedure of Vodkin *et al.* (8). The modifications of the procedure were: (*a*) the disruption of the cells in a Bronwill homogenizer for 4 min prior to the phenol extraction; (*b*) each precipitation in ethanol was repeated twice; and (*c*) as a control the samples were treated with 50 μg/ml of bovine pancreatic ribonuclease (30 min, 37°) at high ionic strength (0.3 M NaCl–0.03 M sodium citrate) and at low ionic strength (0.15×10^{-3} M NaCl–0.15×10^{-4} M sodium citrate). All the bands revealed by electrophoresis were resistant to RNase at high ionic strength and sensitive at low ionic strength.

Gel electrophoresis was performed in 5% polyacrylamide gel, 0.125% methylene-bisacrylamide, 0.03% ammonium persulfate, and 0.11% TEMED (*N,N,N′,N′*-tetramethylethylenediamine), 2 mA per gel for 8 and 16 hr in 0.04 M Tris-acetate buffer with 10^{-3} M EDTA. Gels were preelectrophoresed at 4 mA per gel for 1 hr to remove the persulfate. Electrophoresis for 8 hr enabled the detection of components with a molecular weight (M_r) as low as 0.2×10^6, whereas electrophoresis for 16 hr provided clear resolution of components within the range of 1.0×10^6 to 4.0×10^6. The gels were fixed for 1 hr in 1 M acetic acid and stained for 5 hr in 2% methylene blue in 0.4 M Na-acetate pH 5.0. Molecular weight determinations for dsRNA were made by coelectrophoresis with reovirus type 3 dsRNA (11, 12) (kindly supplied by A. J. Shatkin, Roche Institute of Molecular Biology, Nutley, N.J.).

RESULTS AND DISCUSSION

In all crosses between killer and sensitive strains there is a preponderance of killers in random sporidial progenies (Table 2). These results alone do not exclude nuclear genes as killer determinants. However, the results obtained from tetrad analysis strongly support a cytoplasmic factor as the killer determinant. The tetrads are generally uniform, all the products of each tetrad displaying one phenotype either killer or nonkiller (Table 2). The percentage of killers is similar in both random progeny (86–94%) and tetrads (76–97%).

Table 2. Segregation of the killer and immunity functions

Cross			Random sample*				Tetrad analysis				Diploids	
Killer		Sensitive	Total	K	I	S	Total	K	I	S	K	NK
P1	×	P2†	762	718(94.2)	7	37	—	—	—	—	—	—
P4	×	P2	583	502(86.1)	43	38	29	22(75.9)	3	4	18	0
P6	×	P2	759	717(94.4)	20	18	33	32(96.9)	1	0	9	0
P3(1)	×	P2	407	—	280†(68.5)	127	11	—	11(100.0)	0	—	—
P3(4)	×	P2	245	—	235 (96.0)	10	17	—	15 (88.2)	2	—	—
P3(6)	×	P2‡	—	—		—	12	—	3	9	—	—

* K = killer; I = immune P3; S = sensitive P2; NK = nonkiller either immune or sensitive. Number in parentheses refers to percent.
† From ref. 1, I and S figures estimated from percentages given.
‡ Low germination, but each tetrad was uniformly either immune or sensitive.

We conclude that all three killer specificities, P1, P4, and P6, are cytoplasmically inherited.

The recovery of diploids from unreduced tetrads produced in crosses between strains with various P states allowed us to examine the phenotypes of zygotes with mixed cytoplasms. All diploids recovered from crosses between sensitive and killer strains displayed killer phenotypes (Table 2). Evidently all three killer states (P1, P4, and P6) are suppressive to the sensitive state (P2).

Two distinct classes can be distinguished among the nonkiller progeny from random sporidia and tetrads from crosses of all three killers with sensitive strains: (*a*) nonkillers that are sensitive to the parental killer strain and (*b*) nonkillers that are immune to the parental killer strain. Crosses among sensitive strains yield only sensitive progeny (1); however, immunity to P1 is inherited as a cytoplasmic character in a similar way to the killer phenotype. Immunity to P4 and to P6 killer specificities are also inherited cytoplasmically. Progeny immune to P4 predominate in a cross between a strain immune to P4 and a sensitive strain. This result is evident from analysis of random sporidia and tetrads. Also, in spite of low germination in the cross of P3(6) × P2, the tetrads were of only one phenotype, being either all immune or all sensitive.

The cytoplasmic nature of P4 and P6 was confirmed by heterokaryon transfer of the killer phenotype between killer and sensitive strains. A mixed sporidial suspension of an auxotrophic P6 strain and a compatible prototrophic P2 strain (with nuclear resistance to P6) was streaked on double-strength complete medium (3). Hyphal tips of the heterokaryon were transferred to minimal medium and prototrophic sporidial colonies were picked, restreaked on minimal medium, and tested for killer activity. Of 215 colonies tested, 174 were killers. Each killer was incompatible with the P2 parent strain and compatible with the P6 killer strain. The same procedure was followed with P4. Of 316 colonies with the nuclear markers of the P2 parent, 243 were killers after the cytoplasmic mixing in the heterokaryon. Thus, the killer specificities of P4 and P6 can be transferred by cytoplasmic mixing without any exchange of nuclear genetic information. Immunity to all three killers is also cytoplasmically inherited.

Virus-like particles about 41 nm in diameter were isolated from P4 and P6 strains after precipitation with urea and centrifugation on a sucrose density gradient. The particles were similar in appearance to those described in P1 and P3(1) by Wood and Bozarth (4). Absorbance peaks at 260 nm were obtained at a density of 1.095 and 1.124 g/cm^3 from both P4 and P6 killers. VLPs were seen in the light fraction from P6 and in both fractions from P4. The same isolation procedure carried out with the sensitive strains 5 and 1003 used in the crosses resulted in no comparable peaks in the sucrose gradient. To exclude the possibility that VLPs in the sensitive strains are present in very low undetectable titers, fractions of the same density as those in which VLPs from the killer strains were found were negatively stained and examined in the electron microscope. Again, no evidence for VLPs was found.

The strain immune to P1 [P3(1)] carries VLPs similar in size to those in the killer strains (4). The pattern of segregation of the individual immunities to each of the three killers suggests that all immune strains carry VLPs, perhaps with only part of the information contained in the particles found in killer strains that display both the killing property and immunity.

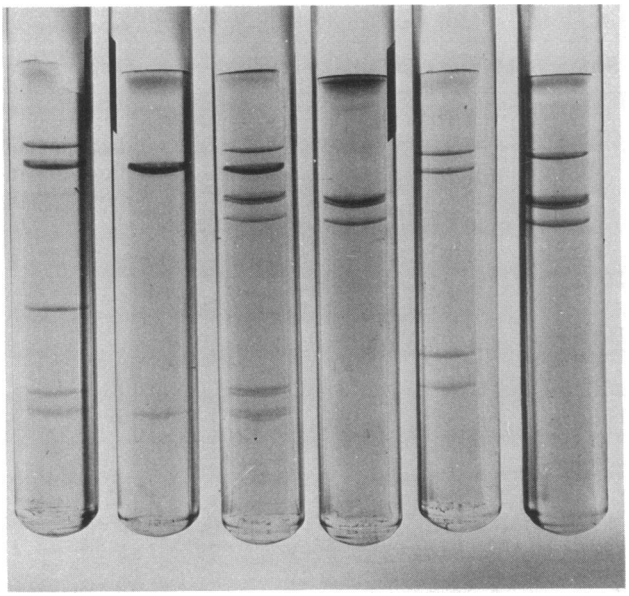

FIG. 2. dsRNA components of killer and immune strains separated on 5% acrylamide gel (8 hr). Left to right: P1, P3(1), P4, P3(4), P6, and P3(6). Gels stained with methylene blue.

The correlation between the killer characteristic and the presence of VLPs suggests a further characterization of the differences between the strains through comparisons of the dsRNA in each of the killer, immune, and sensitive strains. Once again a perfect correlation was found between the presence of dsRNA in killer and immune strains and the absence of dsRNA in the sensitive strains 5, 79, and 1003 used in the crosses (Fig. 2). Coelectrophoresis of dsRNA extracted from the various killers and the immune strains and molecular weight determination using reovirus (type 3) dsRNA as a standard indicate the presence of a multicomponent dsRNA of at least nine species (Table 3). Some of the same species are found in killer strains with different specificities, but the three killer strains differ in the size distribution of dsRNA.

Two species (2.9 × 10^6 and 2.6 × 10^6 M_r) are common to all three killers (Table 3). P1 has in addition a medium molecular weight dsRNA, about 0.9 × 10^6, and two light components, 0.54 × 10^6 and 0.45 × 10^6 M_r. These molecular

Table 3. Species of dsRNA in killer and immune strains*

M_r † \ P state	P1	P4	P6	P3(1)	P3(4)	P3(6)
Killer	P1	P4	P6	—	—	—
Immunity	P1	P4	P6	P1	P4	P6
4.7	—	—	—	—	+	—
2.9	+	+	+	—	—	+
2.6	+	+	+	+	—	—
2.1	—	+	—	—	+	+
1.8	—	+	—	—	+	+
0.94	+	—	—	—	—	—
0.70	—	—	+	—	—	—
0.56	—	—	+	—	—	—
0.54	+	+	—	—	—	—
0.46	+	+	—	+	—	—

+ Indicates specific dsRNA component present.
* Sensitive strains 5, 79, and 1003 lack dsRNA.
† Molecular weight (× 10^{-6}) determined with reovirus type 3 as marker (11).

Table 4. Segregation of the killer function in crosses between killer strains

Killer parents			Random sample					Tetrad analysis					Diploids	
			Total	P1	P4	P6	NK	Total	P1	P4	P6	NK	K	NK
P1	×	P4(I)‡	—	—	—	—	—	13	2	10(92.3)†	—	1	—	—
		(II)	73	0	73(100.0)*	—	0	39	0	38(97.4)†	—	1	2	0
P1	×	P6(I)	272	0	—	0	272(100.0)	10	0	—	0	10(100.0)	0	1
		(II)	—	—	—	—	—	10	0	—	0	10(100.0)	—	—
P4	×	P6(I)	1070	—	991(92.6)	0	80	46	—	43(93.4)	0	3	—	—
		(II)	450	—	449(99.7)	0	1	18	—	15(83.3)	0	3	—	—

* Percent in parentheses.
† Either pure P4 or P4/P1, but clearly not pure P1.
‡ Roman numerals designate reciprocal crosses with regard to *a* incompatibility factor.

weights are in general agreement with Wood and Bozarth (4) after extraction of dsRNA from VLPs isolated from a strain with P1 specificity. One additional very light component (0.06×10^6 M_r) detected by Wood and Bozarth was not compared in the present study.

Since the correlation between the killer phenotype and dsRNA was established, it was anticipated that killer progeny from a cross between any killer and a sensitive strain would possess the dsRNA components of its progenitor. Therefore, dsRNA was extracted from 1268, a P1 strain derived earlier by heterokaryon transfer, and from one randomly selected meiotic product with killer activity from each of the crosses P2 × P4 and P2 × P6. The meiotic products with killer activity were compatible with their killer parents. Each killer gave a pattern identical to its progenitor.

The immune strains found among the progeny of crosses between killer and sensitive strains (Table 2) carry dsRNAs, and these appear to share some components found in the killer strains from which they were derived (Table 3). The derivation of P3(1) from P1 is associated with the loss of the 2.9×10^6, 0.94×10^6, and 0.54×10^6 M_r components, with the retention of the 2.6×10^6 and 0.4×10^6 M_r components. The derivation of P3(4) from P4 involves the loss of the two heaviest and the two lightest components, with the retention of the 2.1×10^6 and 1.8×10^6 M_r species typical of P4. In addition P3(4) has a new component that is similar in molecular weight to the sum of the two heavy components that were lost. The derivation of P3(6) from P6 is associated with the loss of the 2.6×10^6, 0.7×10^6, and 0.56×10^6 components and the appearance of two new components, about 2.1×10^6 and 1.8×10^6 M_r.

In general, loss of killing and retention of immunity is associated with a difference in one or both of the common dsRNA components and one or both of the low-molecular-weight components. It is, however, difficult to assign a specific function to the various components that could account jointly for the results obtained with all three killers. However, the similarity in molecular weight may not reflect functional equality. Separation of the fractions and their use in synthesis *in vitro*, followed by a comparison of the amino-acid sequence of the product with that of the killer substance, which is thought to be a protein, could provide a means of assigning roles to the various dsRNA components. Hybridization experiments may also reveal whether some components are merely degradation products and which others carry unique information.

The comparison above shows that dsRNA components are not common to all strains of *U. maydis* examined. Since three sensitive strains had no detectable dsRNA, we conclude that each component probably has some function related to killer, immunity, or the structure of the VLPs.

The presence of three distinct killer specificities all associated with the presence of viral particles raises the question whether a fungal cell can carry more than one type of virus and whether specificities can be combined. Our approach to these problems was limited to examining the outcome of interkiller strain crosses and their progeny phenotypes.

The effect of the *a* incompatibility factor (1) on the direction of nuclear exchange and the degree of cytoplasmic mixing is unknown; therefore, crosses were performed reciprocally with respect to the incompatibility genes. No basic differences were detected in each pair (I and II) of the six reciprocal crosses (Table 4). The uniformity within tetrads detailed in Table 4 again indicates that the killer phenomenon is associated with a cytoplasmically transmitted element. The progeny within each tetrad is either entirely killer or entirely nonkiller. These crosses between killer strains with different specificities provide a demonstration of mutual or partial exclusion in a fungal virus, a phenomenon well known in bacterial viruses (13–15), plant viruses (16), and among different colicinogenic and sex factors of *Escherichia coli* (17).

The cross between P1 and P6 shows absolute mutual exclusion of the killing property with retention of immunity to P6 only. A single case of combined immunity was detected in which one progeny was immune to both P1 and P6. The result displayed by all the meiotic products can already be detected in the heterokaryon, a stage that precedes zygote formation. Vegetative cells isolated from a heterokaryon of P1 and P6 lacked the killing property of either parent but were immune to P6. A diploid recovered from the cross between P1 and P6 also lacked the killing property and was immune to P6. The exclusion mechanism, which is an intracellular phenomenon, evidently recognizes unique species of dsRNA since immunity to P6 can be found among the progeny. The exclusion mechanism also appears to be an inherent or adjunct property of the same determinant that is involved in the killing phenomenon. This suggestion is based on the observation that vegetative cells from a heterokaryon formed between a strain with immunity P3(6) and a P1 killer strain do not show any alteration of the P1 killing function and yet the same cells are immune to P6, thus combining P1 with P3(6) without exclusion of any component. Similarly, in a heterokaryon between a strain with the immunity P3(4) and a P1 killer strain, the P1 killing property is unaffected unlike the interaction in crosses between P1 killer strains and P4 killer strains.

The cross P4 × P6 yielded only P4 progeny among the killers. Killer progeny with dual specificity were not detect-

ed, but some indications suggest that cells can carry dual immunity. The exclusion of one killer specificity was detected in spontaneous diploids recovered from a heterokaryon. The diploid vegetative cells were all phenotypically P4 killers. Thus, all the interactions detected in crosses can also be found in vegetative cells if the proper particles are introduced into a common cytoplasm.

The results of the crosses between P1 and P4 suggest an almost total exclusion of P1. In only one of the two crosses (Table 4) two out of 12 killer tetrads were purely P1, as shown by the inability of their killer progeny to kill cells of a sensitive strain carrying $p1^r$. In the second cross all 39 tetrads examined killed $p1^r$ sensitive cells. Thus, of 51 killer tetrads recovered in the crosses, 49 were P4. However, thus far we have failed to recover a tester that will clearly distinguish between progeny with only the P4 specificity and progeny with the combined specificity of P1 and P4. The difficulty is primarily due to a degree of sensitivity of P4 cells to their own inhibitory substance and the lower activity of the P1 inhibitory product compared with that of P4. Thus, the degree of inhibition of P4 cells by P1 is only slightly more pronounced than the inhibition caused by spotting a high density of P4 cells on a P4 lawn. The results under these conditions are ambiguous, and a rigorous test will have to await the development of other testers. Nevertheless, it is clear that in the interaction between P1 and P4 there is neither mutual exclusion nor absolute unilateral exclusion. Nevertheless, P1 is rarely expressed independently.

The crosses between killer strains show that P4 excludes P6 and, to a high degree, P1. P1 and P6 mutually exclude each other, but immunity to P6 is unaffected. Cell-free systems may resolve the mechanism for these interactions and should show, for example, whether modification restriction systems are associated with these viruses.

In the absence of markers in the viral genomes other than the killer and immunity specificities, a test for exchange of genetic information can only be based on tests for either rare recombined killing functions or the ability to form halos on lawns resistant to all three killer specificities. Samples of the progenies from crosses of P1 × P4 and of P4 × P6 (Table 4) were plated on differential lawns that could detect individuals carrying two specificities or a new specificity. As an example, by the use of two tester lawns, one sensitive only to P4, the other only to P6, strains with combined specificities, or a new specificity, could be detected. New killer specificities can also be identified on a lawn of a sensitive strain in which nuclear resistance to all three killer specificities is combined. No new specificities and no dual specificities were detected from these crosses. Some 1500 progeny from the cross between P4 and P6 that yielded only P4 were replicated to a lawn of P4, but none showed any effect, thus excluding the presence of any new specificity in the progeny from this cross.

Ten thousand tetrads from the cross of P4 and P6 and 5000 tetrads from the cross of P1 with P6 were replicated to a lawn resistant to the three known killer specificities. In neither test were any halos detected. Thus we have no information to suggest any genetic-exchange to generate new killing specificities. However, strains with dual immunity derived from these crosses can be studied to see if the dual immunity is a result of (*i*) the presence of two distinct viral particles in a single cell, (*ii*) recombined molecules, or (*iii*) molecules jointly packaged in a single particle.

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