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Production of Fumonisin B_1 and Moniliformin by *Gibberella fujikuroi* from Rice from Various Geographic Areas

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Gibberella fujikuroi **strains isolated from rice in the United States, Asia, and other geographic areas were tested for sexual fertility with members of mating population D and for production of fumonisin B_1 and moniliformin in culture. Of the 59 field strains tested, 32 (54%) were able to cross with tester strains of mating population D, but only a few ascospores were produced in most of these crosses. Thirty-four strains produced more than 10 μg of fumonisin B_1 per g, but only three strains produced more than 1000 μg/g. Twenty-five strains produced more than 100 μg of moniliformin per g, and 15 produced more than 1,000 μg/g. Seven field strains produced both fumonisin B_1 and moniliformin, but none of these strains produced a high level of fumonisin B_1 (>1,000 μg/g). However, a genetic cross between a strain that produced fumonisin B_1 but no moniliformin and a strain that produced moniliformin but no fumonisin B_1 yielded progeny that produced high levels of both toxins. Strains of *G. fujikuroi* isolated from rice infected with bakanae disease are similar to strains of mating population D isolated from maize in their ability to produce both fumonisins and moniliformin. This finding suggests a potential for contamination of rice with both fumonisins and moniliformin.**

Bakanae disease of rice occurs widely throughout Asia and sporadically in other areas of rice production (25, 29). The most dramatic symptom of this disease is yellowing and abnormal elongation of infected rice seedlings, which led to the name bakanae, a Japanese word meaning "foolish seedling." In older plants, the roots, crowns, stems, leaf sheaths, and panicles can be infected. The disease is seed borne and primarily seed transmitted (25, 29). The sexual stage of the fungus causing bakanae disease was reported in 1919 and named *Lisea fujikuroi*, which was amended in 1931 to *Gibberella fujikuroi* by Ito and Kimura, who also identified the asexual stage as *Fusarium moniliforme* (25).

Although some early authorities considered *G. fujikuroi* to be a single species (24), Nirenberg (17) and Nelson et al. (16) used morphological characters to distinguish a number of species within this group. More recently, Leslie et al. (7–9) and others (3, 6) have used formation of the sexual stage to distinguish mating populations, or biological species, within this group. These different mating populations are associated with preferred host plants (3, 6, 7) and differences in a number of characters, including electrophoretic karyotypes (31), isozyme profiles (4), and DNA sequences (18, 27, 28). Furthermore, the mating populations studied to date differ significantly in their ability to produce a wide range of mycotoxins, such as fumonisins and moniliformin, that are hazardous to human and animal health (7, 8, 10, 11, 15, 26).

Despite the agricultural importance of bakanae disease, little information about the taxonomy and mycotoxicology of the bakanae pathogen is available. Our objectives in this study were to investigate the sexual fertility of strains of *G. fujikuroi* isolated from rice in different geographic areas and their ability to produce the mycotoxins fumonisin B_1 and moniliformin.

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MATERIALS AND METHODS

Strains. The *G. fujikuroi* field strains and tester strains for mating population C used in this study were obtained from the culture collection of the Fusarium Research Center, The Pennsylvania State University. All the strains originated from single microconidial cultures. Morphological descriptions were obtained from strains grown on carnation leaf agar and potato dextrose agar as described previously (16). Crosses for genetic analysis were made on carrot agar as described by Klittich and Leslie (5), but male parents were cultured on V-8 juice agar medium rather than complete medium. Ascospores were obtained at random from crushed perithecia and were dissected freehand at a ×150 magnification under a dissecting microscope as described previously (2). The female fertile tester strains for mating populations A and D were kindly supplied by J. F. Leslie, Plant Pathology Department, Kansas State University.

DNA manipulations. Fungal DNA was isolated in minipreps as described previously (21) and modified (2). Random amplified polymorphic DNA (RAPD) screening of strains was performed as described previously (30). Random 10-mers were purchased from Operon Technologies Inc. (Alameda, Calif.). Nine primers were tested.

Analysis of mycotoxin production. For analysis of field strains, cultures were grown as described previously (14). In brief, 250 g of yellow maize kernels and 250 ml of distilled water were autoclaved in a polyethylene bag and then inoculated with an aqueous suspension from a lyophilized fungal culture. Bags of inoculated maize were incubated in the dark at 20 to 22°C for 4 weeks. For analysis of mating population D tester strains and ascospore progeny, cultures were grown at 25°C in the dark for 4 weeks on autoclaved cracked maize in Erlenmyer flasks as described previously (2, 14). The production of fumonisin B_1 by field strains and ascospore progeny was assessed by high-performance fluorescence liquid chromatography as described previously (14). The detection limits for fumonisins were 5 μg/g of culture material. Fumonisin B_1 was the only fumonisin homolog quantitated in this study. Fumonisin B_2 and B_3 were observed in the extracts at the expected ratios but were not quantitated.

The production of moniliformin by field strains and ascospore progeny was also assessed in cultures grown on autoclaved maize. Moniliformin was efficiently extracted from the cultures by the same acetonitrile-water extraction method used to measure fumonisins. Moniliformin was detected and quantitated in extracts by thin-layer chromatography on silica plates with one of two solvent systems (toluene-acetone 75:25, with a ratio of fronts for fumonisin B_1 of 0.4; or acetonitrile-water 85:15, with a ratio of fronts for fumonisin B_1 of 0.85). The moniliformin was detected by viewing the plate under a shortwave UV lamp, under which moniliformin strongly absorbed the light, yielding a black spot. Quantitation was done by visual comparison to a lane in which 1 μg of purified moniliformin had been applied. The detection limit by this method was approximately 100 μg/g of culture material. Because most moniliformin-producing

strains make high levels of moniliformin (>1,000 μg/g) under these culture conditions, the relatively low sensitivity and precision of the thin-layer chromatography procedure were deemed adequate for this general survey. Some of the moniliformin-positive extracts were confirmed by high-performance liquid chromatography, using a method (1) that has a detection limit of <10 μg/g.

RESULTS

Mating tests. Strains of *G. fujikuroi* were isolated from rice grown in Georgia and California in the United States, several Asian countries (Japan, Malaysia, the Philippines, and Taiwan), Australia, Iran, and Italy (Table 1). The strains from rice were generally characterized by the presence of microconidia in chains on monophialides and on polyphialides, although in some strains the microconidial chains were short and in some strains the polyphialides were rare. These morphological characters associate the rice strains with two mating populations of *G. fujikuroi*: mating population C, which corresponds to the asexual stages *F. fujikuroi* and *F. proliferatum* (7, 17), and mating population D, which corresponds to *F. proliferatum* (7, 16). The presence of polyphialides excludes rice strains from *F. moniliforme* (*G. fujikuroi* mating populations A and F) (7, 8, 16).

To test these associations, a series of crosses was undertaken with mating population C tester strains, which were originally isolated from rice grown in Taiwan (3), and mating population D tester strains, which were developed from U.S. field strains (7a) (Table 2). Despite repeated attempts, all crosses of tester strains of mating population C with each other and with other strains from rice were barren. However, mature perithecia and ascospores were formed in crosses of 32 of the rice strains to tester strains of mating population D: 25 strains crossed as mating type D^- and 7 strains crossed as mating type D^+ (Table 1). In fact, mating population C tester strains M-1148 and M-1150 produced a few ascospores in crosses with mating population D tester strains. Mating population C^- tester strain M-1151 produced abundant ascospores in a cross with a D^- tester strain, but most (>80%) of them were small and were not viable upon subculture. Crosses of mating population C tester strains with *G. fujikuroi* mating population A (asexual stage, *F. moniliforme*) tester strains were completely barren; no mature perithecia or ascospores were produced.

The fertility of crosses between mating population D tester strains and strains from rice was further investigated by determining the viability of ascospores from four crosses and the sexual fertility of ascospores from one of these crosses. As shown in Table 3, ascospore viability ranged from 35 to 68% in crosses between female fertile tester strains from the United States and strains from rice from California, the Philippines, and Malaysia. A total of 20 progeny of cross 394 were tested for sexual fertility with mating population D testers; 5 progeny were not fertile, and 15 progeny shared the D^+ mating type of strain M-6992, the female parent of cross 394. Ascospore viability ranged from 40 to 69% in backcrosses between progeny of cross 394 and the mating type D^- tester strain M-6993; thus, backcrossing one generation did not increase the fertility of the crosses. In a final attempt to obtain more fertile crosses, selected rice strains were mated with each other in various combinations based on mating type and geographic origin. All of these crosses were barren. Crosses of selected rice strains with tester strains of mating population A were also completely barren.

Mycotoxin production. Both fumonisin and moniliformin are produced by strains of *G. fujikuroi* mating population D (*F. proliferatum*) isolated from maize (9–12, 15, 23, 26). We therefore investigated the production of both these mycotoxins by *G. fujikuroi* strains from rice. All but six of the rice field strains

TABLE 1. Fumonisin B_1 and moniliformin production by strains of *G. fujikuroi* from rice

Strain source	Mating type	Strain	Concn (μg/g) of:	
			Fumonisin B_1	Moniliformin
United States	D^+	M-1329	970	ND[a]
	D^-	M-3105	10	ND
	D^-	M-3106	20	ND
	D^-	M-3108	10	ND
	D^-	M-3113	20	ND
	D^-	M-3101	10	1,000
	Not fertile	M-3096	80	ND
	Not fertile	M-3102	10	ND
	Not fertile	M-3104	20	ND
	Not fertile	M-3107	10	ND
	Not fertile	M-3110	20	ND
	Not fertile	M-3111	10	ND
	Not fertile	M-3099	10	1,000
	Not fertile	M-3103	30	5,000
Malaysia	D^-	M-6173	20	ND
	D^-	M-6178	10	ND
	D^-	M-6179	20	ND
	D^-	M-6180	10	ND
	D^-	M-6181	160	ND
	D^-	M-6182	170	ND
	D^-	M-6186	50	ND
	D^-	M-6184	ND	1,000
	D^-	M-6174	100	1,500
	D^-	M-6183	ND	ND
	Not fertile	M-6175	30	ND
Japan	D^+	M-1268	ND	250
	D^+	M-1269	ND	ND
	Not fertile	M-1250	ND	500
	Not fertile	M-1267	250	ND
	Not fertile	M-1271	ND	ND
Philippines	D^-	M-5083	4,860	ND
	D^-	M-1231	ND	1,000
	D^-	M-1234	ND	200
	D^-	M-1236	ND	250
	D^-	M-5086	160	500
	D^-	M-1235	ND	ND
	Not fertile	M-5085	550	ND
	Not fertile	M-1228	ND	2,500
	Not fertile	M-1229	ND	750
	Not fertile	M-1230	ND	750
	Not fertile	M-1232	ND	1,250
	Not fertile	M-1233	ND	750
	Not fertile	M-5087	580	750
Taiwan	D^+	M-1215	ND	750
	D^-	M-1287	ND	5,000
	Not fertile	M-1214	ND	2,500
	Not fertile	M-1283	ND	2,500
	Not fertile	M-4056	690	1,250
Australia	D^+	M-3746	2,220	ND
	D^-	M-3744	1,360	ND
Iran	D^-	M-5167	10	ND
	Not fertile	M-5168	30	ND
Italy	Not fertile	M-3609	ND	ND
Unknown	Not fertile	M-2589	170	ND
	Not fertile	M-2589	ND	ND

[a] ND, not detected. The limits of detection for fumonisin B_1 and moniliformin were 5 and 100 μg/g, respectively.

TABLE 2. Characteristics of tester strains of *G. fujikuroi* mating populations C and D

FRC[a] strain no.	Source	Mating type	Concn (μg/g)[b] of:		Other strain no.[c]
			Fumonisin B_1	Moniliformin	
M-1148	Taiwan, rice	C^+	0	5,000	JFL-C01993, EGK-288, ATCC 38938
M-1149	Taiwan, rice	C^+	0	6,000	JFL-01994, EGK-290, ATCC 38939
M-1150	Taiwan, rice	C^-	0	0	JFL-C01995, EGK-293, ATCC 38940
M-1151	Taiwan, rice	C^-	0	7,000	JFL-C01996, EGK-294, ATCC 38941
M-6992	United States	D^+	1,510	0	JFL-D04853
M-6993	United States	D^-	2,200	0	JFL-D04854

[a] FRC, Fusarium Research Center.
[b] Results of a single test of all strains on autoclaved maize in bags (mating population C strains) or in flasks (mating population D strains). Zero indicates that <5 μg of fumonisin B_1 per g was detected by high-performance liquid chromatography and that <100 μg of moniliformin per g was detected by thin-layer chromatography (mating population C) or by high-performance liquid chromatography (mating population D).
[c] JFL, J. F. Leslie, Kansas State University, Manhattan; EGK, E. J. Kuhlman, USDA Forest Service, Research Triangle Park, N.C.; ATCC, American Type Culture Collection, Rockville, Md.

and one of the mating population C tester strains produced detectable levels of at least one of the two mycotoxins when cultured on autoclaved maize (Table 1). There was no obvious correlation between sexual fertility or mating type and mycotoxin production.

All 14 strains isolated from rice in the United States produced fumonisin B_1 (>5 μg/g). However, only strain, M-1329, from California produced a moderately high level of fumonisin B_1 (970 μg/g). The 13 strains from Georgia produced only low levels of fumonisin B_1 (10 to 80 μg/g). Three strains from Georgia (M-3099, M-3101, and M-3103) produced high levels of moniliformin (1,000 to 5,000 μg/g). The remaining strains from the United States produced no moniliformin (<100 μg/g). Eleven strains from Malaysia showed a similar trend of frequent, but low fumonisin B_1 production (0 to 170 μg/g) and less frequent moniliformin production (Table 1). Only Malaysian strains M-6174 and M-6184 produced high levels of moniliformin (1,000 to 1,500 μg/g). The mating population D tester strains from the United States produced high levels of fumonisin B_1 and little or no moniliformin (<100 μg/g) (Table 2).

Of the 27 strains isolated from rice in Japan, the Philippines, and Taiwan, 22 produced no fumonisin B_1 (<5 μg/g). Five strains produced relatively high levels of fumonisin B_1, ranging from 160 to 4,860 μg/g (the maximum amount was produced by strain M-5083 from the Philippines) (Tables 1 and 2). The majority (74%) of the strains from Japan, the Philippines, and Taiwan produced moniliformin, with a range of 200 to 7,000 μg/g and a mean of 2,000 μg/g. Moniliformin production by 15 of these strains was reported previously (11). Collections from other geographic areas were very limited but included two Australian strains that produced high levels of fumonisin B_1 (1,360 and 2,220 μg/g) and no moniliformin.

Segregation of mycotoxin production. Thirty random ascospore progeny were isolated from a cross between the mating population D tester strain M-6992 and strain M-1231 isolated from rice in the Philippines. In the initial survey, strain M-6992 produced a high level of fumonisin B_1 and little or no moniliformin (Table 2) whereas strain M-1231 produced no fumonisin B_1 and a high level of moniliformin (Table 1). Both parents and 20 progeny of cross 394 were regrown on autoclaved maize and analyzed for the production of fumonisin B_1 and moniliformin (Table 4). In cross 394, fumonisin production segregated as a single gene ($\chi^2 = 0.2$) and moniliformin production segregated as a single gene ($\chi^2 = 0.8$) (Table 4). Recombinant progeny that produced high levels of both fumonisin B_1 and moniliformin were recovered. Recombinant progeny that produced neither mycotoxin were also recovered. Fumonisin production and moniliformin production appeared to segregate independently, but progeny numbers were not sufficient for a χ^2 test of this hypothesis to be valid.

As noted above, mating type did not segregate as expected in cross 394: the 15 fertile progeny all belonged to the D^+ mating type of the female parent M-6992. To confirm that the progeny of cross 394 were derived from both parental strains M-6992 and M-1231, a strain M-1231-specific DNA marker was identified by RAPD. One of the primers tested, OPA-9, resulted in the presence of a strongly amplified fragment of approximately 1.8 kb in strain M-1231 that was absent in strain M-6992. The fragment unique to strain M-1231 was present in 6 of the 10 progeny tested, and 5 of these progeny were the mating type D^+ of strain M-6992 (Table 4). These data confirm the presence of sexual recombination in cross 394.

DISCUSSION

Analysis of 59 strains of *G. fujikuroi* from rice indicated that 58% of the strains produced significant levels of fumonisin B_1 (>5 μg/g) and 42% of the strains produced significant levels of moniliformin (>100 μg/g) when grown on autoclaved maize substrate. Seven strains (12%) produced both fumonisin B_1 and moniliformin, but none of these strains produced high levels of fumonisin B_1 (>1,000 μg/g). In their production of both fumonisin B_1 and moniliformin, bakanae strains from rice

TABLE 3. Fertility of crosses between *G. fujikuroi* tester strains of mating population D and bakanae strains from rice

Cross no.	Female parent	Source	Male parent	Source	No. of ascospores	
					Viable	Nonviable
393	M-6992	United States	M-6179	Malaysia	15	21
394	M-6992	United States	M-1231	Philippines	30	14
395	M-6992	United States	M-6184	Malaysia	30	56
396	M-6993	United States	M-1329	California	61	96

TABLE 4. Segregation of mycotoxin production and other traits in cross 394 of *G. fujikuroi*

Random ascospore progeny no.	Mating type	Concn (μg/g)[a] of:		1.8-kb RAPD marker[b]
		Fumonisin B_1	Moniliformin	
29	Not fertile	8,630	340	
15	D^+	6,850	3,750	−
12	D^+	5,200	2,330	+
25	Not fertile	1,240	410	
10	D^+	610	290	−
11	D^+	4,760	0	+
22	Not fertile	4,200	0	
24	D^+	460	0	
26	D^+	420	0	
30	D^+	0	1,500	
13	Not fertile	0	1,500	+
7	D^+	0	1,110	+
8	D^+	0	1,010	−
6	D^+	0	780	
21	Not fertile	0	220	
3	D^+	0	150	+
4	D^+	0	0	+
16	D^+	0	0	−
17	D^+	0	0	
19	D^+	0	0	
Parent M-6992	D^+	1,510	0	−
Parent M-1231	D^-	0	1,000	+

[a] Results of a single test of all strains on autoclaved maize in culture flasks. Zero indicates that ≤5 μg of fumonisin B_1 per g was detected by high-performance liquid chromatography, and that <100 μg of moniliformin per g was detected by thin-layer chromatography.

[b] Results of agarose gel electrophoresis of PCR products from reactions with RAPD primer OPA9 and DNA templates from parents and progeny. +, a 1.8-kb band is present; −, the band is absent.

are more similar to *G. fujikuroi* mating population D and the asexual stage of *F. proliferatum* than they are to any of the other *G. fujikuroi* mating populations and associated asexual stages. Table 5 summarizes previous surveys of fumonisin and moniliformin production by bakanae strains and by *G. fujikuroi* mating population D and *F. proliferatum*. These other survey data can be directly compared to data from the present study because all of the data were obtained from fungal cultures grown on maize, and many of these surveys were conducted by standard methods in our laboratories. The tabulated data show that in 10 of the 11 fumonisin surveys, more than half of the strains produced significant levels of fumonisins (>5 μg/g) (fumonisin B_2 and fumonisin B_3 were included in some of these studies). Also, 19 to 100% of the strains in each moniliformin survey produced significant levels of moniliformin (>100 μg/g). Strains of *F. proliferatum* that produced both fumonisin B_1 and moniliformin were identified in each of the surveys where both mycotoxins were analyzed. In particular, four strains of *F. proliferatum* that produced high levels of both fumonisin B_1 (1,500 to 2,000 μg/g) and moniliformin (3,300 to 5,300 μg/g) were isolated from maize from Sardinia (10). In an additional survey of mycotoxin production by *F. proliferatum* under liquid culture conditions, more than 90% of 30 strains from Canadian maize produced both fumonisin B_1 and moniliformin (12). All other mating populations of *G. fujikuroi* studied to date show a different pattern of mycotoxin production (7, 8, 10–12, 15, 23, 26). Most strains of mating population A (*F. moniliforme*) produce high levels of fumonisins, but few produce moniliformin. On the other hand, most strains of mating population B (*F. subglutinans*) and mating population F (*F. moniliforme*) produce high levels of moniliformin, but few produce fumonisins.

Although only fumonisin B_1 and moniliformin were quantitated in this study, it is likely that many bakanae strains produce additional mycotoxins and other secondary metabolites. Strains of *F. proliferatum* isolated from maize in Canada have been reported to produce the mutagenic fusarins (12), and strains of *F. proliferatum* isolated from maize, wheat, asparagus, and rice in Italy have been reported to produce the cyclic peptide toxin beauvericin (10, 13).

Comparison of morphological characters and mycotoxin production indicates that bakanae strains of *G. fujikuroi* share many similarities with strains of *G. fujikuroi* mating population D and *F. proliferatum* isolated from maize. Leslie (7) has recently summarized other biochemical and genetic characters that have been used to analyze relationships in the *G. fujikuroi* species complex. Although isozyme differences can distinguish among mating populations A, B, C, D, E, and F, strains from mating populations C and D have been difficult to resolve by this method. Mating populations C and D also had identical restriction maps of PCR-amplified ribosomal DNA internal transcribed spacer sequences. Comparison of one bakanae strain from Taiwan and one *F. proliferatum* strain isolated from an orchid in Germany indicated that the internal transcribed spacer (ITS2) sequences were identical but diverged from those of all other related taxa (28). On the other hand, elec-

TABLE 5. Summary of fumonisin and moniliformin production by *G. fujikuroi* mating population D and related groups

Sample	Origin	Total no. of strains tested	Fumonisin		Moniliformin		Reference
			% Postive	Mean concn of positives (μg/g)	% Positive	Mean concn of positives (μg/g)	
F. proliferatum	Various	8			100	130	12
Bakanae strains	Rice, Japan, Taiwan, and Philippines	15			100	9,800	11
F. proliferatum	Feeds, United States	3	100	2,270			23
F. proliferatum	Grains, Africa and United States	4	100	680			26
F. proliferatum	Various	31	61	1,270			15
G. fujikuroi D	Maize, United States	12	92	680			9
G. fujikuroi D	Sorghum, United States	10	90	580			9
Bakanae strains	Rice, unknown	58	14	403			32
F. proliferatum	Maize, Nepal	8	88	520			20b
F. proliferatum	Maize, China	7	100	3,330			20c
F. proliferatum	Maize, Italy	26	54	1,500	42	770	10
F. proliferatum	Feeds, United States	16	88	3,300	19	460	20a
Bakanae strains	Rice	59	58	400	42	3,260	This study

trophoretic karyotypes of tester strains of mating populations C and D shared 12 chromosomes but differed in estimated chromosome sizes (31). Analysis by RAPD showed that tester strains of mating populations C and D are closely related but belong to different groups (27). Further DNA sequence analysis should clarify the true relationship of bakanae strains within the *G. fujikuroi* species complex.

Leslie (7) has made a strong case for the value of mating tests to assign *Fusarium* strains to mating populations or biological species, defined as interbreeding populations reproductively isolated from other mating populations. Our attempts to resolve the relationship of bakanae strains to *G. fujikuroi* mating populations C and D by using mating tests were hampered by the consistent infertility of crosses with the mating population C tester strains. This infertility is unexplained, but it is not unique to our study. Many other workers have reported difficulties and failures in obtaining fertile crosses in mating population C (3, 6, 13, 22). In the present study, crosses between bakanae strains and mating population D tester strains were of low fertility; in most crosses, viable ascospores were rare. Nevertheless, analysis of progeny from one such cross demonstrated that genes could be transferred by mating. Thus, a cross between parental strains that produced only fumonisin B_1 or only moniliformin yielded progeny that produced high levels of both mycotoxins. Such strains have also been found to occur naturally in maize from Sardinia (10).

Fertility barriers have been widely used to distinguish mating populations and species in well-studied fungal genera such as *Neurospora*. However, even in *Neurospora*, certain strains can cross unusually well with tester strains from *N. intermedia* and *N. crassa*, the most closely related species (19). In addition, poor fertility is not confined to interspecies crosses but is often observed between strains from different natural populations of the same species. Thus, the ability to form a highly fertile cross with a tester strain can determine the mating population or biological species of a fertile strain, but it is more problematic to exclude a strain only because it is infertile or poorly fertile.

In conclusion, some *G. fujikuroi* strains isolated from rice from the United States, various Asian countries, and other geographic areas are able to produce fumonisin B_1 and moniliformin in culture. To date, the occurrence of these mycotoxins in rice and rice-based foods has received little attention (20). Future studies should focus on the natural occurrence of fumonisins, moniliformin, and other relevant mycotoxins in rice infected by bakanae disease.

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